UNIVERSITÀ DEL PIEMONTE ORIENTALE

SCHOOL OF MEDICINE

Department of Health Sciences

Master Degree in Medical Biotechnologies

Master Thesis

Peripheral immune subsets as markers of hepatocellular carcinoma recurrence

Relator: Prof. Mattia Bellan

Co-relator: Prof. David James Pinato

Candidate: Federica Vincenzi

Matricula: 20028271

Firmato digitalmente da Mattia Bellan
Data: 01.07.2024 17:22:01 CEST
Organizzazione: UNIVERSITA' DEGLI STUDI DEL PIEMONTE ORIENTALE/01943490027

Firmato digitalmente da David James Pinato
Data: 01.07.2024 16:36:45 CEST
Organizzazione: UNIVERSITA' DEGLI STUDI DEL PIEMONTE ORIENTALE/01943490027

Academic year 2023/2024

Table of Contents

1. ABBREVIATIONS

AFP, α-fetoprotein; ALBI score, Albumin-Bilirubin score; APC, Adenomatous Polyposis Coli Protein; APCs, Antigen Presenting Cells; BCLC, Barcelona Clinic for Liver Cancer; BMI, Body Mass Index; BSC, Best Supportive Care; CAFs, Cancer-Associated Fibroblasts; CCL25, C-C Motif Chemokine Ligand 25; CCND1, Cyclin D1; CCR9, C-C Motif Chemokine Receptor 9; CD, Cluster Differentiation; Ce^{140}, Cerium 140; CKIα, Casein Kinase I Isoform Alpha; CLEC4D, C-Type Lectin Domain Family 4 Member D; CTCs, Circulating Tumour Cells; CTLA-4, Cytotoxic T-Lymphocyte Associated Protein 4; CTNNB1, Catenin β1; CX3CR1, C-X3-C Motif Chemokine Receptor 1; CXCL16, C-X-C Motif Chemokine Ligand 16; CXCR6, C-X-C Motif Chemokine Receptor 6; DAMPs, Damage Associated Molecular Patterns; ECOG PS, Eastern Cooperative Oncology Group Performance Status; EGFR, Epidermal Growth Factor Receptor; EMT, Epithelial-to-Mesenchymal Transition; EOMES, Eomesodermin; FGF19, Fibroblast Growth Factor 19; FoxP3, Forkhead box P3; GSK-3β, Glycogen Synthase Kinase 3 Beta; HBV, Hepatitis B virus; HCC, Hepatocellular Carcinoma; HCV, Hepatitis C virus; HGF, Hepatocyte Growth Factor; HSCs, Hepatic Stellate Cells; INFγ, Interferon γ; INR, International Normalized Ratio; Ir^{191}, Iridium 191; Ir^{193}, Iridium 193; IRI, Ischemia Reperfusion Injury; JAK1, Janus Kinase 1; KEAP, Kelch Like ECH Associated Protein; LAG-3, Lymphocyte Activation Gene 3; LEF, Lymphoid Enhancer Binding Factor; LncRNAs, Long non-coding RNAs; LRP, LDL Receptor Related Protein; LSECs, Liver Sinusoidal Endothelial Cells; LT, Liver Transplant; MAPK, Mitogen-Activated Protein Kinase; MASLD, Metabolic dysfunction-Associated Steatotic Liver Disease; MDSCs, Myeloid-Derived Suppressor Cells; MELD, Mayo End stage Liver Disease; MMP, Matrix Metalloproteinases Protein; mTOR, mechanistic Target of Rapamycin Kinase; NAFLD, Non-Alcoholic Fatty Liver Disease; NF-KB, Nuclear Factor Kappa B; NK, Natural Killer; NLR, Neutrophil-to-Lymphocyte Ratio; NSCLC, Non-Small Cell Lung Cancer; OS, Overall Survival; PBMCs, Peripheral Blood Mononuclear Cells; PD-L1, Programmed Death-Ligand 1; PD1, Programmed Cell Death 1; PFS, Progression Free Survival; PI3K, Phosphatidylinositol 3-Kinase; PS, Performance Status; ROC, Receiver Operator Characteristics; ROS, Reactive Oxygen Species; SLECs, Short-Lived Effector Cells; STAT, Signal Transducer and Activator of Transcription; TACE, Trans-Arterial Chemo Embolization; TAMs, Tumour Associated Macrophages; TBET, T-box transcription factor 21; TCF, Transcription Factor; TEMRA, T Effector Memory cells re-expressing CD45RA; TERT, Telomerase Reverse Transcriptase; TGF-β, Transforming Growth Factor Beta; TIGIT, T cell Immunoreceptor with Ig and ITIM domains; TIM-3, T-cell immunoglobulin and mucin domain-3; TME, Tumour Microenvironment; TNM, Tumour Nodes Metastasis; TP53, Tumour Protein P53; Tregs, T regulatory cells; VEGF, Vascular Endothelial Growth

2. ABSTRACT

Hepatocellular carcinoma (HCC) is the sixth most common cancer and third leading cause of tumour-related death worldwide. Despite the advances in curative treatments of hepatocellular carcinoma, recurrence remains a major burden and occur in 50% of patients at 5 years after tumour resection. The mechanisms underlying HCC recurrence are not fully understood but it has been reported that modifications of the immune context may play a crucial role. In this preliminary study we evaluated the peripheral immune landscape of resected patients to identify a possible association with recurrence.

In this prospective observational study, 19 HCC patients, eligible for curative treatment, were evaluated at baseline (time of surgery) and at 1 year (no recurrence group) or at time of recurrence (recurrence group), using CyTOF technique.

We found recurrence to be associated with increased levels of NK cells (p=0.033) and $CD8^+$ CM cells (p=0.029). Additionally, NK cells ≥ 27.5% (p=0.040) and $CD8^+$ CM ≥ 9.2% (p=0.021) predict worse recurrence free survival. Moreover, our results show a difference in $CD8^+$ activated (p=0.002), $CD8^+$ $CD186^+$ (p=0.004), $CD8^+$ $CD199^+$ (p=0.009), $CD8^+$ $CX3CR1^+$ (p=0.009), $CLEC4D^+$ (p=0.039), NK $CD199^+$ (p=0.006), $CD4^+$ $CD186^+$ (p=0.004), $CD4^+$ $CD199^+$ (p=0.014), $CD4^+$ $CX3CR1^+$ (p=0.002), $CD4^+$ TEMRA (p=0.031) at the time of recurrence compared to baseline. Furthermore, NK cells showed a negative correlation with both NLR (p=0.003) and PLR (p=0.004), while $CD8^+$ $CD279^+$ cells were positively correlated with NLR (p=0.0009) and PLR (p=0.008).

In conclusion, our results evidence that elevated NK cells and $CD8^+$ CM cells values at baseline may be predictive of tumour recurrence. The heterogeneous immune landscape at time of recurrence, suggests the presence of an active immune response as well as simultaneous exhaustion of effector memory cells. These findings may influence the selection of the therapeutic approach for these patients with recurrence. Additionally, we evidenced a relationship between exhausted phenotype, NLR and PLR. Finally, we identified a correlation between the peripheral immune landscape and liver function. A limitation of this study is the small number of patients enrolled. In the future we would like to apply this knowledge to a wider population, analysing different timepoints to evaluate the trend of these cellular subtypes during time.

3. INTRODUCTION

3.1 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the sixth most common cancer and third leading cause of tumour-related death worldwide [1]. The median age at diagnosis is generally around 60 years old and affects prevalently males, which have higher progression rate and worse response to treatments. HCC has a poor prognosis, with a median overall survival of 6-10 months since diagnosis is frequently made at advanced stages. However, the introduction of novel chemotherapeutic strategies has increased the median OS at 10.7 months [2,3]. In 2018, the age-standardized incidence rate of HCC at global level was 7.3 cases per 100,000 person-year, with an incidence of 11.6 per 100,000 men and 3.4 per 100,000 women. Epidemiology of HCC varies according to the population ethnicity, in 2018 the regions largely contributing to the overall HCC cases were Eastern Asia (58.3%), South-Eastern Asia (9.6%) and North America (4.9%) [4].

Risk factors for HCC development are multiple and include demographic factors (sex, age, ethnicity), severity of underlying liver disease (fibrosis score, inflammation), metabolic and lifestyle factors (diabetes, obesity, alcohol and smoke), as well as genetic alterations. Indeed, HCC usually develops on a background of chronic liver disease or cirrhosis, progressed after the exposure to risk factors which induces a chronic inflammatory status, followed by structural and functional alteration of the liver. The major risk factors include hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD), diabetes, as well as rare conditions such as hereditary hemochromatosis [5].

Chronic hepatitis B infection occurs in 50-60% of global HCC cases and the lifelong risk of developing HCC is approximately 30% and 10% in male and female carriers, respectively. Indeed, HBV is able to integrate its DNA into the chromosomes of liver cells, which clonally expand and are likely to become HCC precursors [6]. Moreover, HBV can induce epigenetic changes and subsequent abnormal gene regulation, increasing the possibility of developing HCC. Currently, the availability of vaccines and antiviral therapy has reduced infection rate and the risk of liver cancer [7].

Hepatitis C virus infection is another well-known risk factor for the development of HCC. Conversely to HBV, HCV does not integrate into the host genome, thus it is not able to directly

induce mutations in the cells [7]. Indeed, the development of HCC in HCV infected patients is mainly due to the chronic inflammation and persistent immune response. Many factors, such as cytokines and chemokines, induce the activation of hepatic stellate cells (HSCs), responsible for fibrosis and subsequent development of cirrhosis, favouring the onset of HCC [8]. Moreover, studies have reported that HCV-related HCC is more aggressive than HCC related to other aetiologies, it is more frequently multifocal and shows an increased recurrence rate after resection [9].

Excessive alcohol consumption, defined as > 40 g of alcohol intake a day over a sustained period, is one of the leading causes of liver disease. In 2019, it was estimated that 19% of liver cancer-related deaths were associated to alcohol. In alcohol-related liver disease, alcohol is metabolized in acetaldehyde, which in turn leads to the production of reactive oxygen species (ROS) and activate inflammation. ROS and inflammation induce the formation of DNA adducts and subsequent hepatocarcinogenesis [10].

Lastly, MASLD, previously known as Non-Alcoholic Fatty Liver Disease (NAFLD), is another risk factor for the development of HCC and encompasses patients with excessive accumulation of hepatic lipids (steatosis), carrying one of five cardiometabolic risk factors. These include Body Mass Index (BMI) $\geq$ 25 kg/m^2, elevated glucose levels or type 2 diabetes mellitus, high blood pressure, elevated plasma triglycerides and cholesterol [11]. In Italy, the majority of HCC patients are defined as MASLD, and the proportion has been significantly increased in the last years [12].

3.2 Pathogenesis

Hepatocarcinogenesis is a multi-step process that starts from the chronic exposure to risk factors, which induces chronic inflammation and eventually leads to the development of cirrhosis. In 80% of the cases, HCC develops on a cirrhotic liver, which has already acquired many molecular alterations (***Figure 1***) [13]. Cirrhotic liver is more susceptible to genomic instability; therefore, the increasing accumulation of somatic alterations and epigenetic modifications could modify metabolic pathways, leading to the development of HCC [14].

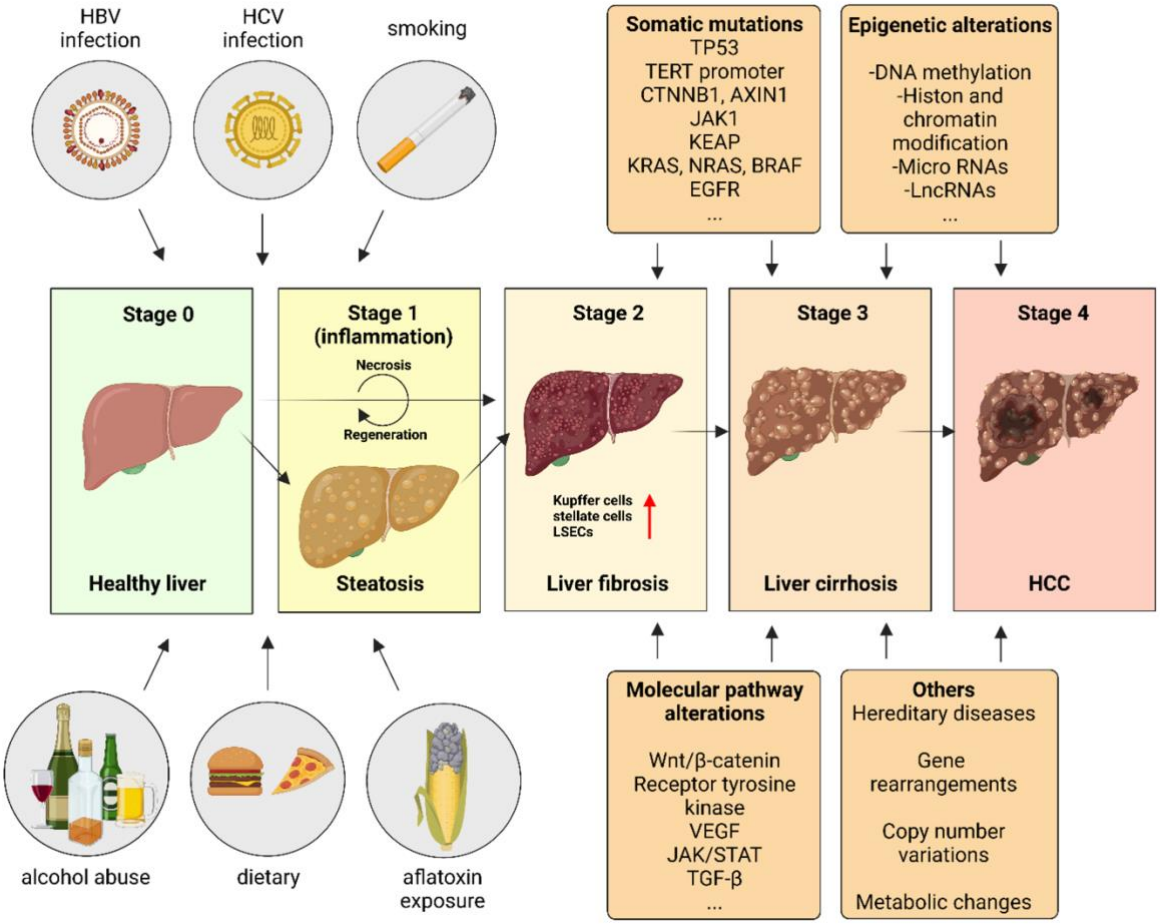

Figure 1: Aetiology, risk factors and pathogenic pathways of HCC development [14]. Abbreviations: HBV, Hepatitis B virus; HCV, Hepatitis C virus; LSECs, Liver Sinusoidal Endothelial Cells; TERT, Telomerase Reverse Transcriptase; CTNNB1, β-catenin; JAK1, Janus Kinase 1; KEAP, Kelch Like ECH Associated Protein; EGFR, Epidermal Growth Factor Receptor; VEGF, Vascular Endothelial Growth Factor; STAT, Signal Transducer and Activator of Transcription; TGF-β, Transforming Growth Factor Beta; LncRNAs, Long non-coding RNAs.

Somatic mutations occur in oncogenes, oncosuppressor genes or genes involved in regulatory pathways that can lead to cell transformation. Among these alterations, mutations in the telomerase reverse transcriptase (TERT) gene promoter have been reported in 40-65% of HCC cases and are considered as an early event in hepatocarcinogenesis. These mutations are responsible for the overexpression of TERT, which in turn leads to cell immortality and malignant transformation through the interaction with many transcription factors such as MYC, β-catenin and NF-KB [15].

Genomic studies on HCC reported that 30-40% of tumours demonstrate aberrant activation of WNT/β-catenin pathway, considered an early signal of HCC pathogenesis [16]. Indeed, frequent mutations involve the gene CTNNB1, which encodes for β-catenin. In particular, the alteration arises in the exon 3 of CTNNB1 gene, preventing the phosphorylation and degradation of β-catenin. This gain of function mutation results in stabilization of β-catenin and its translocation into the nucleus (***Figure 2***), where it can act as a transcription factor for genes involved in cell proliferation, dedifferentiation, and epithelial-to-mesenchymal transition (EMT), thus leading to the development of malignancies [17]. In addition, AXIN1 loss of function mutations have been found in 8% of human HCC. AXIN1 is essential for the degradation of β-catenin, therefore its loss of function induces the overactivation of the WNT/β-catenin pathway and subsequent hepatocarcinogenesis [18].

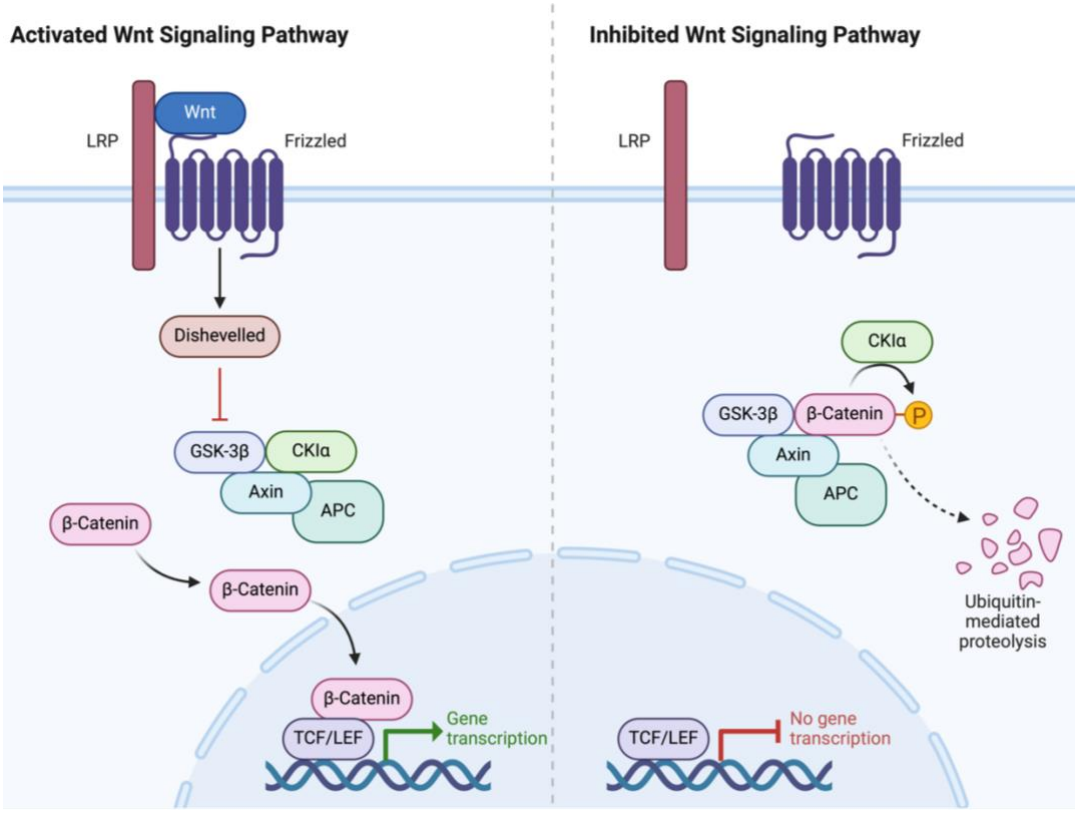

Figure 2: WNT/β-catenin pathway. Genetic alterations of β-catenin or Axin induce a constitutive activation of β-catenin, thus leading to transcription of genes involved in HCC pathogenesis. Created with BioRender. Abbreviations: LRP, LDL Receptor Related Protein; GSK-3β, Glycogen Synthase Kinase 3 Beta; CKIα, Casein Kinase I Isoform Alpha; APC, Adenomatous Polyposis Coli Protein; TCF, Transcription Factor; LEF, Lymphoid Enhancer Binding Factor.

Tumour suppressor TP53 mutations occur in half of human cancers. This gene is pivotal for the regulation of cell cycle arrest and the induction of apoptosis, preventing accumulation of oncogenic mutations and development of malignancies. Loss of function mutations of TP53 are usually followed by loss of heterozygosity in the late stages of tumour development [19]. Mutations in TP53 gene have been reported in 30% of HCC cases[20], a rate which increases to 60% in HBV-related HCC[21]. This alteration correlates with tumour differentiation, serum AFP levels, vascular invasion and tumour stage [22]. Moreover, patients with mutant TP53 have lower overall survival and relapse free survival, compared to wild-type TP53 [21,23]. In addition, in a subset of patients, TP53 mutation occurs in concomitance with c-MET activation[23]. c-MET is a protooncogene that encodes for hepatocyte growth factor (HGF) receptor. After the binding to HGF, c-MET activates downstream pathways, such as RAF/MAPK and PI3K/AKT, that drive tumour dedifferentiation and metastasis [24]. The presence of both TP53 mutations and c-MET activation is associated with shorten overall survival [23].

The genetic background of the tumour allows the classification of HCC into two major molecular subtypes, that may have a potential implication in patient prognosis and treatment [25]. In particular, the proliferative subclass is characterized by the activation of proliferating signalling pathways, such as PI3K-AKT-mTOR, MAPK and MET signalling, enrichment of TP53 inactivation as well as FGF19/CCND1 amplification. This subtype has been associated with HBV-related HCC, high levels of AFP and poor prognosis [13,26]. The non-proliferative subclass is more heterogeneous and is characterized by the activation of the WNT pathway, mainly harbouring the CTNNB1 mutation, and higher TERT promoter mutations [27,28]. This subtype has been associated with alcohol and HCV-related HCC and better prognostic outcome [28]. Unfortunately, most of the mutations cannot be targeted by current therapeutic strategies. Indeed, further research is required in order to understand the genetic background and molecular mechanisms underlying this disease, translating them into prognostic and therapeutic strategies [28].

3.3 Diagnosis and treatment

HCC diagnosis in cirrhotic patients is mostly based on non-invasive criteria that comprise the use of different imaging techniques. These include contrast-enhanced Computer Tomography or Magnetic Resonance Imaging. The diagnosis relies on the detection of typical hallmarks of HCC, which differ according to imaging techniques or contrast agents [29]. While pathology

confirmation is optional in cirrhotic patients, it is mandatory in non-cirrhotic patients. Moreover, the use of tissue biopsy allows the histological evaluation based on TNM classification [30].

The prognosis and treatment strategies of HCC are currently defined by the recommendations given by Barcelona Clinic for Liver Cancer (BCLC) staging system, represented in ***Figure 3***, which allows patient stratification based on tumour burden, liver function and performance status. In particular, liver function can be evaluated using different clinical and laboratory parameters, including Child-Pugh score, AFP serum levels and ALBI score. BCLC system suggests the appropriate therapeutic regimen for each of the five stages: very early stage (BCLC 0), early stage (BCLC A), intermediate stage (BCLC B), advanced stage (BCLC C) and terminal stage (BCLC D) [31].

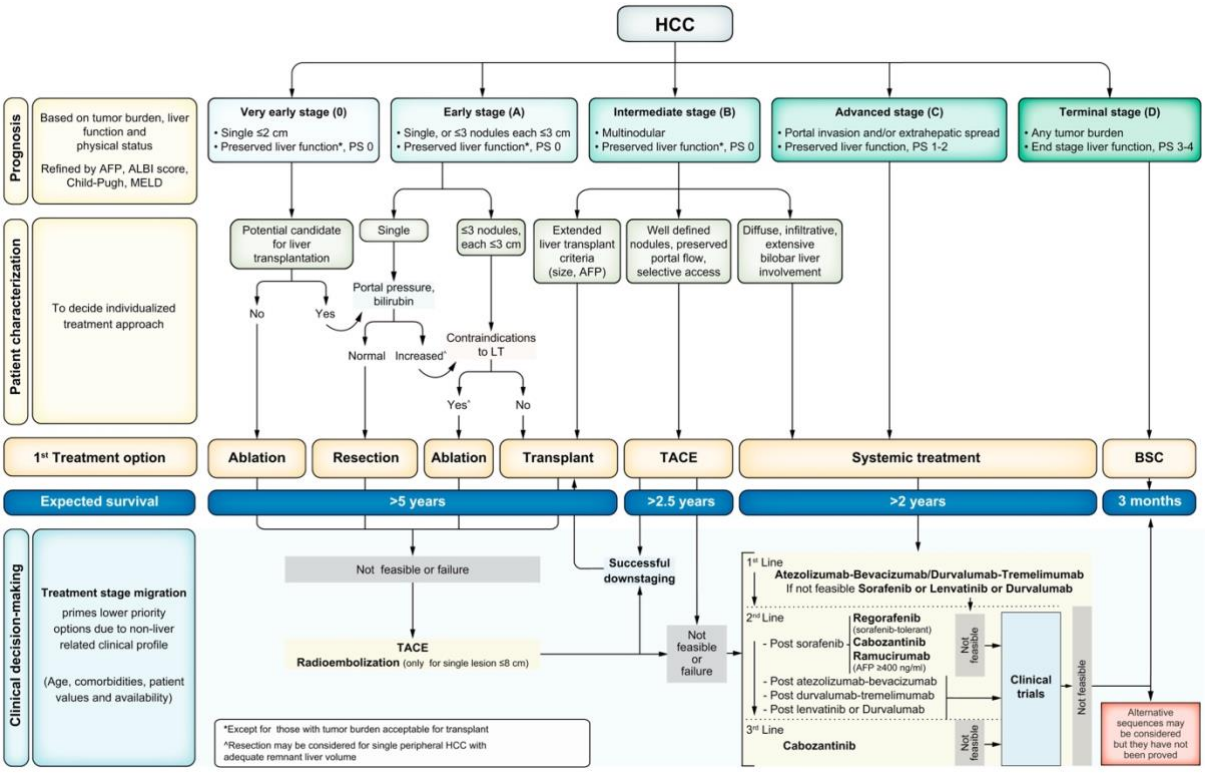

Figure 3: BCLC classification of HCC and relative therapeutic interventions [31]. Abbreviations: AFP, α-fetoprotein; ALBI score, Albumin-Bilirubin score; MELD, Mayo End stage Liver Disease; PS, Performance Status; LT, Liver Transplant; TACE, Trans-Arterial Chemo Embolization; BSC, Best Supportive Care.

HCC curative treatments for early-stages include surgical resection, ablation and liver transplantation and provide a 5-year survival rate of approximately 70%. Liver resection is

reserved for patients belonging to BCLC 0-A, with localized tumour and preserved liver function. The introduction of minimal invasive surgical approaches, such as laparoscopic or robotic-assisted hepatectomy, has extended the characteristics of patients that can undergo surgical resection and has been associated with a decreased 30-day morbidity and surgical blood loss [32]. Even though the progress in the surgical field have expanded eligibility criteria for resection, patients in advanced stages or BCLC B with diffuse bilobar involvement are recommended for systemic therapy using tyrosine kinase inhibitors or immune checkpoint inhibitors, which target pathways involved in angiogenesis and proliferation [31].

3.4 Recurrence

Despite the numerous advances in the treatment of HCC, recurrence is still a major concern and occurs in approximately 70% of patients 5 years after liver resection[33]. Early recurrence is associated with tumour related factors, including tumour size and number of lesions, presence of microvascular invasion and high AFP levels pre- and post-hepatectomy and has a great impact on overall survival [34]. On the contrary, late recurrence does not depend on the characteristics of the resected tumour, but on the underlying liver disease and by de novo development of neoplasms [35].

In this context, surgical stress may play an important role in the induction of inflammation, alteration of the tumour microenvironment (TME) and creation of favourable conditions for the implant and growth of neoplastic cells [36]. To reduce intraoperative complications, the blood supply to the liver is transiently blocked and the subsequent reperfusion of the parenchyma may induce ischemia reperfusion injury (IRI) [37]. IRI increases the production of ROS able to alter macromolecules, including DNA [38]. Moreover, ROS are able to induce necrosis of liver cells with the subsequent release of damage associated molecular patterns (DAMPs) which, in turn, enhance inflammation and recruitment of immune cells [39]. Increasing evidence show that ROS produced following surgery play an important role in the development of local recurrence and distant liver metastases [38].

Furthermore, it has been reported that surgical stress induces a dysfunction of inflammatory cells, such as macrophages and myeloid dendritic cells, and accumulation of Myeloid-Derived Suppressor Cells (MDSCs) which in turn can suppress the antitumor response [40]. In particular, it has been demonstrated that secretion of Interferon-α from dendritic cells induces the mobilization of MDSCs, creating an immunosuppressive environment which consequently

promote early recurrence after surgery [41]. In addition, it has been shown that the induction of an immunosuppressive state following IRI facilitates tumour recurrence, promoting the reactivation of dormant cells and the dissemination of circulating tumour cells (CTCs) [41,42].
In fact, during the progression of a malignancy, tumour cells produce metalloproteinases (MMPs) which are able to break the basal membrane enabling the release of tumour cells in the circulation [43]. These cells undergo EMT acquiring mesenchymal markers, that allow their detachment from the primary tumour and the dissemination in the bloodstream. In this context, the stress-mediated immune suppressive environment, characterized by an enrichment of T regulatory cells (Tregs), helps the immune evasion of CTCs and tissue colonization. Indeed, studies reported that elevated levels of Tregs and CTCs are significant prognostic factors of early recurrence [44].

According to the current literature, the mechanisms underlying HCC recurrence are not fully understood but it has been reported that modifications of the immune context may play a crucial role. Indeed, an immune microenvironment enriched in effector cells confers good prognosis and low risk of recurrence. On the contrary, the repression of anti-tumour immunity mediated by immune escape mechanisms developed by the tumour, promotes poor prognosis and recurrence [45].

3.5 Tumour immune microenvironment

HCC tumour microenvironment is a complex system composed by inflammatory cytokines, extracellular matrix, stromal cells, cancer-associated fibroblasts (CAFs), endothelial cells and HSCs, as well as immune and inflammatory cells, including tumour associated macrophages (TAMs) and lymphocytes [46]. Cancer immune response is initially mediated by Natural Killer (NK) cells, which recognize tumour cells and attack them releasing tumour-associated antigens in the TME. The latter are recognized by antigen presenting cells (APCs) and presented to cytotoxic T lymphocytes which in turn migrate in HCC tissue, destroying tumour cells [47].

In TME, infiltrating immune cells have opposing functions, they can either exert an anti-tumour effect or suppress immune surveillance, contributing to tumour growth [48]. This bidirectional effect is called immunoediting, a dynamic process composed by three phases: elimination, equilibrium and escape [49]. In the elimination phase, the activation of innate and adaptive immune system, including NK and T cells, is able to counteract cancer cell growth. In the equilibrium phase, survived tumour cells start the editing process and enter in equilibrium with

the immune system. Lastly, in the escape phase, cancer cells start growing, becoming clinically evident and creating an immunosuppressive TME [50]. In this context, dysregulation of the immune system, characterized by poor infiltration of effector cells, accumulation of exhausted T cells and recruitment of immunosuppressive cells, has been linked to poor prognosis and promotion of the risk of recurrence [45].

As previously mentioned, the immune response to cancer is dependent on T cell activation. Upon contact with the antigen, short-term memory T cells differentiate into effector T cells, effector memory T cells, central memory T cells and cytotoxic T cells, that can be identified by specific markers [51]. For instance, short-term memory T cells are characterized by an elevated expression of transcription factor TBET as well as lower expression of eomesodermin (EOMES) and Programmed cell Death 1 (PD1) [52]. Chronic antigen exposure induces a mechanism of downregulation of the immune system. This process is controlled by negative regulatory pathways, mediated by the expression of checkpoint molecules on the surface of lymphocytes, like PD1, Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), Lymphocyte-Activation Gene 3 (LAG-3) and T cell Immunoreceptor with Ig and ITIM domains (TIGIT) [53]. Cancer cells express the ligands of these checkpoint molecules, including programmed death-ligand 1 (PD-L1), leading to the progressive exhaustion of T lymphocytes and the subsequent evasion to the anti-tumour immune response[54]. Indeed, increased expression of inhibitory receptors characteristic of exhausted T cells, such as PD1, LAG-3 and T-cell Immunoglobulin and Mucin-domain containing-3 (TIM-3), as well as reduced expression of effector cells markers, such as CX3CR1, have been reported in HCC tissues compared to normal adjacent tissue or peripheral blood [55]. Supporting this concept, Phuong et.al evaluated the immune landscape on tumour tissue, non-tumour liver tissue and peripheral blood of HCC patients following surgical resection. The authors reported an enrichment of exhausted and immunosuppressive cells, such as Tregs ($FoxP3^+$ $TIGIT^+$ $CD152^+$ $CD4^+$) and resident memory T cells ($PD1^+$ $CD103^+$ $CD45RO^+$ $CD8^+$) in the tumour tissue, as well as depletion of APCs and immunoreactive subsets in tumour and non-tumour tissue compared to peripheral blood. These findings delineate the immune modifications that affect different compartments during the stages of HCC [56]. In addition, Shi et.al identified decreased percentage of T cells as well as increased levels of monocytes, $CD4^+$ central memory T cells, Tregs and $PD1^+$ cells in patients' PBMCs at the time of liver resection [57].

Tregs are a subgroup of $CD4^+$ T helper cells able to suppress antitumor immunity, which can be identified by the expression of FoxP3 [58]. In HCC, infiltrating $FoxP3^+$ T cells have been associated with poor survival and high recurrence [59]. Indeed, further studies suggested elevated levels of intra-tumoral Tregs and reduced levels of $CD4^+$ or $CD8^+$ cells as independent risk factors for recurrence after resection [60]. Furthermore, it has been described that Tregs are able to block $CD8^+$ T cells migration into the tumour and suppress granzyme and perforin production [61]. Supporting this concept, it has been demonstrated that increased Tregs in HCC are able to suppress $CD8^+$ T cells activity as well as to induce angiogenesis [62].

The immune landscape of HCC is also composed by innate immune cells, including Tumour Infiltrating Neutrophils (TINs) that can directly activate $CD8^+$ T cells inducing tumour death, or secrete immunoreactive molecules which can potentiate cancer cell growth [63,64]. It has been reported that patients with increased intra-tumoral neutrophils and lower $CD8^+$ T cells have higher risk of early recurrence after resection [65]. Indeed, an elevated Neutrophil-to-Lymphocyte Ratio (NLR), a peripheral marker of inflammation, has been reported as an independent prognostic factor associated with poor recurrence free survival after resection [66,67].

CLEC4D, a C-type lectin receptor mainly expressed on myeloid cells is a mediator of innate immune response. Indeed, it has been observed an inhibitory effect of CLEC4D on the immunosuppressive function of myeloid cells [68]. Moreover, CLEC4D has been positively correlated with infiltration of macrophages, neutrophils, dendritic cells, B cells, $CD4^+$ and $CD8^+$ T cells in HCC tumour tissue [69].

Several chemokines and their receptors are involved in the regulation of the immune response against cancer. For instance, CCR9 (CD199) is the chemokine receptor of CCL25, involved in recruitment and function of immune cells [70]. CCL25 is mainly expressed in thymus and intestinal epithelium, and it is known to promote proliferation and chemotaxis of inflammatory cells expressing CCR9, including $CD4^+$, $CD8^+$ T cells and NK cells [71,72]. Indeed, Berahovich et.al recognized different subsets of NK cells expressing chemokine receptors, including CCR9, which has been thought to mediate homing of mucosal T cells in intestine and lungs [72]. Moreover, studies reported that CCR9 plays a crucial role in tumour proliferation, anti-apoptosis, invasion and drug resistance in melanoma, breast, prostate, pancreatic and liver cancer [70]. Indeed, the presence of CCR9 has been found on non-small cell lung cancer cells and it has been correlated with poor OS and metastasis [73]. These findings have been confirmed

by Rogado et.al which identified poor OS and PFS in NSCLC patients harbouring elevated $CD4^+$ $CCR9^+$ T cells prior to immunotherapy [74].

CXCR6 (CD186) is a well-established marker of T cell differentiation and migration, upon binding to its ligand CXCL16. CXCR6 ligand has been found on the surface of dendritic cells, monocytes, hepatocytes, fibroblasts, keratinocytes, and endothelial cells [75,76]. The CXCR6/CXCL16 axis regulates homing, activation, expansion, and cytotoxic effects of immune cells. Several studies reported the involvement of CXCR6 in T cells anti-tumour effects. Indeed, CXCR6 expression mediates the generation of effector T cytotoxic lymphocyte, which are able to interact with tumour dendritic cells expressing CXCL16 [77]. In HCC, CXCR6 expressing $CD4^+$ T cells and NK T cells have been recognized to promote the activation of cytotoxic immune response [78]. However, HCC patients with elevated tissue expression of CXCR6, as well as CXCL16, have a worse overall survival and increased cancer recurrence [79].

The immune context of tumour development has been widely studied on tissue. Liver biopsy is useful for prognosis and prediction of response, but it is an invasive procedure that could be not feasible for all patients. Moreover, single tissue sampling does not provide an extensive analysis of tumour and immune heterogeneity [80]. However, liquid biopsy may have great potential in monitoring and early detection of tumour recurrence [81]. Liquid biopsy is typically used for the detection of CTCs, circulating tumour DNA (ctDNA), circulating RNA, tumour-educated platelets and exosomes [82]. In lung cancer, liquid biopsy has been used to detect CTCs to point out the mutational profile of tumour-related genes, predictor of treatment response [83]. Moreover, studies have been performed for identification of peripheral markers for HCC diagnosis, including ctDNA, but results on the diagnostic power of these markers have not been conclusive [84]. In this context, it is necessary to identify immune signatures describing the tumour evolution that may help early detection, prediction of treatment response and prognostic outcome.

4. AIM OF THE STUDY

This study aims to evaluate the peripheral immune landscape of resected HCC patients, to identify immunological predictors of tumour recurrence.

5. MATERIALS AND METHODS

5.1 Study population and ethical aspects

The population included in this thesis belongs to the study "Finding the seeds of recurrence: the role of the liquid biopsy to detect circulating tumour cells as markers of advanced disease and prognosis in hepatocarcinoma", which was approved by Ethical Committee of Brianza in date 04/04/2019. All the participants signed an informed consent and were selected according to specific inclusion criteria:

- Age ≥ 18 years old
- First HCC diagnosis and planned to be subjected to surgery (rejection or OLT) as first treatment
- BCLC stage 0, A, B
- Post-surgery histological confirmation of HCC

The exclusion criteria were:

- Other neoplasms
- Autoimmune liver diseases (sclerosing cholangitis, primary biliary cirrhosis, autoimmune hepatitis)
- Surgery with non-curative purpose (palliative)
- Histological diagnosis of combined hepatic neoplasia (hepatocholangiocarcinoma)
- HIV positivity
- Previous HCC treatment

To the purpose of this study, 25 patients affected by HCC were selected, which received surgical intervention in UOC Chirurgia Generale 1, ASST San Gerardo Monza.

5.2 Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood of patients was collected at the time of surgery (baseline) and at the time of recurrence (recurrence group) or at 1 year (recurrence-free group), using EDTA anticoagulant vacutainer. The procedure of isolation of the peripheral blood mononuclear cells (PBMCs) was performed under sterile conditions using the Ficoll gradient (Sigma-Aldrich, Milan, Italy), according to manufacturer instructions.

5.3 Cytometry by Time-Of-Flight (Helios)

The identification of cellular subpopulations was performed by Helios instrument (Standard Biotools, South San Francisco, CA, USA), using Cytometry by Time-Of-Flight (CyTOF) technique. In CyTOF technology, also called mass cytometry, cells are stained with metal-conjugated antibodies direct against surface antigens or intracellular proteins. Inside the Helios instrument, cells are vaporized and atomized in clouds that maintain the metal ions. This cloud passes inside of the TOF chamber where ions are separated according to their mass-to-charge ratio and the detector measures the quantity of each isotope, allowing the classification of the cell. The procedure of sample preparation and acquisition was performed according to manufacturer instruction.

5.4 Antibody conjugation

Antibody conjugation with Cadmium ($^{106\text{-}116}$Cd) metal was performed using Maxpar MCP9 Antibody Labeling Kit, while conjugation with Lanthanide (Ln) metal was performed using Maxpar X8 Antibody Labeling Kit according to manufacturer instructions (Standard Biotools, South San Francisco, CA, USA). The metal tagged antibodies used for cell classification are represented in ***Table 1A*** in appendix.

5.5 Data normalization and gating

Data were normalized by the instrument using EQ beads. Quality control and gating was performed using FlowJo Software v10.10 (BD Life Science, USA). Ir^{191}_DNA, Ir^{193}_DNA, event length and bead specific Ce^{140} were used to differentiate cells from debris and non-cellular events, while cisplatin negativity was used to select live cells. To analyse rare subpopulations, we excluded samples with less than 1000 events. As presented in ***Figure 4***, CD45 positive cells were used to identify T cells ($CD3^+$), B cells ($CD19^+$) and CD3 CD19 double negative cells. The $CD3^-$ $CD19^-$ population was used to identify NK cells ($CD56^+$ $CD16^+$), from which we pointed out three different subpopulations: NK $CD199^+$ cells, NK $CD335^+$ cells and NK $KLRG1^+$ cells. T cell compartment was further evaluated to differentiate helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells, from which we were able to recognize other cellular subsets (***Figure 1A*** and ***Figure 2A*** in appendix).

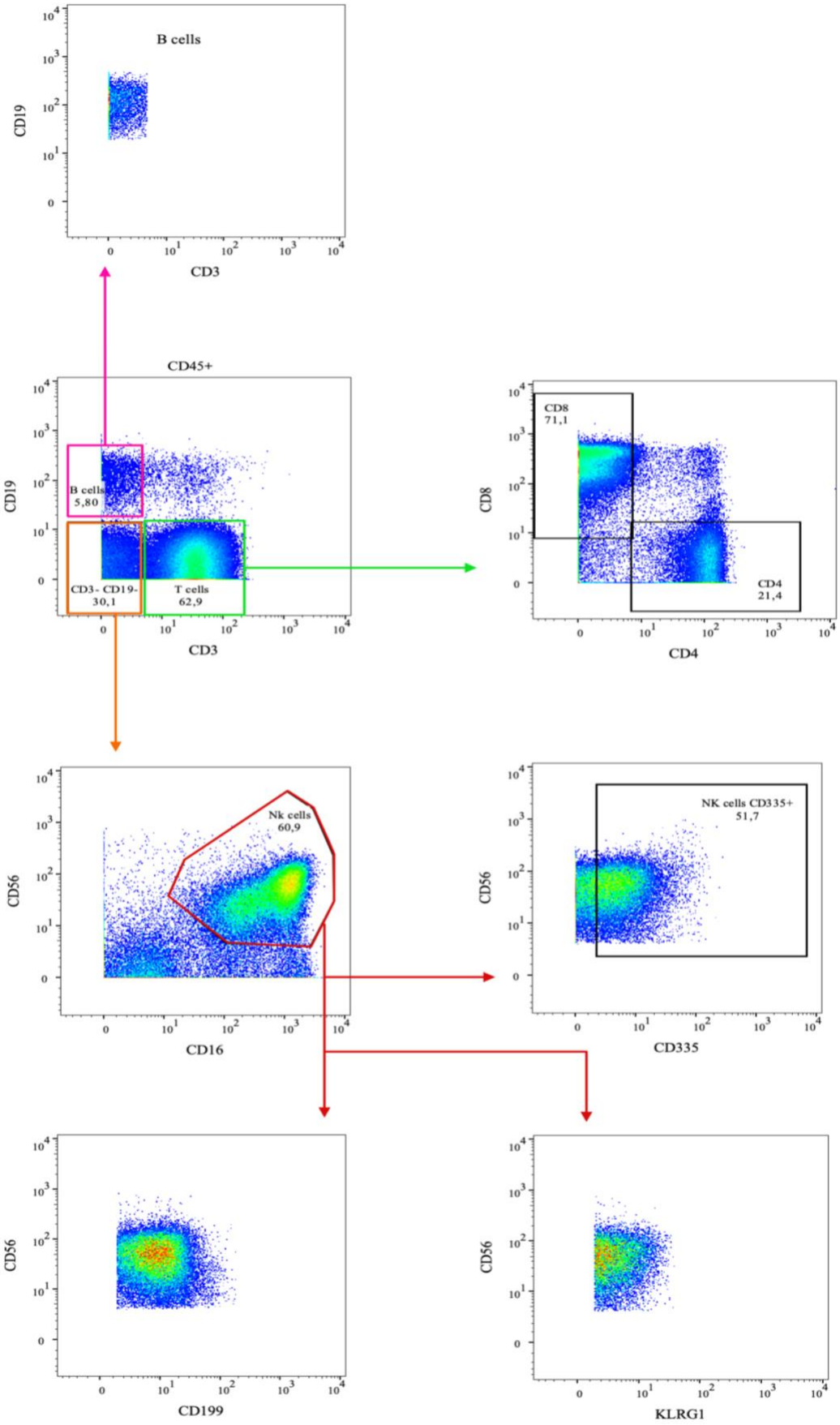

Figure 4: Gating strategy for the identification of cellular subpopulations.

5.6 Data collection

For each patient we collected demographic data (age and sex), along with the main clinical features of the underlying liver disease, including risk factors. We also reported the presence of splenomegaly and oesophageal varices, indicative of portal hypertension [85]. A general biochemical and haematological panel was available for all the patients, including AFP, ALT, total bilirubin, albumin, haemoglobin, creatinine, sodium, White Blood Cells (WBC) count, neutrophils count, lymphocytes count, platelet count and International Normalized Ratio (INR). Moreover, data on prognostic scores were recorded. These include Child-Pugh score, based on serum albumin, bilirubin, ascites, encephalopathy, and prothrombin time [86] and Model of End-Stage Liver Disease (MELD) which evaluates liver functionality according to creatinine, bilirubin, INR for prothrombin time and aetiology of liver disease [87]. Moreover, based on blood parameters we calculated Albumin-Bilirubin score (ALBI), index of liver function [88] as well as Neutrophil-to-Lymphocyte ratio (NLR) and Platelet-Lymphocyte ratio, markers of inflammation [89]. We also collected data on the Eastern Cooperative Oncology Group Performance Status (ECOG PS), which identify patients' fitness [90]. We also collected data regarding the characteristics of the tumour, including number of nodules, histological variant, as well as Edmondson grading, indicator of the degree of hepatocellular carcinoma differentiation [91]. We also evaluated data on surgical intervention, including wedge and anatomic resection which differ according to the extent of resection [92].

5.7 Statistical analysis

Statistical analyses were performed with STATA statistical software version 18.0 (StataCorp, 4905 Lakeway Drive College Station, TX, USA), while graphs were created using GraphPad Prism version 9.4.0 (GraphPad Software, La Jolla, CA, USA). For continuous variables, the measures of centrality and dispersion are presented as medians and interquartile ranges [IQR], while categorical variables are presented as absolute frequency with relative percentages. Comparisons between groups was performed using the Mann–Whitney U test. The optimal cut-off value of NK cells and $CD8^+$ CM cells was determined by Receiver Operator Characteristics (ROC) analysis. Kaplan-Meier survival analysis was used to evaluate recurrence free survival of patients according to NK cells and $CD8^+$ CM cells, followed by log-rank analysis. Recurrence-free survival was defined as the length of time between surgical resection and tumour recurrence or death. Spearman's rank correlation coefficient was used to study correlation between continuous variables. The threshold for statistical significance was 0.05 (two-tailed).

6. RESULTS

6.1 General characteristics of the population

Among the 25 patients, 6 were excluded due to reduced availability of PBMCs, which did not allow a reproducible evaluation of all cellular subpopulations. The analysis was performed on the remaining 19 patients (14 males, 73.7%) with a median age at the time of surgery of 73 [60.7-74.9] years. Clinical characteristics and clinical scores of the population are described in ***Figure 5*** and ***Table 1***, respectively, while blood parameters and related clinical scores are presented in ***Table 2***.

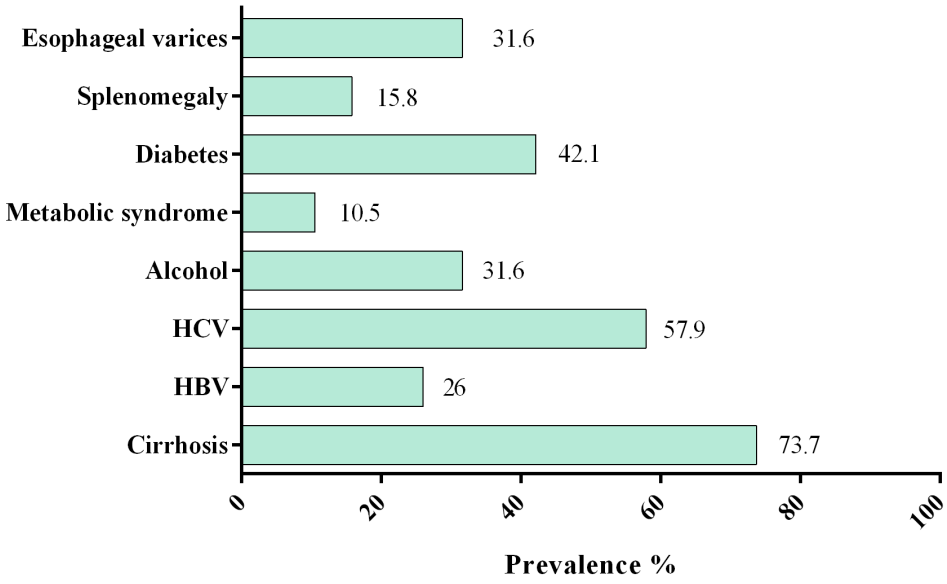

Figure 5: Clinical characteristics of the study population. Variables are presented as absolute frequencies and relative percentages. HCV, Hepatitis C virus; HBV, Hepatitis B virus.

***Table 1**:* Clinical scores of the population. Variables are represented as frequency (%). MELD, Model for End-Stage Liver Disease; ECOG PS, Eastern Cooperative Oncology Group Performance Status; BCLC, Barcelona Clinic Liver Cancer criteria.

Variable	**Frequency (%)**	
Child-Pugh score	5	10 (52.6)
	6	4 (21.1)
	Missing data	5 (26.3)
MELD score	6	1 (5.3)
	7	8 (42.1)
	8	3 (15.8)
	9	2 (10.5)
	10	1 (5.3)
	11	3 (15.8)
	Missing data	1 (5.3)
ECOG PS	0	15 (79)
	1	1 (5.3)
	2	1 (5.3)
	3	2 (10.5)
BCLC	0	7 (36.8)
	A	11 (57.9)
	B	1 (5.3)

Table 2: Blood parameters at baseline and related clinical score. Variables are represented as median and interquartile range [IQR]. AFP, α-fetoprotein; WBC, White Blood Cells; INR, International Normalized Ratio; ALBI, Albumin-Bilirubin ratio; NLR, Neutrophil–Lymphocyte Ratio; PLR, Platelet–Lymphocyte Ratio.

Variable	**Median [IQR]**
AFP, ng/ml	4.8 [3.3-11.5]
ALT, UI/L	22 [15-41]
Total bilirubin, mg/dl	0.6 [0.4-1.0]
WBC, x 10^3/ml	6 [4.2-7.6]
Neutrophils, x 10^3/ml	3.5 [2.6-5.3]
Lymphocytes, x 10^3/ml	1.3 [1.0-1.8]
Platelets, x 10^3/ml	153 [113-187]
INR	1.1 [1.0-1.1]
Albumin, g/dl	4.0 [3.6-4.5]
Haemoglobin	13.9 [11.4-15.4]
Creatinine, mg/dl	0.9 [0.7-1]
Sodium, mEq/L	141 [140-143]
ALBI	-2.7 [-3.3 - -2.4]
NLR	2.5 [1.7-3.7]
PLR	113.5 [83.3-132.5]

In the study population, recurrence occurred in 11 (57.9%) patients, with a median of 17.9 [11.2-24.4] months after surgery. ***Table 3*** lists tumour and intervention characteristics.

Table 3: Characteristics of the tumour and hepatic surgery. Variables are presented as absolute frequencies and relative percentages.

Variable	**Frequency**	
Number of nodules	1	16 (84.2)
	2	1 (5.3)
	3	2 (10.5)
Histological variant	Hepatocellular	13 (68.4)
	Clear cell	4 (21.1)
	Sarcomatous	1 (5.3)
	Missing data	1 (5.3)
Edmonson grading	1	3 (15.8)
	2	10 (52.6)
	3	5 (26.3)
	Missing data	1 (5.3)
Presence of tumor capsule	10 (52.6)	
Major resection	3 (15.8)	
Minor resection	16 (84.2)	
Anatomic resection	7 (36.8)	
Wedge resection	12 (63.2)	
Recurrence	Single	9 (81.8)
	Multiple	2 (18.2)
Number of recurrent nodules	1	9 (81.8)
	2	1 (9.1)
	3	1 (9.1)

6.2 Evaluation of cellular subpopulations at baseline

First, we evaluated the difference between cellular populations at baseline in patients which did and did not develop recurrence, for which median and IQR are presented in ***Table 2A*** in appendix. The results show significantly elevated levels of NK and $CD8^+$ CM cells in the recurrence group compared to the recurrence-free group, as shown in ***Figure 6***.

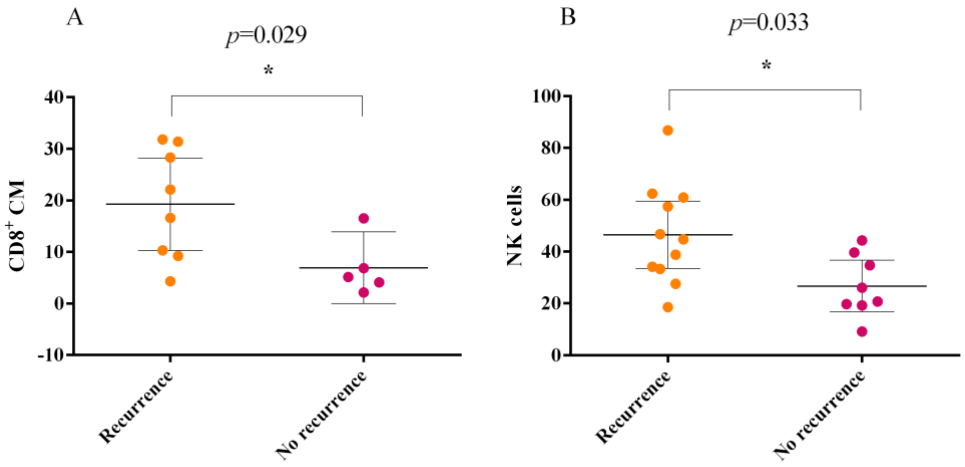

Figure 6: Levels of $CD8^+$ central memory cells (A) and NK cells (B) at baseline in patients with and without recurrence. Data are presented as median and interquartile range [IQR].

Moreover, we found significantly elevated levels of cytotoxic effector memory T cells ($CD8^+$ EM) in patients who did not develop recurrence (***Figure 7***), compared to the recurrence group.

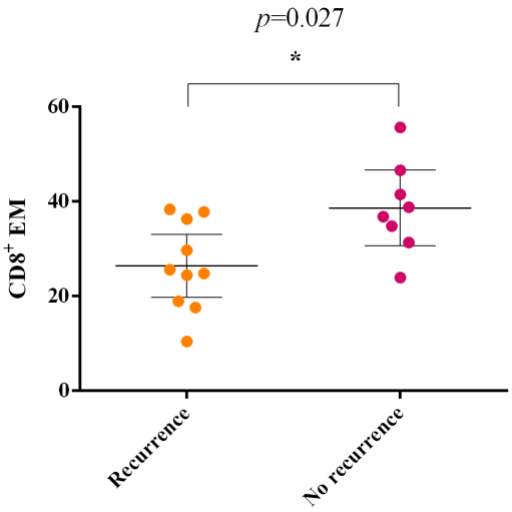

Figure 7: Levels of effector memory ($CD8^+$ EM) T cells baseline in patients with and without recurrence. Data are presented as median and interquartile range [IQR].

6.3 Receiver Operating Characteristic (ROC) curve

On cellular subpopulations identified as significantly different between the recurrence and recurrence-free group, we evaluated their statistical power to identify recurrence using a ROC curve. A presented in ***Figure 8***, our results show that NK cells can differentiate between recurrent and non-recurrent patients at a cut-off ≥ 27.5% (sensitivity: 90.9%, specificity: 62.5%) and $CD8^+$ central memory at a cut off ≥ 9.2% (sensitivity: 87.5%, specificity: 80.0%).

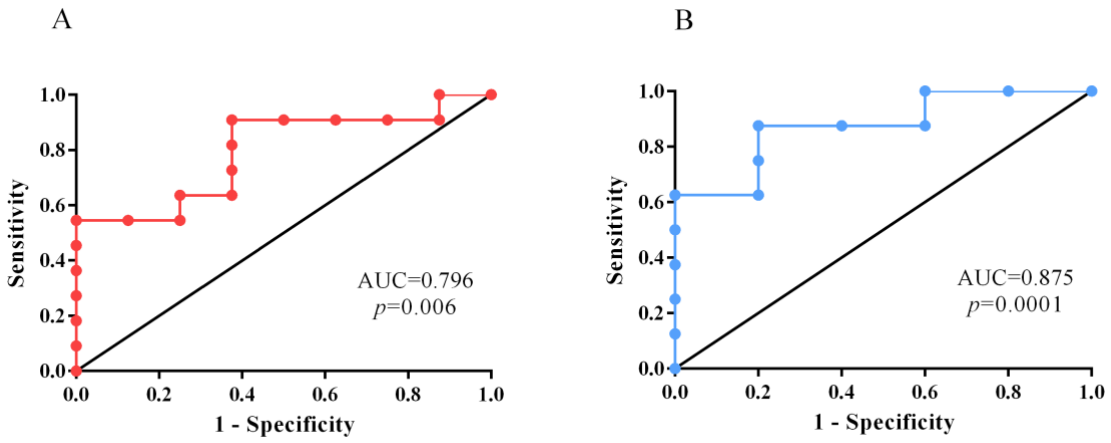

Figure 8: Receiver Operating Characteristic (ROC) curve of NK cells (A) and $CD8^+$ central memory cells (B). Abbreviations: AUC, Area Under Curve.

6.4 Survival analysis

Based on the cut off selected using the ROC analysis, we evaluated patients' recurrence free survival. The Kaplan Meier curves for recurrence free survival are presented in ***Figure 9***. Our results show a worse survival for patients with a percentage of NK cells ≥ 27.5% (p=0.040) and in patients with percentage of $CD8^+$ central memory cells ≥ 9.2% (p=0.021).

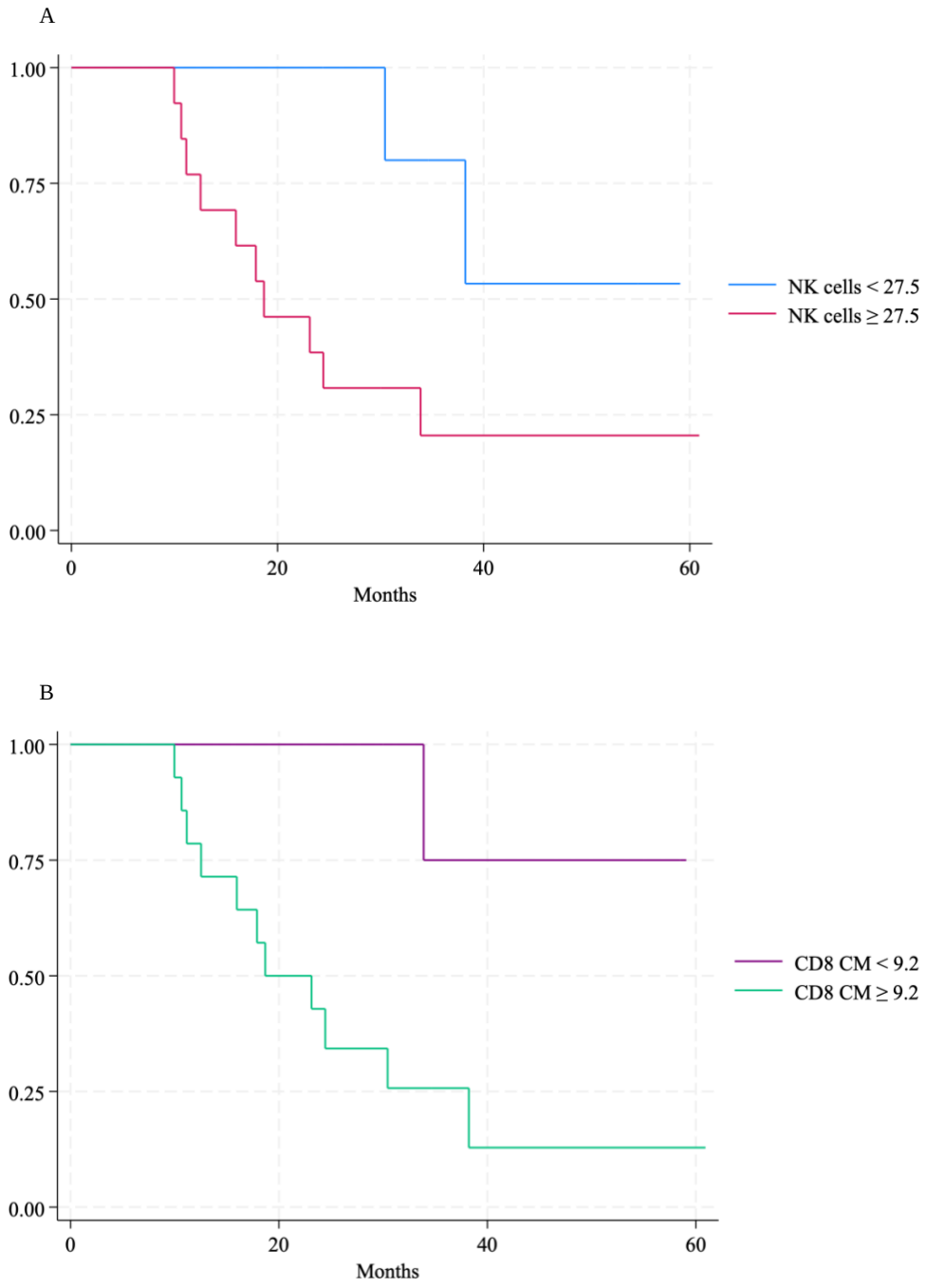

Figure 9: Patients' recurrence free survival curve according to NK cells (A) and $CD8^+$ central memory cells (B) cut-offs. $p<0.05$

6.5 Differences in timepoints

Later, we evaluated the differences between the two timepoints, i.e. baseline and recurrence and follow-up in patients with and without recurrence, respectively. Median, interquartile range and *p*-values for recurrence and the recurrence-free group are presented in ***Table 3A*** and ***Table 4A*** in appendix, respectively. At the time of recurrence we reported increased levels of $CD8^+$

activated (median 22.1% vs 10.9%), $CD8^+$ $CD186^+$ (median 29.6% vs 18.6%), $CD8^+$ $CD199^+$ (median 70.1% vs 52%) and $CD8^+$ $CX3CR1^+$ (median 40.4% vs 23.9%) cells (***Figure 10***) as well as $CLEC4D^+$ (median 60.6% vs 48.4%), NK $CD199^+$ (median 93.9% vs 89.6%), $CD4^+$ $CD186^+$ (median 33% vs 25.4%), $CD4^+$ $CD199^+$ (median 68.3% vs 48.9%), $CD4^+$ $CX3CR1^+$ (median 44.2% vs 25.9%) and $CD4^+$ TEMRA (median 8.1% vs 5.6%) (***Figure 11***).

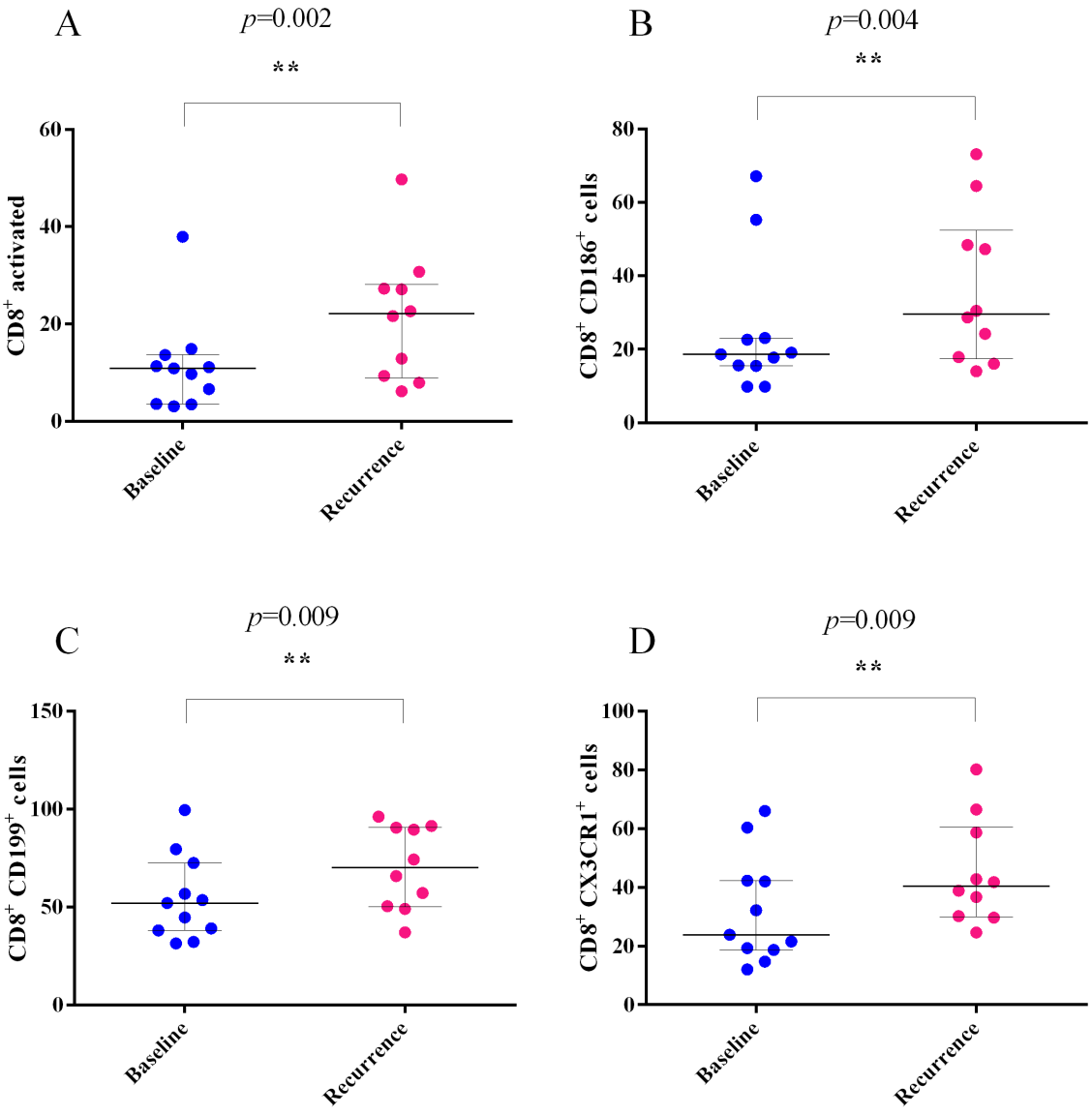

Figure 10: Percentage of CD8 activated (A), $CD8^+$ $CD186^+$ (B), $CD8^+$ $CD199^+$ (C) and $CD8^+$ $CX3CR1^+$ (D) cells in patients at baseline and at the time of recurrence. Data are presented as median and interquartile range [IQR].

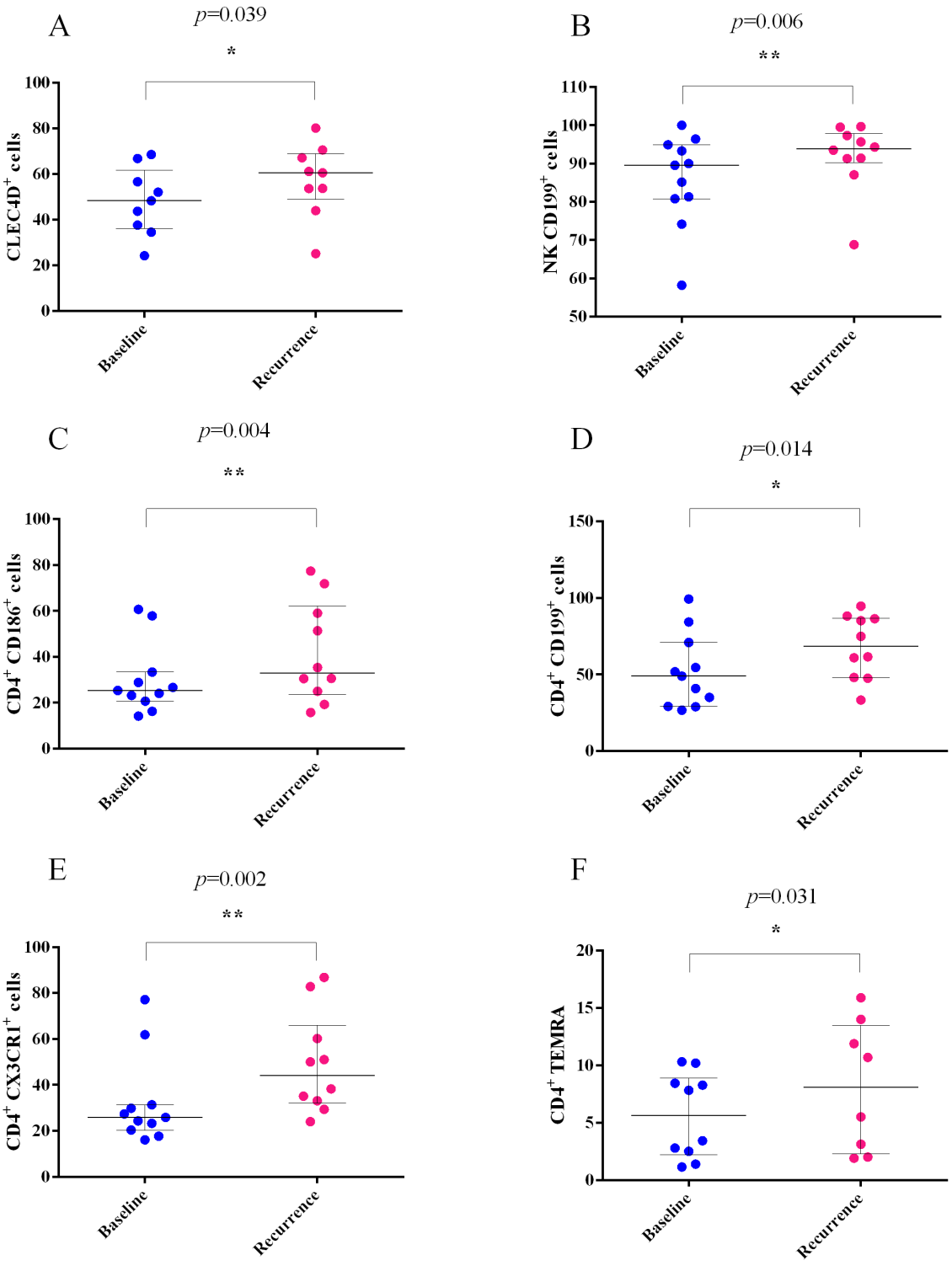

Figure 11: Percentage of CLEC4D$^+$ (A), NK CD199$^+$ (B), CD4$^+$ CD186$^+$ (C), CD4$^+$ CD199$^+$ (D), CD4$^+$ CX3CR1$^+$ (E) and CD4$^+$ TEMRA cells in patients at baseline and at the time of recurrence. Data are presented as median and interquartile range [IQR].

Additionally, we identified slightly elevated levels of $CD4^+$ $CD26^+$ cells (median 68.4% vs 66.7%) in patients who did not develop recurrence at 1 year after tumour resection, as presented in ***Figure 12***.

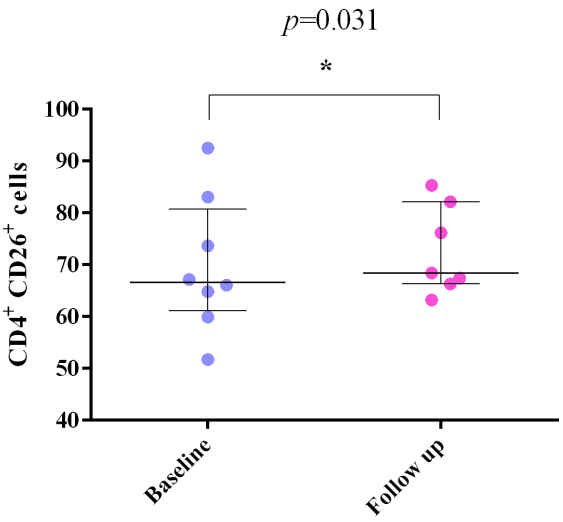

Figure 12: Levels of $CD4^+$ $CD26^+$ cells in recurrence-free group at baseline and 1 year after tumour resection. Data are presented as median and interquartile range [IQR].

6.6 Correlation with clinical scores

Finally, we evaluated the relationship between the cellular populations under study and clinical parameters. We found that ALBI score shows a positive correlation with T helper cells, T cytotoxic SLECs and PD1$^-$ TIGIT$^-$ cytotoxic T cells and a negative correlation with CD8$^+$ TEMRA, as presented in ***Figure 13***.

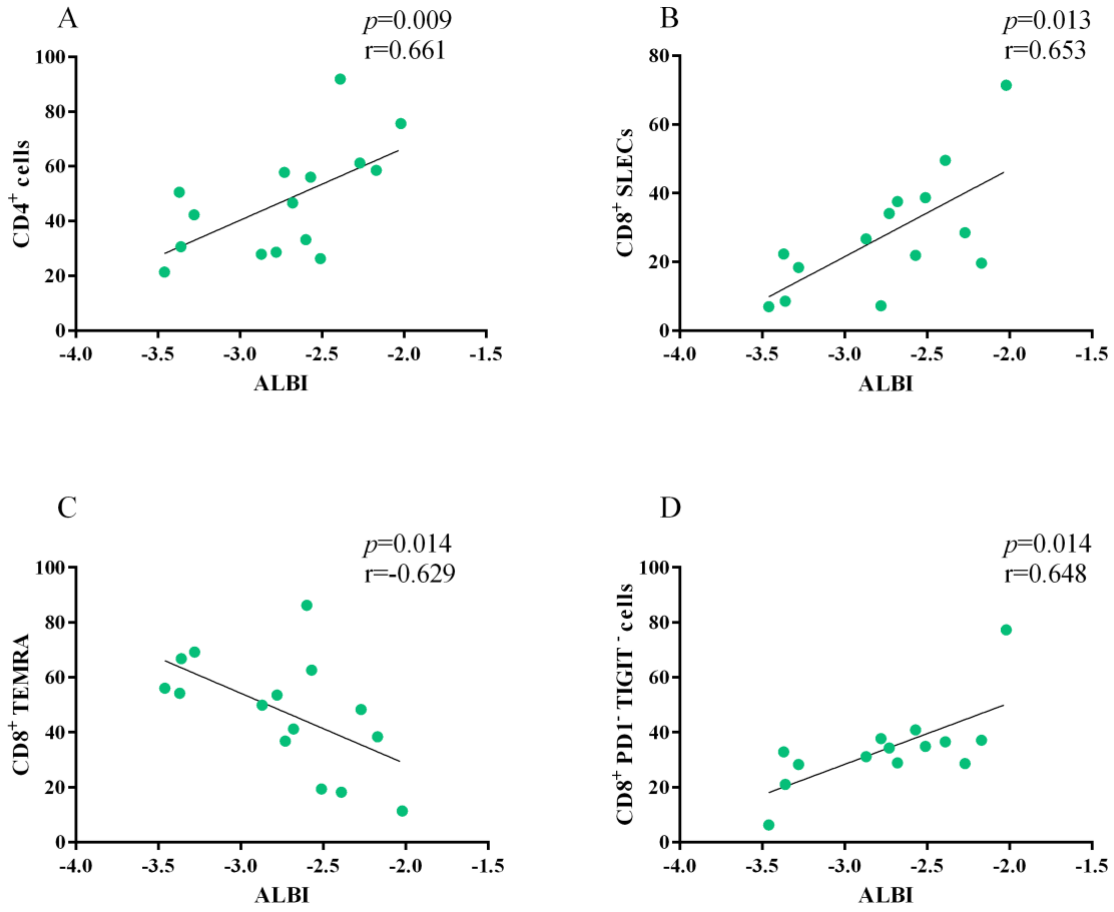

Figure 13: Correlation between ALBI score and CD4$^+$ (A), CD8$^+$ SLECs (B), CD8$^+$ TEMRA (C) and CD8$^+$ PD1$^-$ TIGIT$^-$ (D) cells.

Moreover, NLR results to be negatively correlated with NK cells and positively correlated with CD8$^+$ CD279$^+$ cells and CD8$^+$ PD1$^+$ TIGIT$^+$ cells, as reported in ***Figure 14***.

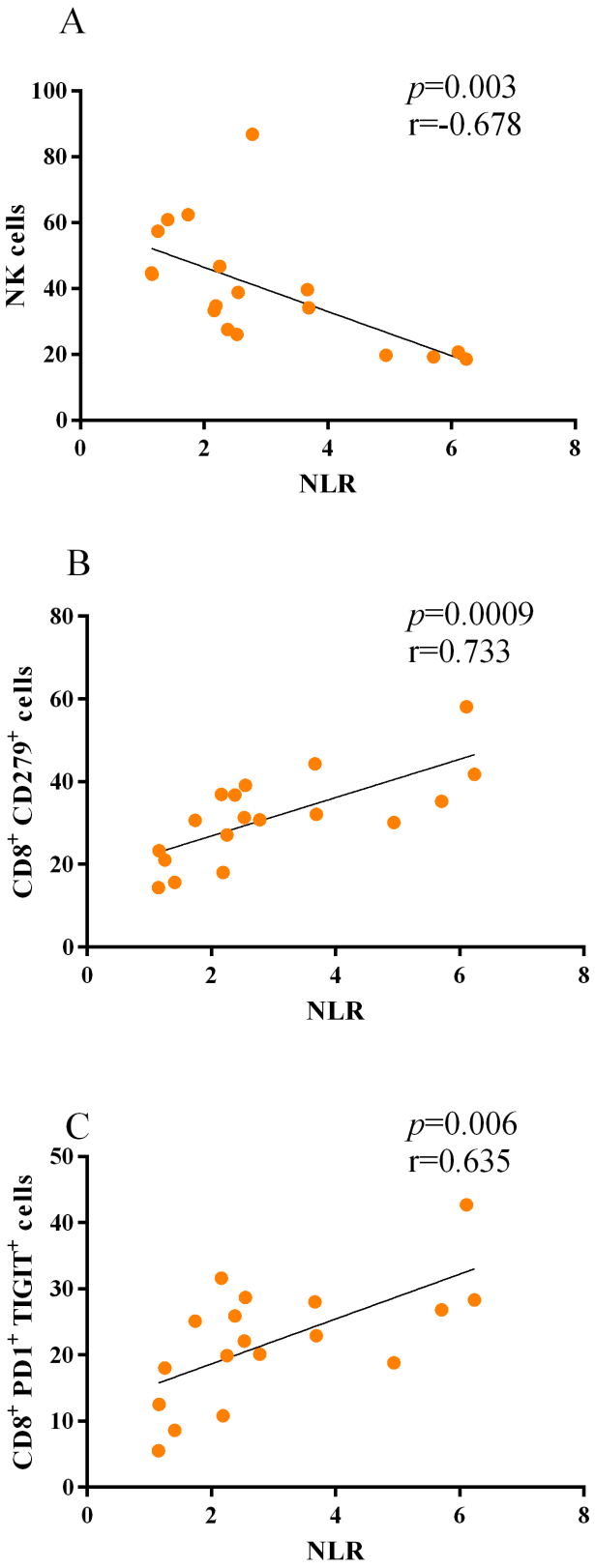

Figure 14: Correlation plot of NLR with NK cells (A), $CD8^+$ $CD279^+$ cells (B) and $CD8^+$ $PD1^+$ $TIGIT^+$ cells (C).

In addition, PLR score seems to correlate negatively with NK cells and positively with $CD8^+$ $CD279^+$ cells, as shown in ***Figure 15***.

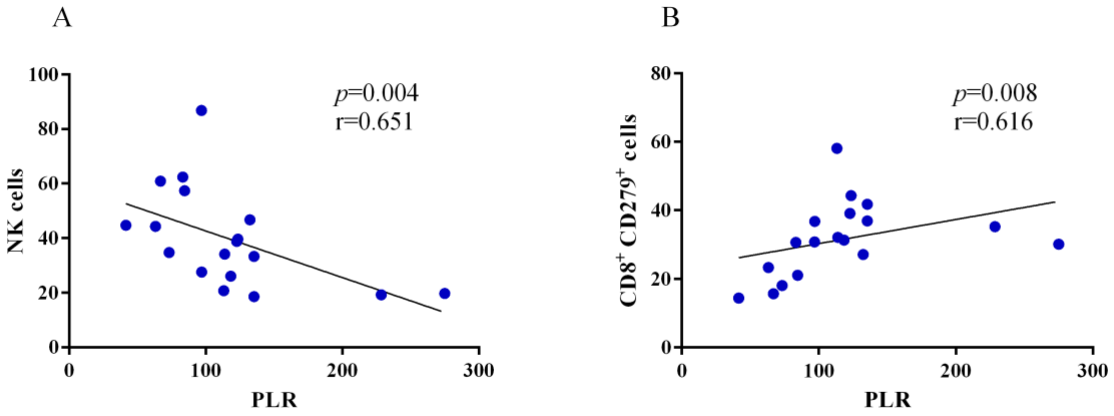

Figure 15: Correlation plot of PLR with NK cells (A) and $CD8^+$ $CD279^+$ cells (B).

7. DISCUSSION

Despite the advances in curative treatments of HCC, recurrence remains a major issue. Since the immunological mechanisms underlying tumour recurrence are not fully understood, the aim of this study was to evaluate the peripheral immune landscape of resected patients to identify a possible association with recurrence.

In this study, 19 patients with HCC of different aetiologies, including HCV (57.9%), HBV (26%) and alcohol (31.6%), were analysed. Cirrhosis was present in 73.7% of the population. In addition, we reported the presence of splenomegaly (15.8%) and oesophageal varices (31.6%), known consequences of portal hypertension [85]. All patients were eligible for surgical resection, since they belonged to BCLC stage 0 (36.8%), stage A (57.9%) and stage B (5.3%). After surgical resection, recurrence occurred in 57.9% of cases with a median of 17.9 months.

First, we compared the baseline levels of cellular subpopulations in the recurrence and in the recurrence-free group. We found increased levels of NK cells and $CD8^+$ central memory (CM) cells in the recurrence group compared to the recurrence-free group, while $CD8^+$ effector memory (EM) levels were higher in the recurrence-free group. Based on these results, we defined the optimal cut-off of NK cells and $CD8^+$ CM cells to identify recurrence. Our results show that NK cells $\geq$ 27.5% have 90.9% sensitivity and 62.5% specificity, while $CD8^+$ CM cells $\geq$ 9.2% have 87.5% sensitivity and 80% specificity in identifying recurrence. $CD8^+$ EM cells did not show to be optimal predictors of recurrence. Additionally, NK cells $\geq$ 27.5% and $CD8^+$ CM $\geq$ 9.2 predict worse recurrence free survival.

In literature, it has been previously reported that the proportion of NK cells before HCC ablation was associated with tumour recurrence, even if the levels' differences in the recurrence and recurrence-free group have not been described [93]. Moreover, it has been demonstrated that NK cells are associated with worse recurrence free survival in HCC patients, independently on the levels [94]. NK cells predominantly reside in the liver and are lower in peripheral blood. Studies in mice reported that liver NK cells exert a higher cytotoxic activity against tumour cells than their peripheral counterpart [95]. Indeed, Lee et al. observed that, even if high levels of NK cells were present in HCC patients at advanced stages, the proportion of cytotoxic NK cells was significantly lower, indicating that circulating NK cells lose their function during the progression of HCC [96].

After the effector response, memory T cells persist in the body as a heterogeneous population, which include central memory (T_{CM}) T cells and effector memory (T_{EM}) T cells. T_{CM} cells reside in the lymph nodes and have high proliferative capacity. On the contrary, T_{EM} cells are excluded by lymph nodes and express molecules important for their localization into the inflamed tissue [97]. Indeed, T_{EM} cells express higher quantity of receptors involved in tissue migration, thus exerting a more potent immediate antitumor response compared to T_{CM} cells [98]. Moreover, studies have evidenced that elevated blood levels of T_{EM} and T_{CM} cells are positive predictors of response to anti-PD1 or anti-CTLA-4 immunotherapy in melanoma [99]. Therefore, in our study, the elevated levels of T_{EM} found in peripheral blood of patients in the recurrence-free group may indicate the presence of a strong immune response, which counteract the development of recurrence.

In our study, $CD8^+$ central memory were elevated in the recurrence group. Literature widely describes the role of central memory T cells in protracted anti-tumour effect, particularly in response to immune checkpoint inhibitors. However, our population is characterized by a reduced tumour burden compared to patients eligible for systemic therapy, therefore the immune landscape of these patients may be different. Moreover, the interplay between NK cells and T_{CM} may create an unfavourable environment. These findings may suggest an implication of persistent inflammation in the development of recurrence. Indeed, it is well known that sustained inflammation induces the release of growth factors and survival factors promoting tumour growth, angiogenesis, as well as dissemination of neoplastic cells and metastasis [100]. However, resection may not provide a complete removal of all tumour cells. In this context, persistent antigen exposure, may reduce memory T cells' function, inducing non-responsiveness to the tumour [101]. Moreover, even NK cells' function depends on activating and inhibitory receptors [102]. Despite the elevated levels of these cellular subpopulations found in our patients, a dysfunctional phenotype may develop overtime, inducing an unbalance of the anti-tumour response. In this context, the increased inflammatory status and decreased anti-tumour response, may be involved in the development of recurrence. Further studies should be performed to evaluate the activation status of peripheral NK cells and central memory T cells, as well as their possible implication in recurrence.

Later, we analysed the differences between cellular subpopulations' levels at baseline (time of surgery) and at the time of recurrence, in the recurrence group, or at 1 year, in the recurrence-free group. According to our data, patients in the recurrence group show significant increase in

the levels of $CD8^+$ activated, $CD8^+$ $CD186^+$, $CD8^+$ $CD199^+$, $CD8^+$ $CX3CR1^+$, $CLEC4D^+$, NK $CD199^+$, $CD4^+$ $CD186^+$, $CD4^+$ $CD199^+$, $CD4^+$ $CX3CR1^+$, $CD4^+$ TEMRA cells at the time of recurrence compared to baseline. These cellular populations regulate innate or adaptive antineoplastic response. Indeed, expression of CXCR6 (CD186) and CX3CR1 on effector $CD8^+$ T cells has been reported to mediate migration on the site of inflammation, thus inducing an antitumor immune response [77,103]. Moreover, it has been reported that CD186 expression is associated with lymphocyte tissue infiltration during liver inflammation [104]. Another chemokine receptor, CCR9 (CD199), regulate T cell development [105], leukocytes activity and secretion of pro-inflammatory cytokines [106]. It has been denoted that $CD199^+$ T cells display an activated phenotype, which may evidence a stronger anti-tumour immune response after infiltration in the tumour microenviornment [107]. Indeed, it has been reported that hepatic aberrant expression of gut specific CCL25 may induce recruitment of $CCR9^+$ cells in the liver [108,109], thus activating an anti-tumour immune response. Our findings suggest that a wide panel of antitumoral immune cells are activated at the time of recurrence. The subsets evidenced by our analysis are mainly infiltrating cells, which can be recruited to tumour site and may induce an anti-tumour response. Moreover, effector memory T cells re-expressing CD45RA (TEMRA) are terminally differentiated cells associated with protracted antigen exposure, involved in immune senescence [110]. These cells exert a reduced proliferative activity, due to expression of senescent markers including PD1 [111]. The presence of elevated $CD4^+$ TEMRA with an exhausted phenotype, at the time of recurrence, may suggest a possible use of immune checkpoint inhibitors for the treatment of these patients.

In addition, we identified slightly elevated levels of $CD4^+$ $CD26^+$ cells one year after tumour resection in the recurrence-free group. Studies have established that the expression of CD26 is restricted to $CD4^+$ memory/helper population [112] and they can secrete effector cytokines and cytotoxic molecules [113]. In metastatic melanoma patients, a low percentage of peripheral $CD4^+$ $CD26^+$ cells has been associated with worse prognosis and survival [113]. Therefore, this result may evidence a protective immune phenotype 1 year after resection in patients that did not develop recurrence.

We also evaluated inflammatory scores that in literature have been associated with worse prognosis. We found a positive correlation between NLR and exhausted T cell phenotype, represented by $CD8^+$ $CD279^+$ (p=0.0009) and $CD8^+$ $PD1^+$ $TIGIT^+$ (p=0.006), and a negative correlation with NK cells (p=0.003). NLR is an inflammatory index widely evaluated as tumour

marker. It has been reported that elevated pre-operative levels of NLR in HCC relate to poor prognosis in terms of overall survival and disease-free survival after tumour resection [67]. Moreover, $CD8^+$ $CD279^+$ cells positively correlated with PLR (p=0.008), another marker of inflammation. Our results may indicate a possible link between exhausted phenotype, NLR and PLR. Therefore, the identification of recurrence risk factors, including elevated inflammation, may be helpful for the choice of the correct therapeutic approach.

Finally, we found that baseline ALBI score was positively correlated with $CD4^+$ cells, $CD8^+$ SLECs and $CD8^+$ $PD1^-$ $TIGIT^-$ cells, as well as negatively correlated with $CD8^+$ TEMRA. ALBI score is an indicator of liver function, and several studies identified its worth in OS prediction of surgically resected patients, both at baseline and post-operative [114,115]. Our results may suggest an involvement of inflammatory cells in liver dysfunction, identified by ALBI score. It has been demonstrated that the presence of chronic inflammation induces immune-related hepatotoxicity in patients treated with immune checkpoint inhibitors [116]. In cirrhosis, DAMPs released by damaged hepatocytes are sensed by immune cells, which produce pro-inflammatory cytokines, leading to activation of systemic inflammation. The immune response changes accordingly to the cirrhosis state (compensated or decompensated) and the extent of liver injury [117]. In very late stages of cirrhosis, i.e. acute-on-chronic liver failure, elevated systemic inflammation has been found in concomitance with profound immune suppression [118]. In acute decompensated cirrhosis, Khamri et.al identified a suppressive subtype of $CD4^+$ cells expressing HLA-G, which correlated positively with Child-Pugh and MELD score indicators of liver function [119]. In this study, we did not evaluate whether these cellular subpopulations were properly functioning. Therefore, we can only speculate that, in the context of tumour-induced immune dysregulation, activated immune subtypes may acquire a suppressive phenotype, that could induce liver damage, reflected by ALBI score. The evaluation of the immune landscape in concomitance with ALBI score may underline the functional status of the liver, thus exerting a prognostic function and providing indications on the correct therapeutic approaches.

In conclusion, our results evidence that elevated NK cells and $CD8^+$ CM cells values at baseline may be predictive of tumour recurrence. The heterogeneous immune landscape at time of recurrence, suggests the presence of an active immune response as well as simultaneous exhaustion of effector memory cells. These findings may influence the selection of the therapeutic approach for these patients with recurrence. Additionally, we evidenced a

relationship between exhausted phenotype, NLR and PLR. Finally, we identified a correlation between the peripheral immune landscape and liver functionality.

This study presents some limitations. Firstly, the small number of patients enrolled does not allow to draw conclusive results and these preliminary data should be validated in larger cohorts. Moreover, they should be validated in independent datasets. Additionally, some samples have been removed due the low number of PMBCs, reducing the number of available samples for final analysis. Despite these limitations, this study explores an interesting field that may be helpful for the management of tumour recurrence. These preliminary results may open a window for the evaluation of specific cellular subpopulations as predictive markers of recurrence.

In the future we would like to apply this knowledge to a wider population, analysing different timepoints to evaluate the trend of these cellular subtypes during time. Moreover, we could evaluate whether different aetiologies harbour the same pattern of inflammation, in order to understand the best therapeutic approach. In this study, we evaluated only the phenotype of peripheral cells, while it could be interesting to study their function in the context of tumour recurrence. Additionally, we could match peripheral blood with tumour tissue to understand the possible presence of a phenotype indicator of recurrence. Further studies should be performed to better comprehend the intricate network that composes HCC immune landscape. The evaluation of tumour and peripheral immune subsets may provide a deeper understanding of the mechanisms underlaying progression and recurrence, as well as improve the therapeutic strategies for HCC.

8. BIBLIOGRAPHY

1. Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., and Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin *71*. https://doi.org/10.3322/caac.21660.
2. Toh, M.R., Wong, E.Y.T., Wong, S.H., Ng, A.W.T., Loo, L.H., Chow, P.K.H., and Ngeow, J. (2023). Global Epidemiology and Genetics of Hepatocellular Carcinoma. Gastroenterology *164*. https://doi.org/10.1053/j.gastro.2023.01.033.
3. Llovet, J.M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.-F., de Oliveira, A.C., Santoro, A., Raoul, J.-L., Forner, A., et al. (2008). Sorafenib in Advanced Hepatocellular Carcinoma. New England Journal of Medicine *359*. https://doi.org/10.1056/nejmoa0708857.
4. Rumgay, H., Ferlay, J., de Martel, C., Georges, D., Ibrahim, A.S., Zheng, R., Wei, W., Lemmens, V.E.P.P., and Soerjomataram, I. (2022). Global, regional and national burden of primary liver cancer by subtype. Eur J Cancer *161*. https://doi.org/10.1016/j.ejca.2021.11.023.
5. Vogel, A., Meyer, T., Sapisochin, G., Salem, R., and Saborowski, A. (2022). Hepatocellular carcinoma. The Lancet *400*, 1345–1362. https://doi.org/10.1016/S0140-6736(22)01200-4.
6. Yeh, S.H., Li, C.L., Lin, Y.Y., Ho, M.C., Wang, Y.C., Tseng, S.T., and Chen, P.J. (2023). Hepatitis B Virus DNA Integration Drives Carcinogenesis and Provides a New Biomarker for HBV-related HCC. https://doi.org/10.1016/j.jcmgh.2023.01.001.
7. Shen, C., Jiang, X., Li, M., and Luo, Y. (2023). Hepatitis Virus and Hepatocellular Carcinoma: Recent Advances. https://doi.org/10.3390/cancers15020533.
8. Khatun, M., and Ray, R.B. (2019). Mechanisms underlying hepatitis C virus-associated hepatic fibrosis. https://doi.org/10.3390/cells8101249.
9. Ninio, L., Nissani, A., Meirson, T., Domovitz, T., Genna, A., Twafra, S., Srikanth, K.D., Dabour, R., Avraham, E., Davidovich, A., et al. (2019). Hepatitis C virus enhances the invasiveness of hepatocellular carcinoma via EGFR-mediated invadopodia formation and activation. Cells *8*. https://doi.org/10.3390/cells8111395.
10. Huang, D.Q., Mathurin, P., Cortez-Pinto, H., and Loomba, R. (2023). Global epidemiology of alcohol-associated cirrhosis and HCC: trends, projections and risk factors. https://doi.org/10.1038/s41575-022-00688-6.
11. Rinella, M.E., Lazarus, J. V., Ratziu, V., Francque, S.M., Sanyal, A.J., Kanwal, F., Romero, D., Abdelmalek, M.F., Anstee, Q.M., Arab, J.P., et al. (2023). A multisociety Delphi consensus statement on new fatty liver disease nomenclature. Hepatology *78*. https://doi.org/10.1097/HEP.0000000000000520.
12. Vitale, A., Svegliati-Baroni, G., Ortolani, A., Cucco, M., Riva, G.V.D., Giannini, E.G., Piscaglia, F., Rapaccini, G., Di Marco, M., Caturelli, E., et al. (2023). Epidemiological trends

and trajectories of MAFLD-associated hepatocellular carcinoma 2002-2033: the ITA.LI.CA database. Gut *72*, 141–152. https://doi.org/10.1136/gutjnl-2021-324915.

13. Llovet, J.M., Pinyol, R., Kelley, R.K., El-Khoueiry, A., Reeves, H.L., Wang, X.W., Gores, G.J., and Villanueva, A. (2022). Molecular pathogenesis and systemic therapies for hepatocellular carcinoma. https://doi.org/10.1038/s43018-022-00357-2.
14. Tümen, D., Heumann, P., Gülow, K., Demirci, C.N., Cosma, L.S., Müller, M., and Kandulski, A. (2022). Pathogenesis and Current Treatment Strategies of Hepatocellular Carcinoma. https://doi.org/10.3390/biomedicines10123202.
15. Dhanasekaran, R., Nault, J.C., Roberts, L.R., and Zucman-Rossi, J. (2019). Genomic Medicine and Implications for Hepatocellular Carcinoma Prevention and Therapy. Gastroenterology *156*, 492–509. https://doi.org/10.1053/j.gastro.2018.11.001.
16. Xu, C., Xu, Z., Zhang, Y., Evert, M., Calvisi, D.F., and Chen, X. (2022). β-Catenin signaling in hepatocellular carcinoma. https://doi.org/10.1172/JCI154515.
17. Gajos-Michniewicz, A., and Czyz, M. (2024). WNT/β-catenin signaling in hepatocellular carcinoma: The aberrant activation, pathogenic roles, and therapeutic opportunities. https://doi.org/10.1016/j.gendis.2023.02.050.
18. Krutsenko, Y., Singhi, A.D., and Monga, S.P. (2021). β-Catenin Activation in Hepatocellular Cancer: Implications in Biology and Therapy. https://doi.org/10.3390/cancers13081830.
19. Zhu, G., Pan, C., Bei, J.X., Li, B., Liang, C., Xu, Y., and Fu, X. (2020). Mutant p53 in Cancer Progression and Targeted Therapies. https://doi.org/10.3389/fonc.2020.595187.
20. Schulze, K., Nault, J.C., and Villanueva, A. (2016). Genetic profiling of hepatocellular carcinoma using next-generation sequencing. https://doi.org/10.1016/j.jhep.2016.05.035.
21. Yang, C., Huang, X., Li, Y., Chen, J., Lv, Y., and Dai, S. (2021). Prognosis and personalized treatment prediction in TP53-mutant hepatocellular carcinoma: An in silico strategy towards precision oncology. Brief Bioinform *22*. https://doi.org/10.1093/bib/bbaa164.
22. Long, J., Wang, A., Bai, Y., Lin, J., Yang, X., Wang, D., Yang, X., Jiang, Y., and Zhao, H. (2019). Development and validation of a TP53-associated immune prognostic model for hepatocellular carcinoma. EBioMedicine *42*, 363–374. https://doi.org/10.1016/j.ebiom.2019.03.022.
23. Zhou, Y., Cui, G., Xu, H., Chun, J., Yang, D., Zhang, Z., Yang, L., Wang, J., Wan, M., Calvisi, D.F., et al. (2023). Loss of TP53 cooperates with c-MET overexpression to drive hepatocarcinogenesis. Cell Death Dis *14*, 476. https://doi.org/10.1038/s41419-023-05958-y.
24. Giordano, S., and Columbano, A. (2014). Met as a therapeutic target in HCC: Facts and hopes. https://doi.org/10.1016/j.jhep.2013.09.009.
25. Sia, D., Villanueva, A., Friedman, S.L., and Llovet, J.M. (2017). Liver Cancer Cell of Origin, Molecular Class, and Effects on Patient Prognosis. https://doi.org/10.1053/j.gastro.2016.11.048.

26. Désert, R., Nieto, N., and Musso, O. (2018). Dimensions of hepatocellular carcinoma phenotypic diversity. https://doi.org/10.3748/wjg.v24.i40.4536.
27. Lachenmayer, A., Alsinet, C., Savic, R., Cabellos, L., Toffanin, S., Hoshida, Y., Villanueva, A., Minguez, B., Newell, P., Tsai, H.W., et al. (2012). Wnt-pathway activation in two molecular classes of hepatocellular carcinoma and experimental modulation by sorafenib. Clinical Cancer Research *18*, 4997–5007. https://doi.org/10.1158/1078-0432.CCR-11-2322.
28. Llovet, J.M., Montal, R., Sia, D., and Finn, R.S. (2018). Molecular therapies and precision medicine for hepatocellular carcinoma. https://doi.org/10.1038/s41571-018-0073-4.
29. Galle, P.R., Forner, A., Llovet, J.M., Mazzaferro, V., Piscaglia, F., Raoul, J.L., Schirmacher, P., and Vilgrain, V. (2018). EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J Hepatol *69*, 182–236. https://doi.org/10.1016/j.jhep.2018.03.019.
30. Vogel, A., Meyer, T., Sapisochin, G., Salem, R., and Saborowski, A. (2022). Hepatocellular carcinoma. The Lancet *400*, 1345–1362. https://doi.org/10.1016/S0140-6736(22)01200-4.
31. Reig, M., Forner, A., Rimola, J., Ferrer-Fàbrega, J., Burrel, M., Garcia-Criado, Á., Kelley, R.K., Galle, P.R., Mazzaferro, V., Salem, R., et al. (2022). BCLC strategy for prognosis prediction and treatment recommendation: The 2022 update. J Hepatol *76*, 681–693. https://doi.org/10.1016/j.jhep.2021.11.018.
32. Singal, A.G., Kudo, M., and Bruix, J. (2023). Breakthroughs in Hepatocellular Carcinoma Therapies. Clinical Gastroenterology and Hepatology *21*, 2135–2149. https://doi.org/10.1016/j.cgh.2023.01.039.
33. Roayaie, S., Obeidat, K., Sposito, C., Mariani, L., Bhoori, S., Pellegrinelli, A., Labow, D., Llovet, J.M., Schwartz, M., and Mazzaferro, V. (2013). Resection of hepatocellular cancer ≤2 cm: Results from two Western centers. Hepatology *57*, 1426–1435. https://doi.org/10.1002/hep.25832.
34. Jung, S.M., Kim, J.M., Choi, G.S., Kwon, C.H.D., Yi, N.J., Lee, K.W., Suh, K.S., and Joh, J.W. (2019). Characteristics of Early Recurrence After Curative Liver Resection for Solitary Hepatocellular Carcinoma. Journal of Gastrointestinal Surgery *23*, 304–311. https://doi.org/10.1007/s11605-018-3927-2.
35. Nevola, R., Ruocco, R., Criscuolo, L., Villani, A., Alfano, M., Beccia, D., Imbriani, S., Claar, E., Cozzolino, D., Sasso, F.C., et al. (2023). Predictors of early and late hepatocellular carcinoma recurrence. https://doi.org/10.3748/wjg.v29.i8.1243.
36. Wu, J., Chan, Y.T., Lu, Y., Wang, N., and Feng, Y. (2023). The tumor microenvironment in the postsurgical liver: Mechanisms and potential targets of postoperative recurrence in human hepatocellular carcinoma. Med Res Rev *43*, 1946–1973. https://doi.org/10.1002/med.21967.
37. Ajamieh, H., Merino, N., Candelario-Jalil, E., Menéndez, S., Martinez-Sanchez, G., Re, L., Giuliani, A., and Leon, O.S. (2002). Similar protective effect of ischaemic and ozone oxidative

preconditionings in liver ischaemia/reperfusion injury. Pharmacol Res *45*, 333–339. https://doi.org/10.1006/phrs.2002.0952.

38. Senoner, T., Schindler, S., Stättner, S., Öfner, D., Troppmair, J., and Primavesi, F. (2019). Associations of Oxidative Stress and Postoperative Outcome in Liver Surgery with an Outlook to Future Potential Therapeutic Options. Oxid Med Cell Longev *2019*. https://doi.org/10.1155/2019/3950818.
39. Kaltenmeier, C., Wang, R., Popp, B., Geller, D., Tohme, S., and Yazdani, H.O. (2022). Role of Immuno-Inflammatory Signals in Liver Ischemia-Reperfusion Injury. Cells *11*, 2222. https://doi.org/10.3390/cells11142222.
40. Tang, F., Tie, Y., Tu, C., and Wei, X. (2020). Surgical trauma-induced immunosuppression in cancer: Recent advances and the potential therapies. Clin Transl Med *10*, 199–223. https://doi.org/10.1002/ctm2.24.
41. Pang, L., Yeung, O.W.H., Ng, K.T.P., Liu, H., Zhu, J., Liu, J., Yang, X., Ding, T., Qiu, W., Wang, Y., et al. (2022). Postoperative Plasmacytoid Dendritic Cells Secrete IFNa to Promote Recruitment of Myeloid-Derived Suppressor Cells and Drive Hepatocellular Carcinoma Recurrence. Cancer Res *82*, 4206–4218. https://doi.org/10.1158/0008-5472.CAN-22-1199.
42. Tohme, S., Simmons, R.L., and Tsung, A. (2017). Surgery for cancer: A trigger for metastases. Cancer Res *77*, 1548–1552. https://doi.org/10.1158/0008-5472.CAN-16-1536.
43. Niland, S., Riscanevo, A.X., and Eble, J.A. (2022). Matrix metalloproteinases shape the tumor microenvironment in cancer progression. Int J Mol Sci *23*, 146. https://doi.org/10.3390/ijms23010146.
44. Zhou, Y., Wang, B., Wu, J., Zhang, C., Zhou, Y., Yang, X.R., Zhou, J., Guo, W., and Fan, J. (2016). Association of preoperative EpCAM Circulating Tumor Cells and peripheral Treg cell levels with early recurrence of hepatocellular carcinoma following radical hepatic resection. BMC Cancer *16*, 506. https://doi.org/10.1186/s12885-016-2526-4.
45. Devan, A.R., Nair, B., Aryan, M.K., Liju, V.B., Koshy, J.J., Mathew, B., Valsan, A., Kim, H., and Nath, L.R. (2023). Decoding Immune Signature to Detect the Risk for Early-Stage HCC Recurrence. Cancers (Basel) *15*, 2729. https://doi.org/10.3390/cancers15102729.
46. Birgani, M.T., and Carloni, V. (2017). Tumor microenvironment, a paradigm in hepatocellular carcinoma progression and therapy. Int J Mol Sci *18*, 405. https://doi.org/10.3390/ijms18020405.
47. Chen, C., Wang, Z., Ding, Y., and Qin, Y. (2023). Tumor microenvironment-mediated immune evasion in hepatocellular carcinoma. Front Immunol *14*. https://doi.org/10.3389/fimmu.2023.1133308.
48. de Visser, K.E., and Joyce, J.A. (2023). The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth. Cancer Cell *41*, 374–403. https://doi.org/10.1016/j.ccell.2023.02.016.

49. O'Donnell, J.S., Teng, M.W.L., and Smyth, M.J. (2019). Cancer immunoediting and resistance to T cell-based immunotherapy. Nat Rev Clin Oncol *16*, 151–167. https://doi.org/10.1038/s41571-018-0142-8.

50. Rabinovich, G.A., Gabrilovich, D., and Sotomayor, E.M. (2007). Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol *25*, 267–296. https://doi.org/10.1146/annurev.immunol.25.022106.141609.

51. Golubovskaya, V., and Wu, L. (2016). Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. Cancers (Basel) *8*, 36. https://doi.org/10.3390/cancers8030036.

52. Chen, D.S., and Mellman, I. (2017). Elements of cancer immunity and the cancer-immune set point. Nature *541*, 321–330. https://doi.org/10.1038/nature21349.

53. Curdy, N., Lanvin, O., Laurent, C., Fournié, J.J., and Franchini, D.M. (2019). Regulatory Mechanisms of Inhibitory Immune Checkpoint Receptors Expression. Trends Cell Biol *29*, 777–790. https://doi.org/10.1016/j.tcb.2019.07.002.

54. Mellman, I., Chen, D.S., Powles, T., and Turley, S.J. (2023). The cancer-immunity cycle: Indication, genotype, and immunotype. Immunity *56*, 2188–2205. https://doi.org/10.1016/j.immuni.2023.09.011.

55. Bian, J., Lin, J., Long, J., Yang, X., Yang, X., Lu, X., Sang, X., and Zhao, H. (2020). T lymphocytes in hepatocellular carcinoma immune microenvironment: insights into human immunology and immunotherapy. Am J Cancer Res *10*, 4585–4606.

56. Nguyen, P.H.D., Wasser, M., Tan, C.T., Lim, C.J., Lai, H.L.H., Seow, J.J.W., DasGupta, R., Phua, C.Z.J., Ma, S., Yang, J., et al. (2022). Trajectory of immune evasion and cancer progression in hepatocellular carcinoma. Nat Commun *13*, 1441. https://doi.org/10.1038/s41467-022-29122-w.

57. Shi, J., Liu, J., Tu, X., Li, B., Tong, Z., Wang, T., Zheng, Y., Shi, H., Zeng, X., Chen, W., et al. (2022). Single-cell immune signature for detecting early-stage HCC and early assessing anti-PD-1 immunotherapy efficacy. J Immunother Cancer *10*. https://doi.org/10.1136/jitc-2021-003133.

58. Curotto de Lafaille, M.A., and Lafaille, J.J. (2009). Natural and Adaptive Foxp3+ Regulatory T Cells: More of the Same or a Division of Labor? Immunity *30*, 626–635. https://doi.org/10.1016/j.immuni.2009.05.002.

59. Huang, Y., Liao, H., Zhang, Y., Yuan, R., Wang, F., Gao, Y., Wang, P., and Du, Z. (2014). Prognostic value of tumor-infiltrating FoxP3+ T cells in gastrointestinal cancers: A meta analysis. PLoS One *9*. https://doi.org/10.1371/journal.pone.0094376.

60. Lee, S.K., Lee, S.W., Jang, J.W., Bae, S.H., Choi, J.Y., and Yoon, S.K. (2021). Immunological markers, prognostic factors and challenges following curative treatments for hepatocellular carcinoma. Int J Mol Sci *22*, 10271. https://doi.org/10.3390/ijms221910271.

61. Fu, J., Xu, D., Liu, Z., Shi, M., Zhao, P., Fu, B., Zhang, Z., Yang, H., Zhang, H., Zhou, C., et al. (2007). Increased Regulatory T Cells Correlate With CD8 T-Cell Impairment and Poor Survival in Hepatocellular Carcinoma Patients. Gastroenterology *132*, 2328–2339. https://doi.org/10.1053/j.gastro.2007.03.102.

62. Huang, Y., Wang, F.M., Wang, T., Wang, Y.J., Zhu, Z.Y., Gao, Y.T., and Du, Z. (2013). Tumor-infiltrating FoxP3+ tregs and CD8+ T cells affect the prognosis of hepatocellular carcinoma patients. Digestion *86*, 329–337. https://doi.org/10.1159/000342801.

63. Kousis, P.C., Henderson, B.W., Maier, P.G., and Gollnick, S.O. (2007). Photodynamic therapy enhancement of antitumor immunity is regulated by neutrophils. Cancer Res *67*, 10501–10510. https://doi.org/10.1158/0008-5472.CAN-07-1778.

64. Tazzyman, S., Lewis, C.E., and Murdoch, C. (2009). Neutrophils: Key mediators of tumour angiogenesis. Int J Exp Pathol *90*, 222–231. https://doi.org/10.1111/j.1365-2613.2009.00641.x.

65. Li, Y.W., Qiu, S.J., Fan, J., Zhou, J., Gao, Q., Xiao, Y.S., and Xu, Y.F. (2011). Intratumoral neutrophils: A poor prognostic factor for hepatocellular carcinoma following resection. J Hepatol *54*, 497–505. https://doi.org/10.1016/j.jhep.2010.07.044.

66. Yu, Y., Song, J., Zhang, R., Liu, Z., Li, Q., Shi, Y., Chen, Y., and Chen, J. (2017). Preoperative neutrophil-to-lymphocyte ratio and tumor-related factors to predict microvascular invasion in patients with hepatocellular carcinoma. Oncotarget *8*, 79722–79730. https://doi.org/10.18632/oncotarget.19178.

67. Gomez, D., Farid, S., Malik, H.Z., Young, A.L., Toogood, G.J., Lodge, J.P.A., and Prasad, K.R. (2008). Preoperative neutrophil-to-lymphocyte ratio as a prognostic predictor after curative resection for hepatocellular carcinoma. World J Surg *32*. https://doi.org/10.1007/s00268-008-9552-6.

68. Yang, N., Ji, F., Cheng, L., Lu, J., Sun, X., Lin, X., and Lan, X. (2021). Knockout of immunotherapy prognostic marker genes eliminates the effect of the anti-PD-1 treatment. NPJ Precis Oncol *5*, 37. https://doi.org/10.1038/s41698-021-00175-2.

69. Zhang, Y., Wei, H., Fan, L., Fang, M., He, X., Lu, B., and Pang, Z. (2021). CLEC4s as Potential Therapeutic Targets in Hepatocellular Carcinoma Microenvironment. Front Cell Dev Biol *9*, 681372. https://doi.org/10.3389/fcell.2021.681372.

70. Zhang, Z., Sun, T., Chen, Y., Gong, S., Sun, X., Zou, F., and Peng, R. (2016). CCL25/CCR9 signal promotes migration and invasion in hepatocellular and breast cancer cell lines. DNA Cell Biol *35*, 348–357. https://doi.org/10.1089/dna.2015.3104.

71. Ran, G. he, Lin, Y. qing, Tian, L., Zhang, T., Yan, D. mei, Yu, J. hua, and Deng, Y. cai (2022). Natural killer cell homing and trafficking in tissues and tumors: from biology to application. Signal Transduct Target Ther *7*, 205. https://doi.org/10.1038/s41392-022-01058-z.

72. Berahovich, R.D., Lai, N.L., Wei, Z., Lanier, L.L., and Schall, T.J. (2006). Evidence for NK Cell Subsets Based on Chemokine Receptor Expression. The Journal of Immunology *177*, 7833–7840. https://doi.org/10.4049/jimmunol.177.11.7833.

73. Lu, L., Du, H., Huang, H., Wang, C., Wang, P., Zha, Z., Wu, Y., Liu, X., Weng, C., Fang, X., et al. (2020). CCR9 promotes migration and invasion of lung adenocarcinoma cancer stem cells. Int J Med Sci *17*, 912–920. https://doi.org/10.7150/ijms.40864.

74. Rogado, J., Pozo, F., Troule, K., Sánchez-Torres, J.M., Romero-Laorden, N., Mondejar, R., Donnay, O., Ballesteros, A., Pacheco-Barcia, V., Aspa, J., et al. (2022). Peripheral Blood Mononuclear Cells Predict Therapeutic Efficacy of Immunotherapy in NSCLC. Cancers (Basel) *14*, 2898. https://doi.org/10.3390/cancers14122898.

75. Han, X. (2014). Constitutively active chemokine CXC receptors. In Advances in Pharmacology, pp. 265–301. https://doi.org/10.1016/B978-0-12-417197-8.00009-2.

76. Ji, G., Ma, L., Yao, H., Ma, S., Si, X., Wang, Y., Bao, X., Ma, L., Chen, F., Ma, C., et al. (2020). Precise delivery of obeticholic acid via nanoapproach for triggering natural killer T cell-mediated liver cancer immunotherapy. Acta Pharm Sin B *10*, 2171–2182. https://doi.org/10.1016/j.apsb.2020.09.004.

77. Di Pilato, M., Kfuri-Rubens, R., Pruessmann, J.N., Ozga, A.J., Messemaker, M., Cadilha, B.L., Sivakumar, R., Cianciaruso, C., Warner, R.D., Marangoni, F., et al. (2021). CXCR6 positions cytotoxic T cells to receive critical survival signals in the tumor microenvironment. Cell *184*, 4512–4530. https://doi.org/10.1016/j.cell.2021.07.015.

78. Li, T., Pan, J., Chen, H., Fang, Y., and Sun, Y. (2022). CXCR6-based immunotherapy in autoimmune, cancer and inflammatory infliction. Acta Pharm Sin B *12*, 3255–3262. https://doi.org/10.1016/j.apsb.2022.03.012.

79. Gao, Q., Zhao, Y.J., Wang, X.Y., Qiu, S.J., Shi, Y.H., Sun, J., Yi, Y., Shi, J.Y., Shi, G.M., Ding, Z. Bin, et al. (2012). CXCR6 upregulation contributes to a proinflammatory tumor microenvironment that drives metastasis and poor patient outcomes in hepatocellular carcinoma. Cancer Res *72*, 3546–3556. https://doi.org/10.1158/0008-5472.CAN-11-4032.

80. Di Tommaso, L., Spadaccini, M., Donadon, M., Personeni, N., Elamin, A., Aghemo, A., and Lleo, A. (2019). Role of liver biopsy in hepatocellular carcinoma. World J Gastroenterol *25*, 6041–6052. https://doi.org/10.3748/wjg.v25.i40.6041.

81. Ye, Q., Ling, S., Zheng, S., and Xu, X. (2019). Liquid biopsy in hepatocellular carcinoma: Circulating tumor cells and circulating tumor DNA. Mol Cancer *18*, 114. https://doi.org/10.1186/s12943-019-1043-x.

82. Li, W., Liu, J. Bin, Hou, L.K., Yu, F., Zhang, J., Wu, W., Tang, X.M., Sun, F., Lu, H.M., Deng, J., et al. (2022). Liquid biopsy in lung cancer: significance in diagnostics, prediction, and treatment monitoring. Mol Cancer *21*, 25. https://doi.org/10.1186/s12943-022-01505-z.

83. Lone, S.N., Nisar, S., Masoodi, T., Singh, M., Rizwan, A., Hashem, S., El-Rifai, W., Bedognetti, D., Batra, S.K., Haris, M., et al. (2022). Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments. Mol Cancer *21*, 79. https://doi.org/10.1186/s12943-022-01543-7.

84. Howell, J., Atkinson, S.R., Pinato, D.J., Knapp, S., Ward, C., Minisini, R., Burlone, M.E., Leutner, M., Pirisi, M., Büttner, R., et al. (2019). Identification of mutations in circulating cell-free tumour DNA as a biomarker in hepatocellular carcinoma. Eur J Cancer *116*. https://doi.org/10.1016/j.ejca.2019.04.014.

85. Buob, S., Johnston, A.N., and Webster, C.R.L. (2011). Portal Hypertension: Pathophysiology, Diagnosis, and Treatment. J Vet Intern Med *25*, 169–186. https://doi.org/10.1111/j.1939-1676.2011.00691.x.

86. Ruf, A., Dirchwolf, M., and Freeman, R.B. (2022). From Child-Pugh to MELD score and beyond: Taking a walk down memory lane. Ann Hepatol *27*, 100535. https://doi.org/10.1016/j.aohep.2021.100535.

87. Kamath, P.S., Wiesner, R.H., Malinchoc, M., Kremers, W.K., Therneau, T.M., D'Amico, G., Kosberg, C.L., Dickson, E.R., and Kim, W.R. (2001). A model to predict survival in patients with end-stage liver disease. Gastroenterology *120*, 464–470. https://doi.org/10.1016/s0016-5085(08)80377-0.

88. Toyoda, H., and Johnson, P.J. (2022). The ALBI score: From liver function in patients with HCC to a general measure of liver function. JHEP Reports *4*, 100557. https://doi.org/10.1016/j.jhepr.2022.100557.

89. Wang, D., Bai, N., Hu, X., OuYang, X.W., Yao, L., Tao, Y.M., and Wang, Z.M. (2019). Preoperative inflammatory markers of NLR and PLR as indicators of poor prognosis in resectable HCC. PeerJ *2019*. https://doi.org/10.7717/peerj.7132.

90. Simcock, R., and Wright, J. (2020). Beyond Performance Status. Clin Oncol *32*, 553–561. https://doi.org/10.1016/j.clon.2020.06.016.

91. Zhou, Z., Cao, S., Chen, C., Chen, J., Xu, X., Liu, Y., Liu, Q., Wang, K., Han, B., and Yin, Y. (2023). A Novel Nomogram for the Preoperative Prediction of Edmondson-Steiner Grade III-IV in Hepatocellular Carcinoma Patients. J Hepatocell Carcinoma *10*, 1399–1409. https://doi.org/10.2147/JHC.S417878.

92. Lee, K.F., Wong, J., Cheung, Y.S., Ip, P., Wong, J., and Lai, P.B.S. (2010). Resection margin in laparoscopic hepatectomy: A comparative study between wedge resection and anatomic left lateral sectionectomy. HPB *12*, 649–653. https://doi.org/10.1111/j.1477-2574.2010.00221.x.

93. Wang, H.Y., Cui, X.W., Zhang, Y.H., Chen, Y., Lu, N.N., Bai, L., and Duan, Z.P. (2023). Dynamic changes of phenotype and function of natural killer cells in peripheral blood before and after thermal ablation of hepatitis B associated hepatocellular carcinoma and their

correlation with tumor recurrence. BMC Cancer *23*, 486. https://doi.org/10.1186/s12885-023-10823-4.

94. Zhou, C., Lu, Z., Sun, B., Yi, Y., Zhang, B., Wang, Z., and Qiu, S.J. (2024). Peripheral Lymphocytes in Primary Liver Cancers: Elevated NK and CD8+ T Cells and Dysregulated Selenium Metabolism. Biomolecules *14*, 222. https://doi.org/10.3390/biom14020222.
95. Cai, L., Zhang, Z., Zhou, L., Wang, H., Fu, J., Zhang, S., Shi, M., Zhang, H., Yang, Y., Wu, H., et al. (2008). Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. Clinical Immunology *129*, 428–437. https://doi.org/10.1016/j.clim.2008.08.012.
96. Lee, H.A., Goh, H.G., Lee, Y.S., Jung, Y.K., Kim, J.H., Yim, H.J., Lee, M.G., An, H., Jeen, Y.T., Yeon, J.E., et al. (2021). Natural killer cell activity is a risk factor for the recurrence risk after curative treatment of hepatocellular carcinoma. BMC Gastroenterol *21*, 258. https://doi.org/10.1186/s12876-021-01833-2.
97. Roberts, A.D., Ely, K.H., and Woodland, D.L. (2005). Differential contributions of central and effector memory T cells to recall responses. Journal of Experimental Medicine *202*, 123–133. https://doi.org/10.1084/jem.20050137.
98. Liu, Q., Sun, Z., and Chen, L. (2020). Memory T cells: strategies for optimizing tumor immunotherapy. Protein Cell *11*, 549–564. https://doi.org/10.1007/s13238-020-00707-9.
99. Han, J., Khatwani, N., Searles, T.G., Turk, M.J., and Angeles, C. V. (2020). Memory CD8+ T cell responses to cancer. Semin Immunol *49*, 101435. https://doi.org/10.1016/j.smim.2020.101435.
100. Coussens LM, and Werb Z (2002). Inflammation and cancer. Nature *420*, 860–867. https://doi.org/10.1038/nature01322.
101. Sarkar, I., Pati, S., Dutta, A., Basak, U., and Sa, G. (2019). T-memory cells against cancer: Remembering the enemy. Cell Immunol *338*, 27–31. https://doi.org/10.1016/j.cellimm.2019.03.002.
102. Han, C., Jiang, Y., Wang, Z., and Wang, H. (2019). Natural killer cells involved in tumour immune escape of hepatocellular carcinomar. Int Immunopharmacol *73*, 10–16. https://doi.org/10.1016/j.intimp.2019.04.057.
103. Griffith, J.W., Sokol, C.L., and Luster, A.D. (2014). Chemokines and chemokine receptors: Positioning cells for host defense and immunity. Annu Rev Immunol *32*, 659–702. https://doi.org/10.1146/annurev-immunol-032713-120145.
104. Heydtmann, M., Lalor, P.F., Eksteen, J.A., Hübscher, S.G., Briskin, M., and Adams, D.H. (2005). CXC Chemokine Ligand 16 Promotes Integrin-Mediated Adhesion of Liver-Infiltrating Lymphocytes to Cholangiocytes and Hepatocytes within the Inflamed Human Liver. The Journal of Immunology *174*, 1055–1062. https://doi.org/10.4049/jimmunol.174.2.1055.

105. Wang, C., Liu, Z., Xu, Z., Wu, X., Zhang, D., Zhang, Z., and Wei, J. (2018). The role of chemokine receptor 9/chemokine ligand 25 signaling: From immune cells to cancer cells (Review). Oncol Lett *16*, 2071–2077. https://doi.org/10.3892/ol.2018.8896.

106. Spinnen, J., Ringe, J., and Sittinger, M. (2018). CCL25 chemokine-guided stem cell attraction: An assessment of possible benefits and risks. Regenerative Med *13*, 833–844. https://doi.org/10.2217/rme-2018-0016.

107. Chen, H., Cong, X., Wu, C., Wu, X., Wang, J., Mao, K., Li, J., Zhu, G., Liu, F., Meng, X., et al. (2020). Intratumoral delivery of CCL25 enhances immunotherapy against triple-negative breast cancer by recruiting CCR9+ T cells. Sci Adv *6*. https://doi.org/10.1126/sciadv.aax4690.

108. Guidotti, L.G., and Iannacone, M. (2013). Effector CD8 T cell trafficking within the liver. Mol Immunol *55*, 94–99. https://doi.org/10.1016/j.molimm.2012.10.032.

109. Eksteen, B., Grant, A.J., Miles, A., Curbishley, S.M., Lalor, P.F., Hübscher, S.G., Briskin, M., Salmon, M., and Adams, D.H. (2004). Hepatic endothelial CCL25 mediates the recruitment of CCR9+ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. Journal of Experimental Medicine *200*, 1511–1517. https://doi.org/10.1084/jem.20041035.

110. Guo, L., Liu, X., and Su, X. (2023). The role of TEMRA cell-mediated immune senescence in the development and treatment of HIV disease. Front Immunol *14*, 1284293. https://doi.org/10.3389/fimmu.2023.1284293.

111. Strickland, M., Lee, S., Neo, S.Y., Balachander, A., Low, I., Mustafah, S., Goh, W.I., Wright, G.D., Larbi, A., and Pender, S.L.F. (2023). Mitochondrial Dysfunction in CD4+ T Effector Memory RA+ Cells. Biology (Basel) *12*, 597. https://doi.org/10.3390/biology12040597.

112. Morimoto, C., and Schlossman, S.F. (1998). The structure and function of CD26 in the T-cell immune response. Immunol Rev *161*, 55–70. https://doi.org/10.1111/j.1600-065X.1998.tb01571.x.

113. Galati, D., Zanotta, S., Capone, M., Madonna, G., Mallardo, D., Romanelli, M., Simeone, E., Festino, L., Sparano, F., Azzaro, R., et al. (2023). Potential clinical implications of CD4+CD26high T cells for nivolumab treated melanoma patients. J Transl Med *21*, 318. https://doi.org/10.1186/s12967-023-04184-6.

114. Ho, S.Y., Liu, P.H., Hsu, C.Y., Hsia, C.Y., Su, C.W., Lee, Y.H., Huang, Y.H., Lee, F.Y., Hou, M.C., and Huo, T.I. (2018). Comparison of twelve liver functional reserve models for outcome prediction in patients with hepatocellular carcinoma undergoing surgical resection. Sci Rep *8*, 4773. https://doi.org/10.1038/s41598-018-22923-4.

115. Wang, Y.Y., Zhong, J.H., Su, Z.Y., Huang, J.F., Lu, S.D., Xiang, B.D., Ma, L., Qi, L.N., Ou, B.N., and Li, L.Q. (2016). Albumin-bilirubin versus Child-Pugh score as a predictor of outcome after liver resection for hepatocellular carcinoma. British Journal of Surgery *103*, 725–734. https://doi.org/10.1002/bjs.10095.

116. Demirtas, C.O., D'Alessio, A., Rimassa, L., Sharma, R., and Pinato, D.J. (2021). ALBI grade: Evidence for an improved model for liver functional estimation in patients with hepatocellular carcinoma. JHEP Reports *3*, 100347. https://doi.org/10.1016/j.jhepr.2021.100347.

117. Albillos, A., Lario, M., and Álvarez-Mon, M. (2014). Cirrhosis-associated immune dysfunction: Distinctive features and clinical relevance. J Hepatol *61*, 1385–1396. https://doi.org/10.1016/j.jhep.2014.08.010.

118. Rueschenbaum, S., Ciesek, S., Queck, A., Widera, M., Schwarzkopf, K., Brüne, B., Welsch, C., Wedemeyer, H., Zeuzem, S., Weigert, A., et al. (2021). Dysregulated Adaptive Immunity Is an Early Event in Liver Cirrhosis Preceding Acute-on-Chronic Liver Failure. Front Immunol *11*, 534731. https://doi.org/10.3389/fimmu.2020.534731.

119. Khamri, W., Gudd, C., Liu, T., Nathwani, R., Krasniqi, M., Azam, S., Barbera, T., Trovato, F.M., Possamai, L., Triantafyllou, E., et al. (2022). Suppressor CD4+T cells expressing HLA-G are expanded in the peripheral blood from patients with acute decompensation of cirrhosis. Gut *71*, 1192–1202. https://doi.org/10.1136/gutjnl-2021-324071.

9. APPENDIX

Table 1A part A: Antibody panel used for cell classification. In bold are underlined intranuclear markers.

Metal	**Marker**
^{113}Cd	**Ki-67**
^{161}Dy	**Tbet**
^{162}Dy	**FoxP3**
^{169}Tm	**OPN**
^{174}Yb	**TCF1**
^{175}Lu	**EOMES**
^{089}Y	CD45
^{106}Cd	CD3
^{110}Cd	CD4
^{112}Cd	CD39
^{114}Cd	CD45RO
^{141}Pr	CD196 (CCR6)
^{142}Nd	CD19
^{143}Nd	CD45RA
^{144}Nd	CD69
^{146}Nd	CD335 (NKp46)
^{147}Sm	KLRG1
^{148}Nd	CD278/ICOS
^{149}Sm	CD25 (IL-2R)
^{150}Nd	CD127 (IL-7Ra)
^{151}Eu	CD103
^{152}Sm	CD21
^{153}Eu	TCR Va7.2
^{154}Sm	TIGIT
^{155}Gd	CD279 (PD-1)
^{156}Gd	CD183 (CXCR3)
^{158}Gd	CD194 (CCR4)
^{159}Tb	CD197 (CCR7)

Table 1A part B: Antibody panel used for cell classification. In bold are underlined intranuclear markers.

Metal	**Marker**
^{160}Gd	CD28
^{163}Dy	CD294 (CRTH2)
^{164}Dy	CD161
^{165}Ho	ICOSL (CD275)
^{166}Er	CXCR6 (CD186)
^{167}Er	CD38
^{168}Er	CD199 (CCR9)
^{170}Er	CLEC4D (CD368)
^{172}Yb	CX3CR1
^{173}Yb	TCRgd
^{176}Yb	CD56 (NCAM)
^{209}Bi	CD16
^{145}Nd	CD26
^{171}Yb	CD185 (CXCR5)
^{116}Cd	CD8a

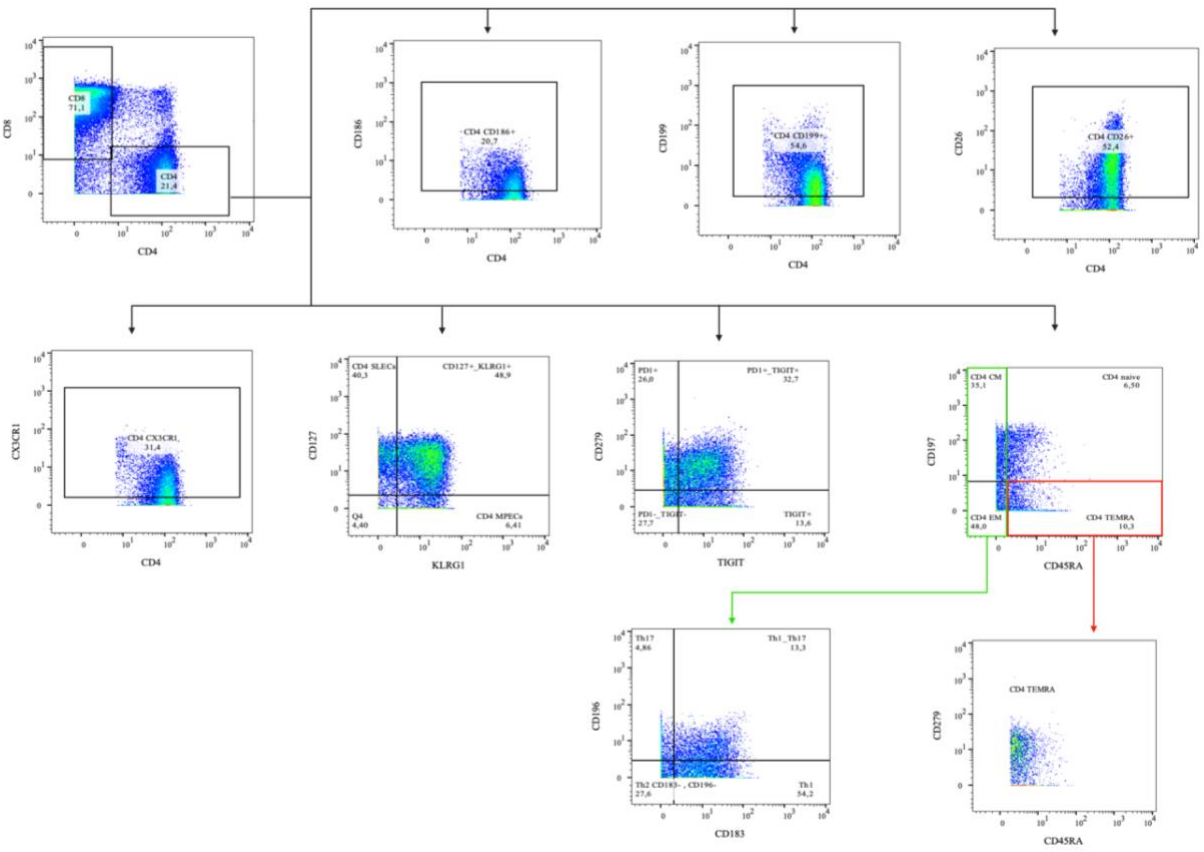

Figure 1A: $CD4^+$ cells gating strategy. Total CD4 T cells were characterized in $CD186^+$, $CD199^+$, $CD26^+$, $CX3CR1^+$ cells. CD127 and KLRG1 were able to identify $CD4^+$ SLECs ($CD127^+$ $KLRG^-$), $CD127^+$ KLG^+ cells and $CD4^+$ MPECs. CD279 (PD1) and TIGIT identified exhausted T cells as $PD1^+$, $PD1^+$ $TIGIT^+$, $TIGIT^+$ and $PD1^-$ $TIGIT^-$. CD197 and CD45RA identified $CD4^+$ CM ($CD197^+$ $CD45RA^-$), $CD4^+$ naïve ($CD197^+$ $CD45RA^+$), CD4 EM ($CD197^-$ $CD45RA^-$) and $CD4^+$ TEMRA ($CD197^-$ $CD45RA^+$). From $CD4^+$ CM/$CD4^+$ EM group were identified T helper subsets: Th17 ($CD196^+$ $CD183^-$), Th1 ($CD197^-$ $CD183^+$) and Th2 ($CD197^-$ $CD183^-$). CD4 TEMRA were further evaluated for the expression of CD279.

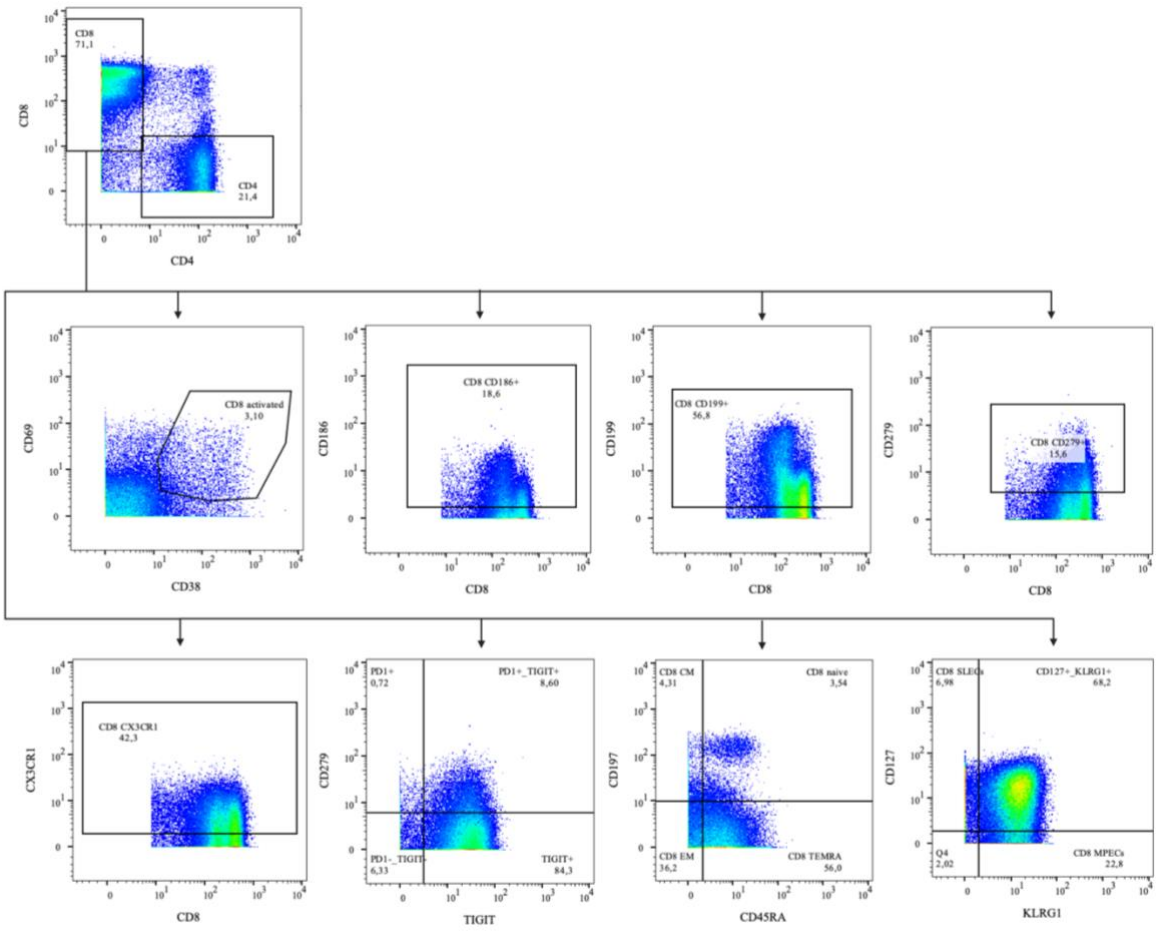

Figure 2A: $CD8^+$ cells gating strategy. Total CD8 T cells were characterized in $CD186^+$, $CD199^+$, $CX3CR1^+$ cells. CD279 (PD1) and TIGIT identified exhausted T cells as $PD1^+$, $PD1^+$ $TIGIT^+$, $TIGIT^+$ and $PD1^-$ $TIGIT^-$. CD197 and CD45RA identified $CD4^+$ CM ($CD197^+$ $CD45RA^-$), $CD4^+$ naïve ($CD197^+$ $CD45RA^+$), $CD4^+$ EM ($CD197^-$ $CD45RA^-$) and $CD4^+$ TEMRA ($CD197^-$ $CD45RA^+$). CD127 and KLRG1 were able to identify $CD8^+$ SLECs ($CD127^+$ $KLRG^-$), $CD127^+$ KLG^+ cells and $CD8^+$ MPECs.

Table 2A part A: Median and interquartile range [IQR] of cellular populations at baseline in patients with and without recurrence. Statistically significant results are presented in bold (p<0.05).

Variable	**No recurrence (n=9)**	**Recurrence (n=10)**	***p*-value**
B cells	10.3 [5.8-11.8]	6.5 [3.5-11.5]	0.530
Not CD3_CD19	30.5 [29.2-33.6]	30.1 [19.8-20.2]	0.615
NK cells	23.4 [19.5-37.2]	44.7 [33.3-60.9]	**0.033**
NK cells $CD199^+$	78.9 [68.4-91.2]	89.6 [80.8-94.9]	0.246
NK cells $CD335^+$	63.1 [53.7-70.2]	68.5 [51.7-73.9]	0.929
NK cells $KLRG1^+$	30.6 [20.6-47.7]	30.8 [26.8-33.7]	0.887
Not NK cells	76.7 [62.9-80.6]	55.3 [39.1-66.7]	**0.033**
$CLEC4D^+$ cells	52.9 [48-58.6]	48.4 [37.7-56.2]	0.541
T cells	60 [54.4-65.6]	62.9 [49.9-72.4]	0.531
$CD4^+$	40.6 [28.3-53.1]	57.8 [42.3-71.5]	0.091
$CD4^+$ $CD26^+$	66.7 [62.4-78.4]	79.4 [60.8-89.1]	0.545
$CD4^+$ $CD186^+$	29.7 [24.8-40.5]	25.4 [20.7-33.4]	0.395
$CD4^+$ $CD199^+$	32.2 [24.8-52.9]	48.9 [29.2-70.9]	0.177
$CD4^+$ CM	40.7 [31.6-52.4]	45.7 [40.4-51.8]	0.657
$CD4^+$ $CX3CR1^+$	30.8 [21.5-48.3]	25.9 [20.4-31.4]	0.733
$CD4^+$ EM	37.3 [17.7-44.4]	17.1 [4.8-32.8]	0.086
$CD4^+$ MPECs	3.4 [0.5-5.2]	2.3 [0.8-6.4]	0.955
$CD4^+$ naive	17.2 [10.5-22.2]	28.1 [11.5-41.8]	0.272
$CD4^+$ SLECs	66.5 [53.7-74.1]	65.7 [55.1-83.8]	0.545
$CD4^+$ TEMRA	10.3 [1.8-12.3]	5.6 [2.5-8.5]	0.203
$CD4^+$ $CD127^+$ $KLRG1^+$	24.7 [16-35.2]	23.2 [11.8-28.8]	0.599
$CD4^+$ $PD1^+$	21.4 [18.4-33.4]	26 [16-33.9]	0.968
$CD4^+$ $PD1^+$ $TIGIT^+$	16.7 [10.3-18.8]	16.8 [12.8-23.1]	0.701
$CD4^+$ $PD1^-$ $TIGIT^-$	51.2 [45.5-55.5]	50.7 [34.6-61.9]	0.904
$CD4^+$ $KLRG1^-$ $CD127^-$	5.7 [4.7-7.8]	4.5 [3.7-8]	0.541

Table 2A part B: Median and interquartile range [IQR] of cellular populations at baseline in patients with and without recurrence. Statistically significant results are presented in bold (p<0.05).

Variable	**No recurrence (n=9)**	**Recurrence (n=10)**	***p*-value**
$CD4^+$ $TIGIT^+$	8.8 [7-9.8]	8.3 [6.7-12.8]	0.965
$CD4^+$ not naive	75.5 [70.1-78.7]	69.1 [56.7-79.6]	0.320
$CD4^+$ Th1	40.2 [34.7-46]	37.4 [27.9-44.2]	0.515
$CD4^+$ Th17	6.2 [4.7-8.1]	9 [8.6-11.8]	0.203
$CD4^+$ Th1_Th17	21.7 [15.1-36.3]	26.2[16.5-34.5]	0.778
$CD8^+$	42.3 [37.1-64.7]	35.7 [22.2-44.2]	0.152
$CD8^+$ activated	9.4 [6.4-12.6]	10.9 [3.6-13.6]	0.795
$CD8^+$ $CD186^+$	21.3 [17.5-32.1]	18.6 [15.5-23.1]	0.531
$CD8^+$ $CD199^+$	33 [23.5-52.4]	52 [38.1-72.5]	0.095
$CD8^+$ $CD279^+$	30.7 [20.7-40]	30.8 [21-36.9]	0.904
$CD8^+$ CM	5.2 [4.1-6.9]	19.4 [9.8-29.9]	**0.029**
$CD8^+$ $CX3CR1^+$	42.3 [20.4-51.8]	23.9 [18.7-42.3]	0.442
$CD8^+$ EM	37.7 [33-44]	25.1 [18.9-36.2]	**0.027**
$CD8^+$ MPECs	23.9 [18.6-41.5]	22.8 [18.2-41.6]	0.887
$CD8^+$ SLECs	22.1 [12.9-32.2]	31.9 [19.6-37.6]	0.315
$CD8^+$ TEMRA	53.9 [41.2-62.3]	41.2 [31.8-56.6]	0.492
$CD8^+$ $CD127^+$ $KLRG1^+$	40.6 [25.5-46.9]	23.7 [21-53.7]	0.657
$CD8^+$ $PD1^+$	10.2 [7.1-14.4]	9.5 [3.1-13.4]	0.364
$CD8^+$ $PD1^+$ $TIGIT^+$	20.5 [11.7-27.4]	22.9 [18-28.3]	0.840
$CD8^+$ $PD1^-$ $TIGIT^-$	33.9 [28.7-39.3]	29.9 [28.3-36.5]	0.408
$CD8^+$ $KLRG1^-$ $CD127^-$	5.2 [3.2-12.6]	9.7 [5.2-11.5]	0.743
$CD8^+$ $TIGIT^+$	29.9 [23.1-47.6]	33.4 [26.5-49.1]	0.717

Table 3A part A: Median and interquartile range [IQR] of cellular populations at the time of surgery (baseline) and at the time of recurrence. Statistically significant results are presented in bold ($p<0.05$).

Variable	**Baseline (n=10)**	**Recurrence**	***p*-value**
B cells	6.5 [3.5-11.5]	5.6 [3.6-14.5]	0.625
Not CD3_CD19	30.1 [19.8-20.2]	30 [20.9-37.4]	0.922
NK cells	44.7 [33.3-60.9]	34.5 [15.2-49.6]	0.131
NK cells $CD199^+$	89.6 [80.8-94.9]	93.9 [91.3-97.3]	**0.006**
NK cells $CD335^+$	68.5 [51.7-73.9]	63.1 [47.8-71.6]	0.375
NK cells $KLRG1^+$	30.8 [26.8-33.7]	39.1 [30-43]	0.084
Not NK cells	55.3 [39.1-66.7]	65.5 [50.4-84.8]	0.131
$CLEC4D^+$ cells	48.4 [37.7-56.2]	60.6 [53.7-67.2]	**0.039**
T cells	62.9 [49.9-72.4]	55.7 [44.5-68.9]	0.695
$CD4^+$	57.8 [42.3-71.5]	59.1 [42-69.6]	0.922
$CD4^+$ $CD26^+$	79.4 [60.8-89.1]	75.8 [65.6-87.5]	0.625
$CD4^+$ $CD186^+$	25.4 [20.7-33.4]	33 [25-59]	**0.004**
$CD4^+$ $CD199^+$	48.9 [29.2-70.9]	68.3 [48-86.4]	**0.014**
$CD4^+$ CM	45.7 [40.4-51.8]	49.9 [34.2-60.7]	0.922
$CD4^+$ $CX3CR1^+$	25.9 [20.4-31.4]	44.2 [33.1-60.2]	**0.002**
$CD4^+$ EM	17.1 [4.8-32.8]	23.5 [12.1-35.6]	0.375
$CD4^+$ MPECs	2.3 [0.8-6.4]	2 [0.7-4.7]	0.844
$CD4^+$ naive	28.1 [11.5-41.8]	18.2 [16.4-25.1]	0.275
$CD4^+$ SLECs	65.7 [55.1-83.8]	71.2 [53.6-78.3]	0.275
$CD4^+$ TEMRA	5.6 [2.5-8.5]	8.1 [2.6-13]	**0.031**
$CD4^+$ $CD127^+$ $KLRG1^+$	23.2 [11.8-28.8]	24.4 [18.5-31.2]	0.322
$CD4^+$ $PD1^+$	26 [16-33.9]	25.4 [18.3-30.7]	0.625
$CD4^+$ $PD1^+$ $TIGIT^+$	16.8 [12.8-23.1]	16.1 [12.6-22.8]	0.734
$CD4^+$ $PD1^-$ $TIGIT^-$	50.7 [34.6-61.9]	47.5 [34.2-53.7]	0.078
$CD4^+$ $KLRG1^-$ $CD127^-$	4.5 [3.7-8]	5 [2.1-5.9]	0.469

Table 3A part B: Median and interquartile range [IQR] of cellular populations at the time of surgery (baseline) and at the time of recurrence. Statistically significant results are presented in bold ($p<0.05$).

Variable	**Baseline (n=10)**	**Recurrence**	***p*-value**
$CD4^+$ $TIGIT^+$	8.3 [6.7-12.8]	10.3 [7.4-14.3]	0.203
$CD4^+$ not naive	69.1 [56.7-79.6]	75 [67.7-78.9]	0.625
$CD4^+$ Th1	37.4 [27.9-44.2]	45.8 [31.1-51.9]	0.301
$CD4^+$ Th17	9 [8.6-11.8]	7.5 [4.7-16.9]	0.910
$CD4^+$ Th1_Th17	26.2[16.5-34.5]	26.6 [20.6-31.9]	0.375
$CD8^+$	35.7 [22.2-44.2]	30.9 [22.7-46.7]	0.695
$CD8^+$ activated	10.9 [3.6-13.6]	22.1 [9.3-27.3]	**0.002**
$CD8^+$ $CD186^+$	18.6 [15.5-23.1]	29.6 [17.9-48.4]	**0.004**
$CD8^+$ $CD199^+$	52 [38.1-72.5]	70.1 [50.4-90.6]	**0.009**
$CD8^+$ $CD279^+$	30.8 [21-36.9]	27 [22.7-34.3]	0.922
$CD8^+$ CM	19.4 [9.8-29.9]	11.8 [7.8-19.5]	0.688
$CD8^+$ $CX3CR1^+$	23.9 [18.7-42.3]	40.4 [30.2-58.7]	**0.009**
$CD8^+$ EM	25.1 [18.9-36.2]	33.3 [21.8-38.5]	0.164
$CD8^+$ MPECs	22.8 [18.2-41.6]	25 [16.2-31.8]	0.176
$CD8^+$ SLECs	31.9 [19.6-37.6]	30.4 [27.2-41.7]	0.301
$CD8^+$ TEMRA	41.2 [31.8-56.6]	43 [34.3-54.4]	0.769
$CD8^+$ $CD127^+$ $KLRG1^+$	23.7 [21-53.7]	35.4 [25.9-44.3]	0.084
$CD8^+$ $PD1^+$	9.5 [3.1-13.4]	6.7 [3.3-10.9	0.652
$CD8^+$ $PD1^+$ $TIGIT^+$	22.9 [18-28.3]	16.7 [12.1-21.9]	0.160
$CD8^+$ $PD1^-$ $TIGIT^-$	29.9 [28.3-36.5]	32.6 [25.1-36.4]	1.000
$CD8^+$ $KLRG1^-$ $CD127^-$	9.7 [5.2-11.5]	7 [5.2-10.4]	0.547
$CD8^+$ $TIGIT^+$	33.4 [26.5-49.1]	46.3 [26.1-54.4]	0.695

***Table 4A part A*:** Median and interquartile range [IQR] of cellular populations at the time of surgery (baseline) and at one year in patients without recurrence. Statistically significant results are presented in bold (p<0.05).

Variable	**Baseline (n=9)**	**Follow up (1 year)**	***p*-value**
B cells	10.3 [5.8-11.8]	7.4 [4.5-16.2]	0.937
Not CD3_CD19	30.5 [29.2-33.6]	31.1 [26.4-38.7]	0.781
NK cells	23.4 [19.5-37.2]	25.8 [15.8-50.4]	0.469
NK cells $CD199^+$	78.9 [68.4-91.2]	71.2 [50.2-98.1]	0.437
NK cells $CD335^+$	63.1 [53.7-70.2]	54.9 [48.9-77]	1.000
NK cells $KLRG1^+$	30.6 [20.6-47.7]	29.4 [20.9-50.4]	0.219
Not NK cells	76.7 [62.9-80.6]	74.2 [49.6-84.2]	0.469
$CLEC4D^+$ cells	52.9 [48-58.6]	56.1 [43.7-66.8]	0.469
T cells	60 [54.4-65.6]	60.5 [26.9-62.5]	0.297
$CD4^+$	40.6 [28.3-53.1]	43.3 [27-49.6]	1.000
$CD4^+$ $CD26^+$	66.7 [62.4-78.4]	68.4 [66.3-82.2]	**0.031**
$CD4^+$ $CD186^+$	29.7 [24.8-40.5]	27.8 [17.1-36.2]	0.578
$CD4^+$ $CD199^+$	32.2 [24.8-52.9]	34.3 [20.9-90.8]	0.375
$CD4^+$ CM	40.7 [31.6-52.4]	48.7 [37-62.4]	1.000
$CD4^+$ $CX3CR1^+$	30.8 [21.5-48.3]	36.8 [18.3-41.4]	1.000
$CD4^+$ EM	37.3 [17.7-44.4]	21.1 [16.8-30.2]	0.578
$CD4^+$ MPECs	3.4 [0.5-5.2]	2.7 [1.2-7.2]	0.875
$CD4^+$ naive	17.2 [10.5-22.2]	19.8 [13.6-31]	0.469
$CD4^+$ SLECs	66.5 [53.7-74.1]	67.7 [65.8-76.3]	0.469
$CD4^+$ TEMRA	10.3 [1.8-12.3]	3.9 [2.5-11.4]	1.000
$CD4^+$ $CD127^+$ $KLRG1^+$	24.7 [16-35.2]	18 [14.9-27.1]	0.156
$CD4^+$ $PD1^+$	21.4 [18.4-33.4]	23.3 [21.8-24.8]	0.937
$CD4^+$ $PD1^+$ $TIGIT^+$	16.7 [10.3-18.8]	18.2 [12.3-24.4]	0.297
$CD4^+$ $PD1^-$ $TIGIT^-$	51.2 [45.5-55.5]	45.8 [40.4-54.8]	0.469
$CD4^+$ $KLRG1^-$ $CD127^-$	5.7 [4.7-7.8]	5.9 [4.1-8.1]	0.812
$CD4^+$ $TIGIT^+$	8.8 [7-9.8]	8.5 [7.1-10.7]	0.937
$CD4^+$ not naive	75.5 [70.1-78.7]	70.3 [66.1-82.5]	0.469

***Table 4A part B*:** Median and interquartile range [IQR] of cellular populations at the time of surgery (baseline) and at one year in patients without recurrence. Statistically significant results are presented in bold ($p<0.05$).

Variable	**Baseline (n=9)**	**Follow up (1 year)**	***p*-value**
$CD4^+$ Th1	40.2 [34.7-46]	42.1 [37.7-48.1]	0.375
$CD4^+$ Th17	6.2 [4.7-8.1]	8.7 [6.2-9.9]	0.687
$CD4^+$ Th1_Th17	21.7 [15.1-36.3]	21.7 [13.7-26.6]	0.687
$CD8^+$	42.3 [37.1-64.7]	49.2 [34.8-63.6]	0.937
$CD8^+$ activated	9.4 [6.4-12.6]	9.4 [6.7-20.6]	0.578
$CD8^+$ $CD186^+$	21.3 [17.5-32.1]	18.8 [13.1-28.6]	0.812
$CD8^+$ $CD199^+$	33 [23.5-52.4]	34.5 [18.2-91.9]	0.687
$CD8^+$ $CD279^+$	30.7 [20.7-40]	20.2 [18.2-45]	0.812
$CD8^+$ CM	5.2 [4.1-6.9]	3.6 [1.5-9.4]	0.875
$CD8^+$ $CX3CR1^+$	42.3 [20.4-51.8]	31.6 [28.9-40.8]	0.812
$CD8^+$ EM	37.7 [33-44]	30.9 [30.2-44]	0.812
$CD8^+$ MPECs	23.9 [18.6-41.5]	32.6 [25.5-46.8]	1.000
$CD8^+$ SLECs	22.1 [12.9-32.2]	23.4 [15.5-26.7]	0.812
$CD8^+$ TEMRA	53.9 [41.2-62.3]	59.3 [37.6-61.7]	0.812
$CD8^+$ $CD127^+$ $KLRG1^+$	40.6 [25.5-46.9]	33.7 [17.6-52.9]	0.812
$CD8^+$ $PD1^+$	10.2 [7.1-14.4]	6.7 [3.9-12.7]	0.219
$CD8^+$ $PD1^+$ $TIGIT^+$	20.5 [11.7-27.4]	13.6 [7.8-29.6]	1.000
$CD8^+$ $PD1^-$ $TIGIT^-$	33.9 [28.7-39.3]	29 [20.1-47.1]	0.812
$CD8^+$ $KLRG1^-$ $CD127^-$	5.2 [3.2-12.6]	12.6 [1.7-18.3]	0.687
$CD8^+$ $TIGIT^+$	29.9 [23.1-47.6]	39.5 [22.8-55.4]	0.375