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Influence of Perivascular Tumor Infiltrating Leukocytes on Survival after Resection of Early Hepatocellular Carcinoma

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II. List of Abbreviations

%	percentage
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
ABC-AP	VECTASTAIN® ABC-AP Kit
AFP	alpha-fetoprotein
APC	antigen presenting cell
B-cell	B-lymphocyte
BCLC	Barcelona Clinic Liver Cancer staging
BSA	bovine serum albumin
CD	cluster of differentiation
CI	confidence interval
CRP	C-reactive protein
CTL	cytotoxic T lymphocytes
DC	dendritic cells
DFS	disease free survival
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FLR	future liver remnant
Foxp3	Forkhead-Box-Protein P3
GB	gigabyte
GPS	Glasgow prognostic score
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HR	hazard ratio
HTCR	human tissue and cell research foundation
ICC	intraclass correlation coefficient

ICD	ImageJ software with color deconvolution
IF	immunofluorescence
IFN	interferon
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IM	invasive margin
IST	ImageJ software with subjective threshold
LR	liver resection
LT	liver transplantation
MB	megabyte
mCRC	metastatic colorectal cancer
MCT	mast cell tryptase
mGPS	modified Glasgow prognostic score
MHC	major histocompatibility complex
min	minutes
ml	milliliter
mm	millimeter
n	sample size, the number
N.A.	not available
N.S.	no significance
NaCl	sodium chloride
NASH	nonalcoholic steatohepatitis
NK cells	natural killer cells
NKT cells	natural killer T cells
NLR	neutrophil-to-lymphocyte ratio
OS	overall survival
OvCa	ovarian cancer
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1

PDAC	pancreatic ductal adenocarcinoma
PLT	platelet
PS	performance status
PV	perivascular
QTS	quantification of the tumor stroma
RFA	radio frequency ablation
ROC	receiver operating characteristic
SD	standard deviation
SPSS	Statistical Product and Service Solutions
TACE	transarterial chemoembolization
TAM	tumor-associated macrophage
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
T-cell	T-lymphocyte
Th1	Type 1 helper T cells
Th17	Type 17 helper T cells
TIL	tumor infiltrating leukocytes
TMA	tissue microarray
TNM	TNM Classification of Malignant Tumors
Treg	regulatory T cells
VEGF	vascular endothelial growth factor
ZAC	ZEN2 software automated counting
ZEN	ZEISS Efficient Navigation software
χ2-test	chi-square test

1. Introduction

In this dissertation, I aimed to elucidate the influence of tumor infiltrating leukocytes on the survival of patients with resected hepatocellular carcinoma (HCC). For that purpose, this work was conducted in three phases. First, through a literature review, I identified the relevant players of tumor infiltrating leukocytes. Then in an interdisciplinary effort, we developed a novel algorithm for quantification of the tumor stroma. Finally, these components were used for an experimental investigation of an HCC cohort from our institution.

1.1. Epidemiology of Hepatocellular Carcinoma

HCC is a major type of all primary liver cancer. It is the most common type, accounting for about 70% to 90%, of primary liver neoplasms. It ranks as fifth in men and ninth in women regarding the incidence and is the second leading reason of cancer death globally.[1] The occurrence of HCC has a clear geographical distribution, as shown in Figure 1, sub-Saharan Africa and Eastern Asia are the two areas with high rates for HCC. Moreover, about 50% of the worldwide HCC cases occur in China alone. In contrast, North and South America, Northern Europe, and Oceania have low rates of HCC occurrence.[2]

During 2012, an estimated 782,500 new cases and 745,500 deaths occurred over the world.[3] The burden of HCC is still increasing worldwide. It is appraised that the incidence rates will reach 78,000 in Europe and 27,000 in the United States by 2020, respectively, in comparison to the number of cases of 65,000 and 21,000 in 2008, this is a significant increase.[1] This increase might be most likely due to the Hepatitis C virus (HCV) infection in Euromerican area during the period 1940-70.[4]

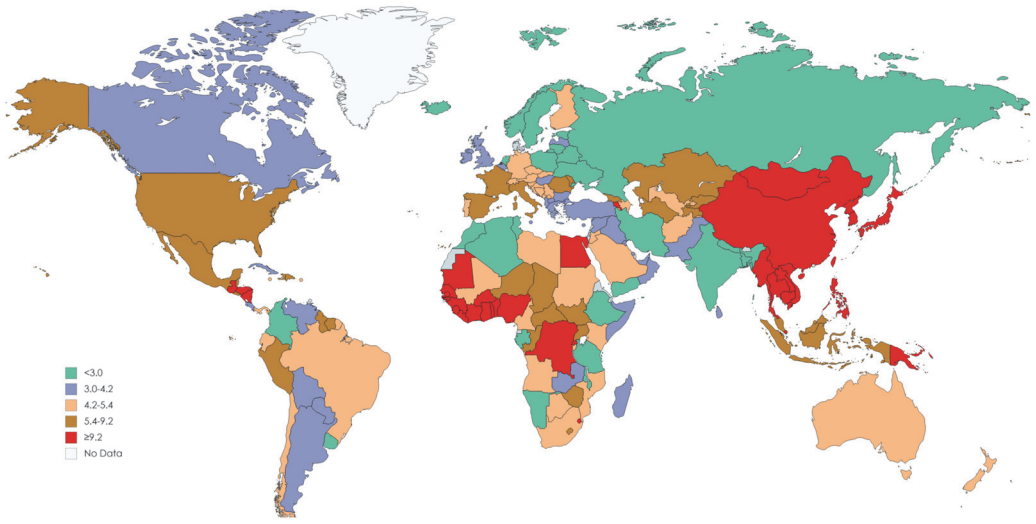

Figure 1. Estimated Age-standardized Rates (World) of Incident Cases of HCC Worldwide in 2012. Data from World Health Organization.[1]

1.2. Treatment of Hepatocellular Carcinoma

Surgery, including liver resection (LR) and liver transplantation (LT), remain the most effective curative treatment of HCC.

The most available way to remove the malignant tumor of the liver is LR. With the improvement of understanding of liver segmental anatomy, control of bleeding, perioperative care, and anesthesia techniques, the outcomes of LR have improved over time, also more and more previously considered unresectable patients have an opportunity to undergo curative resection.[5] In dependence of different tumor locations and size, curative LR can be performed in several types including segmental resection, right or left hepatectomy, and extended right or left hepatectomy.[6] In recent years, minimally invasive techniques like laparoscopic and robotic approaches for LR have increasingly been gaining in popularity for HCC due to the improved short-term outcome.[7] However, long-term tumor specific outcome remains to be validated.[8, 9] However LR, albeit being widely available, is restricted regarding the future liver remnant (FLR). In non-cirrhotic patients, the remaining FLR can be as low as 20% in selected patients. However, in cirrhosis, which is the precursor for most HCCs, the FLR should always be above 30-40% depending on the severity of the cirrhosis and

the fitness of the patient. As soon the patient presents with portal hypertension, LR should not be performed at all.

In the case of severe cirrhosis, however, LT remains an option. To that LT radically removes the tumor and cirrhotic liver and reconstitutes liver function. Therefore it should be considered as the ideal treatment in this situation.[10] However livers for transplantation are not readily available for all patients, and therefore the allocation of these organs for transplantation is limited and subsidiary. In this complex situation, the best treatment option needs to be found individually for all patients.

The Barcelona Clinic Liver Cancer (BCLC) staging system, as shown in Figure 2, is the most standard system globally for the management of HCC patients in clinical practice currently. According to this guideline, the surgical therapy depends mainly on the functional status of the patient, his underlying liver disease, and the tumor size.[11] It is reported that only 30% of diagnosed HCC patients are suitable for surgery (LR and LT).[12] Even though, outcomes vary greatly depending on the surgery. Approximate 70% of patients undergoing LR[13] and 20% of LT recipients[14] develop recurrences within five years after surgery.

Besides resection and transplantation, other recommended nonsurgical treatments include radiofrequency ablation (RFA), transarterial chemoembolization (TACE), and molecularly targeted therapy. RFA is the standard treatment for those HCC patients in BCLC stage 0-A but not suitable for surgery. TACE is recommended as a palliative treatment for patients with multinodular HCC. Sorafenib was approved for use for those with vascular invasion or extrahepatic metastasis based on evidence in prolonged survival compared with placebo.[15]

Because of this fact and the relative scarceness of organs for LT careful and meticulous risk stratification is needed to treat the right patients with the appropriate method.

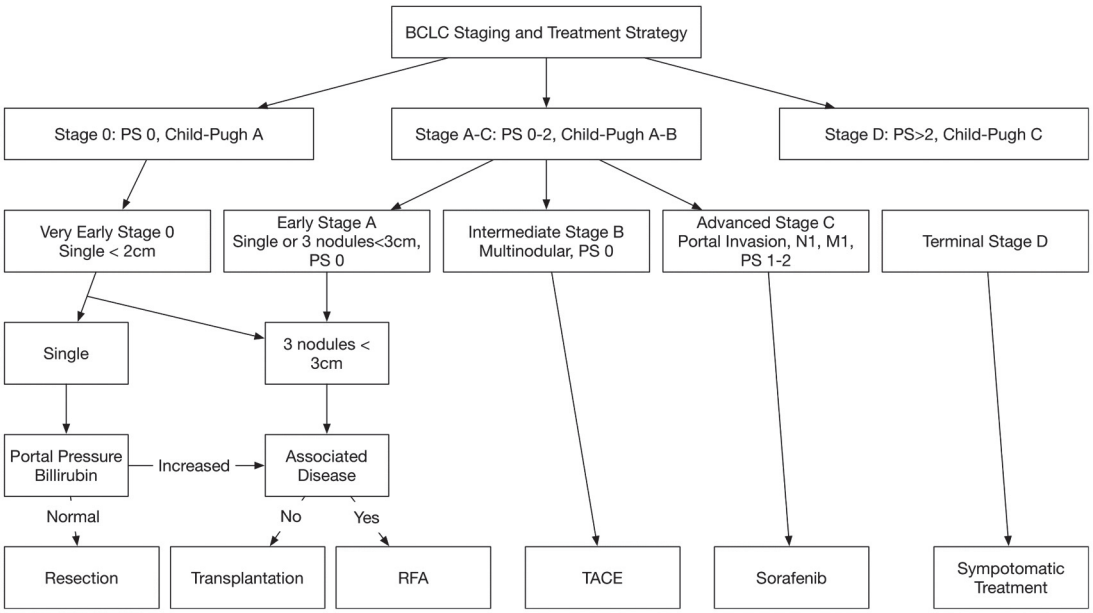

Figure 2. BCLC Staging and Treatment Strategy Flow Chart, modified from [11]. (Abbreviations: HCC, hepatocellular carcinoma; PS, performance status; RFA, radiofrequency ablation; TACE, transarterial chemoembolization)

1.3. Hepatocellular Carcinoma Recurrence after Resection

Despite the continuous improvements of in curative resection, the postoperative recurrence of HCC remains as high as 70% within five years.[13]

Various well-known clinicopathological factors that influencing the risk of postoperative recurrence have been reported, including tumor size and number,[16-19] microscopic and macroscopic vascular invasion,[18, 20, 21] presence of satellite nodules,[21] tumor encapsulation,[22] histopathological grade,[23] and underlying hepatitis and cirrhosis.[19, 21] As well, the types of surgery including the extent of hepatectomy,[19] negative margin,[22] and anatomic resection[24, 25] have been reported as independent risk factors, respectively. Also, numerous biological parameters have been revealed to be predictive, such as pre-operative serum alpha-fetoprotein (AFP) level,[26] glypican-3,[27] and cancer stem cell markers including CD44,[28] CD90,[28] and epithelial cell adhesion molecule.[29]

The mechanisms of recurrence after hepatectomy can be intrahepatic metastasis of the primary tumor or a de novo tumor.[30] Studies have shown that early recurrence within two years is due to the intrahepatic metastasis associating with more aggressive tumor biological behaviors. However, late recurrence after two years is more likely the consequence of de novo tumor related to the worse underlying liver conditions.[31-33]

Strategies to prevent postresection HCC recurrence have been studied over decades, but remain difficult challenges. Because of the inherent chemo resistance of HCC cells, usual systemic chemotherapies have not been proved beneficial to HCC, in compared with many other solid cancers. TACE, which has been reported as a neoadjuvant treatment before resection, could improve staging of HCC, therefore, reduce the recurrence of postresection recurrence.[34] Adoption of interferon-alpha was reported to reduce the late recurrence in hepatitis related HCC patients after resection.[35] Although Sorafenib has been validated to improve overall survival in patients with advanced HCC, the validation of its benefits as adjuvant treatment to prevent postoperative recurrence failed in the recent randomized controlled trial.[15, 36]

Because recurrence remains the primary cause of death after resection for HCC, it is also critical to adopt an efficacious treatment for patients with recurrent HCC promptly. The curative options include salvage liver transplantation, repeated hepatic resection, and radiofrequency ablation.[37-39] Patients who are ineligible for the above treatments still can get prolonged survival by receiving Sorafenib or TACE.[40]

1.4. Immunology in the context of the Liver and HCC

The immune system comprises two parts, innate and adaptive immunity. Innate immunity is the first-line of defense against pathogens or tumor cells nonspecifically. The innate immune cells are comprised of neutrophils, natural killer (NK) cells, macrophages, and dendritic cells (DC). After stimulation, the innate immune cells are enabled to start a rapid response. Eventually, activation of innate immunity can result in stimulation of adaptive immunity by secreting of cytokine and presenting antigens.

The two principal types of adaptive immune cells, T- and B- lymphocyte, express highly specific cell-surface receptors to recognize antigens. Activation procedures of both innate and adaptive immunity are essential for battling pathogens or malignant cells, but overplayed immune reactions can severely damage tissue. Thus, many regulatory mechanisms control the immune system to distinguish self from non-self. Cell types like regulatory T cells regulate immune responses and prevent autoimmunity. As well, immunoinhibitory receptors, for example, programmed death-1 (PD-1), are crucial modulators of T-cell, working with suppressor cells to limit immune responses.[41]

The liver is a vital metabolic organ receiving 75% of its blood supply from the gut.[42] In the absorption of nutrients at the same time, the liver also receives a substantial amount of immunostimulatory products. It must clear these ingested exogenous antigens by extraordinary immune reactions, but also has to establish a tolerogenic state by controlling immune response to protect itself. Intrahepatic tolerance is a basic feature of liver immunology. In liver, there are a considerable amount of innate and adaptive immune cells. Notably, these intrahepatic immune cells demonstrate exclusive functional properties that tend to maintain a tolerogenic environment.[43]

T cells (commonly expressing marker CD3) are a main type of adaptive immune cells. Helper T (Th) cells (commonly expressing marker CD4) arrange immune responses, including main subtypes of Th1 cells (producing interferon-gamma (IFN-γ)), Th2 cells (producing interleukin (IL)-4, IL-5, and IL-13), and Th17 cells (producing IL-17), regulatory T cells (commonly expressing marker FoxP3 and CD25) play an immunomodulatory role, and cytotoxic T cells (commonly expressing marker CD8) destroy targeted cells. Also, memory T cells (commonly expressing marker CD45RO) trigger a faster response for antigens which they have encountered previously.[43]

B cell (commonly expressing marker CD20), accompanied with T cells, mediates adaptive immunity. They are responsible for humoral immunity via producing antibody. B cells could also play a role as antigen presenting cells (APCs) under certain conditions. They make up a considerable proportion of intrahepatic adaptive immune cells and play important roles in liver diseases.[44]

NK cells (commonly expressing marker CD57) and neutrophils (commonly expressing marker CD66b) are innate responders, unlike T or B cells, and do not possess specific antigen receptors. NK cells are a central component of liver lymphocytes and mediate inflammations in viral and autoimmune hepatitis. By releasing of lytic granules or cytokines such as interferon-γ, NK cells kill the target cells in a major histocompatibility complex (MHC) unrestricted fashion.[43] In contrast with NK cells, neutrophils are the most abundant leukocyte type in circulation, once any inciting inflammatory signals are detected, they can infiltrate into liver as an acute response and function as phagocytosis. Inappropriate homing or activation of neutrophils contributes to many kinds of liver disease, including viral hepatitis, liver failure, and tumors.[45]

Dendritic cells (DCs) (commonly expressing marker CD1a or CD83) and liver macrophages (also known as Kupffer cells, commonly expressing marker CD68) play a role in antigen presenting in the liver, constituting another major type of intrahepatic immune cells. Experimental and clinical observations showed that the context of antigen presentation could dramatically alter the response of T cells. Precisely, T cells proliferate into an immunogenic phenotypic when antigen presentation happens together with the appropriate stimulatory molecules. Conversely, antigens presented without stimulatory or with inhibitory signals lead to apoptosis or anergy of T cells.[43, 46, 47]

Especially regarding HCC, the immune system has also been widely acknowledged to play a vital role in the development and progression.[48] To that occurrence, prognosis and treatment are closely related to the host's immune system. Alpha-fetoprotein (AFP) is an oncofetal antigen which produced by immature hepatic cells in the fetus or undifferentiated HCC cells. AFP was the first antigen that was discovered and clinically used as a tumor marker for HCC screening and diagnosis.[49] HCC is considered as an inflammation-induced malignancy. Most HCC cases relate to chronic hepatitis B or hepatitis C infections.[50] The continuous damages of the liver by these virus infections lead to cirrhosis, and eventually hepatocarcinogenesis. These processes are frequently attributed to immune-mediated mechanisms, such as the overreaction of natural killer

(NK) cells and depletion of T cells.[51] Also, the implementation of vaccination against hepatitis B has significantly decreased the incidence of HCC in Asia.[52] Besides the virus infection, the nonalcoholic steatohepatitis (NASH) caused by obesity is also known as a risk factor for HCC.[53] A recent mouse model showed that activated cytotoxic T-cells and natural killer T-cells (NKT) are complicit in the genesis of NASH and concomitantly HCC.[54]

The immune responses against HCC have been well investigated in several aspects. Clinically the systemic inflammation-based Glasgow prognostic score (GPS) and its modified version (mGPS), which combine C-reactive protein and albumin, have shown their prognostic values in HCC, indicating a strong relationship between inflammatory responses and prognosis of HCC.[55] Also, the circulating neutrophil-to-lymphocyte ratio has been validated as another inflammation related predictor of HCC's outcome in recent years.[56] Apart from innate immunity, the adaptive immune responses, including cellular and humoral immunity, have also been well described in HCC. The alternations of number and function of different cell types, cytokine and chemokine levels have been studied both clinically and experimentally in HCC for decades.[48] Among these studies, the identification of tumor infiltrating leukocytes (TILs) has been becoming a milestone in oncologic immunology.

The development of immunomodulation treatments, including antibody-based therapies, immune checkpoint blockade, cytokine targeting, and adoptive cell therapy, have been tested for many years.[57] Some of these immunotherapies have obtained positive results. For instance, in the latest finding, nivolumab, an immune checkpoint inhibitor of PD-1, has shown potential for treatment of advanced HCC.[58]

Because HCC can be ideally treated with liver transplantation (LT) post treatment immunosuppression also needs to be considered. It is widely known that immunosuppression is in part responsible for tumor recurrence.[59] Additionally, immunosuppression is also responsible for the development of de-novo malignancies and other serious complications.[60-63] Hence, an optimized immunosuppression for

those transplanted because of a malignancy benefits the recipients and requires the in-depth assessments of immunological parameters individually as a precondition.[64]

Out of these reasons, this makes having a profound understanding of HCC immune system a priority which could enable us to establish clinical evaluations of the immune status of cancer for diagnostic, prognostic, and therapeutic purposes.

1.5. Quantification of Immune Cells

In recent years, the understanding of tumors regarding their dynamic, growth and so their composition has changed. It has become increasingly clear that malignant neoplasms are also influenced by cellular and non-cellular tumor components, so-called tumor stroma. The tumor stroma influences carcinogenesis and tumor biology.[65] This is in part why a mere description of tumor burden, such as in the TNM tumor staging system, does not always have a high predictive value.[66, 67] Therefore, immune cell infiltration, the most common examined tumor stromal cells have become a focus of intense research.[68, 69] In several tumor entities it has been reported that stromal cells such as fibroblasts may also have a regulatory function in the biologic behavior of malignancies.[70]

The immune components of the tumor stroma especially $CD3^+$ and $CD8^+$ infiltrating cells have frequently been reported in different tumor entities.[71, 72] In fact, some studies suggest that peri- and intratumoral immune cell infiltration exceeds the established staging systems (i.e. TNM) in predictability.[73] Therefore, quantification of cancer infiltrating immune cells has been described as a new clinical score across different tumor entities.[65, 67, 74, 75] Although many publications describe influence on survival, description of section, preparation, staining, and counting methods have often been vague or not mentioned.[73, 76] Contrary to that, there are quantification methods in immunohistochemistry (IHC) which are widely standardized. The Ki67 index, for example, is essential for neuroendocrine tumors in clinical practice. However, since its establishment, different counting concepts have been adopted. Because of

these differences, methodological studies were needed to identify the best counting methods.[76, 77] Similarly, to provide predictive scoring of the tumor stroma across tumor entities, the processes need to be well defined, reproducible, and readily available. In this way results reported in the literature can be put into perspective and compared directly.

Independent from the type and the number of tumor-infiltrating leukocytes (TILs), the location should also be considered when predicting the outcome. Recent years, studies have suggested that high immune cell densities at the invasive margin (IM) might have a better prognostic value than intratumoral (IT) leukocytes in colorectal cancers.[73, 78] Because colorectal cancers inherently have a polarity to the lumen and the basis of the tumor, the invasive margin represents a battlefront at the border of the healthy immune system.[79] However, studies could not confirm that the so-called IM is the equally relevant for HCC. [71, 80] This might be due to the predominant histological growth pattern of HCC. HCC shows an expansive growth within a protective fibrous capsule instead of an infiltrative pattern.[81, 82] Additionally, the normal liver tissue has a unique immunological tolerance property.[83] The tumor encapsulation prevents cancer cells from invading but also blocks the infiltrating leukocytes.[84]

As described above 75% of blood supply in the normal liver comes from the hepatic portal veins, while the remaining 25% comes from the hepatic arteries.[42] In contrast to that, HCCs are predominantly supplied by neovascularized arteries.[85] The formation of these new vessels is essential for the tumor growth and progression by providing nourishment and oxygen. Conversely, however, it can also promote immune cell infiltration which is logically dependent on the network of blood vessels. It has been evidenced that the high degree of endothelial venules within solid human tumors is a strong predictor of the infiltrating T and B cells.[86] In this setting, migration of the TILs from the normal immune system to HCC will not originate at the IM but through extravasation in the intratumoral vessels.[87] The migration of lymphocyte from the blood to the target tissue has been well described. This migration process

includes rolling, adhesion, extravasation, and chemotaxis. Because of minimized shear forces, extravasation mainly occurs at the post-capillary venules. [87-89]

Fittingly, HCC has a high degree of angiogenesis, and the surface area of these vessels represents the largest border from malignant tumor to the normal immune system.[88] However, no previous study has provided any information on the perivascular infiltration.

1.6. Aim of this Study

This study aimed to quantify the tumor-infiltrating leukocytes and to show the connection between perivascularly infiltrating leukocytes and overall and disease-free survival in patients that received liver resection for HCC with curative intent. The measured leukocyte markers were chosen to represent the cellular, humoral, and innate immunity.

Furthermore, these results were correlated and compared with demographics, clinical characteristics, pathological results, laboratory values, overall and disease-free survival.

2. Material and Methods

2.1. Materials

2.1.1. Laboratory Equipment

Electronic pH meter	Knick, Germany
Microtome	Thermo Scientific, USA
Microscope	Olympus, Japan
Pipettes	Eppendorf, Germany
Micro-Centrifuge	NeoLab, Germany
Shaker	Edmund Bühler, Germany
4°C fridge	Liebherr, Germany
-20°C fridge	Bosch, Germany
37°C incubator	Binder, Germany
54°C incubator	Memmert, Germany
Water bath	Julaba, Germany
Electronic balance	Chyo, Japan
Magnetic mixer	GLW, Germany

2.1.2. Computer and Software

Computer hardware	HP, USA
ZEN software	Version 2.0, Carl Zeiss, Germany
Prism	Version 7.0a, GraphPad Software, USA
ImageJ	Version 1.51h, National Institutes of Health, USA
SPSS Statistics	Version 24.0, IBM, USA

2.1.3. Consumables

Microscope slides	Superfrost Plus, Thermo Fisher, USA
Cover slips	Menzel, Thermo Fisher, USA
Hydrophobic pen	S2002, Dako Pen, Agilent Technologies, USA
Aqueous mounting agent	1.08562.0050, Merck Chemicals, Germany
Gloves	ecoSHIELD, USA
Safe-Lock tubes	Eppendorf, Germany
Pipettes reloads	Eppendorf, Germany
Serological pipettes	SIGMA-ALORICH, USA
Parafilm	Pechiney, USA
Centrifuge tube	TPP, Switzerland

2.1.4. Chemicals

99% Ethanol	603-002-00-5, SAV LIQUID PRODUCTION, Germany
96% Ethanol	1000463926011, CLN GmbH Chemikalien Laborbedarf, Germany
70% Ethanol	1004051526001, CLN GmbH Chemikalien Laborbedarf, Germany
Xylene	9713.4, CARL ROTH, Germany
Mayer's hemalum solution	109249, Merck Chemicals, Germany
Citric acid	X863.2, CARL ROTH, Germany
Trisodium citrate dehydrate	3580.3, CARL ROTH, Germany
Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate	E5513, SIGMA-ALORICH, USA
TRIZMA base	T6066, SIGMA-ALORICH, USA
Sodium chloride	71380, SIGMA-ALORICH, USA
Tween 20	37470, SERVA, Germany
PBS buffer (10X Dulbecco's)	Power BC, PanReac AppliChem, Germany

Albumin fraction V	0163.4, CARL ROTH, Germany
Horse Serum	H1270, SIGMA-ALORICH, USA

2.1.5. Buffers and Solutions

Citrate Buffer Solution A

10.5g	Citric acid
500ml	Distilled water

Citrate Buffer Solution B

14.7g	Trisodium citrate dehydrate
500ml	Distilled water

Citrate Buffer

18ml	Solution A
82ml	Solution B
pH	6.0

EDTA solution

0.372g	EDTA disodium salt dihydrate
1L	Distilled water
pH	8.0

10x TBS buffer

24.2g	TRIZMA base
80g	Sodium chloride
1L	Distilled water

1x TBS buffer

100ml	10x TBS buffer
900ml	Distilled water

1x TBS-T buffer

1L	1x TBS buffer
1ml	Tween 20

10x PBS buffer

95.5g	PBS buffer (10X Dulbecco's)
1L	Distilled water

1x PBS buffer

100ml	10x PBS buffer
900ml	Distilled water
pH	7.5

5% BSA (bovine serum albumin) /PBS solution

2g	Albumin fraction V, biotin-free
40ml	1x PBS buffer

2.1.6. Antibodies

Anti-CD3	Ab5960, Abcam, UK
Anti-CD8	Ab17147, Abcam, UK
Anti-CD20	Ab78237, Abcam, UK
Anti-CD66b	Ab197678, Abcam, UK
Biotinylated Horse Anti-Rabbit IgG	BA-1100, VECTOR Laboratories, USA
Biotinylated Horse Anti-Mouse IgG	BA-2000, VECTOR Laboratories, USA

2.1.7. Blocking, Staining, and Substrate kits

Avidin/Biotin Blocking Kit	SP-2001, VECTOR Laboratories, USA

VECTASTAIN ABC-AP Staining Kit (Alkaline Phosphatase)	AK-5000, VECTOR Laboratories, USA
ImmPACT Red Alkaline Phosphatase Substrate Kit	SK-5105, VECTOR Laboratories, USA
Levamisole Solution	SP-5000, VECTOR Laboratories, USA

2.2. Methods

2.2.1. Literature Review

A comprehensive search was performed in PubMed database up to November 2016 for all available studies to assess the prognostic value of tumor-infiltrating leukocytes in patients with HCC after surgery. Since the introduction of Milan criteria in 1996, and the implementation in the following years, the year of 2000 has been widely considered as a historical watershed of surgical practice of HCC.[90-92] The studies before 2000, therefore, were excluded in this review. I used the following search terms ("Carcinoma, Hepatocellular"[Mesh] OR "Liver Neoplasms"[Mesh]) AND ("Survival"[Mesh] OR "Prognosis"[Mesh] OR "Survival Analysis"[Mesh] OR "Survival Rate"[Mesh] OR "Disease-Free Survival"[Mesh]) AND ("Lymphocytes, Tumor-Infiltrating"[Mesh] OR "Neutrophil Infiltration"[Mesh] OR "Leukocytes"[Mesh]). To implement our search, studies in relevant references were also included. Following exclusion criteria were used: 1) Published before January 2000; 2) Published not in English; 3) Not human primary HCC; 4) Immunotherapy studies; 5) Not intratumoral infiltration; 6) Case reports or reviews; 7) Genetic or functional studies.

The required data was obtained from all available studies, including titles and abstracts to recognize the potentially available publications and then the full-texts for further investigations. Following data were extracted: the author's name, study regions, publication year, sample size, types of immune cells, results regarding clinical information, such as demographics, etiology, tumor characteristics, and survival

analysis. Additionally, technical aspects such as slide preparation, counting methods, and grouping methods were analyzed.

2.2.2. Patients and Clinical Data

Tissue samples and annotated data were obtained, and experimental procedures were performed within the framework of the non-profit Foundation Human Tissue and Cell Research (HTCR), including the informed patient's consent. [93] As required by the HTCR Foundation and in accordance with the Declaration of Helsinki the tumor samples were anonymously coded. This study was approved by the institutional review board of the Ludwig-Maximilians University in Munich (EK266-16, EK258-16).

As mentioned above prospectively stored tumor samples of 60 patients who underwent curative resection from November 2004 to October 2015 in our institution were used. Additionally, clinical characteristics including gender, age, hepatitis, the presence of cirrhosis, tumor number, microvascular and macrovascular invasion, Milan staging. As well, routine lab values including serum alpha-fetoprotein (categorized by 20ng/ml and 400ng/ml which are recommended cut-off values for HCC investigation and diagnosis, respectively.[4] negative: ≤20ng/ml, low: >20ng/ml and ≤400ng/ml, high: >400ng/ml), bilirubin, albumin, alanine transaminase (ALT), aspartate transaminase (AST), activated partial thromboplastin time (APTT), creatinine, c-reactive protein (CRP), leukocytes count, and platelets count of all patients were examined by laboratory medicine department and collected in our database.

2.2.3. Immunohistochemistry Staining

Paraffin blocks were stored in the -20°C fridge for 30 minutes in advance of cutting, then the 4μm sections were cut on a microtome, and were floated in a 40°C water bath. Then the slices were mounted onto the microscope slides. The slides were dried and then stored them in 54°C incubator for 1 hour and 37°C incubator overnight before the staining. De-paraffinization and re-hydration were performed gradually in Xylene,

100%, 96%, 70% Ethanol, and distilled water. For CD3, CD20 and CD66b staining, the antigen retrieval was performed in citrate buffer in the 96°C water bath for 30 minutes. For CD8 staining, EDTA buffer was used exclusively instead of citrate buffer; and the heating time in the water bath was shortened to 15 minutes to avoid tissue deconstruction. Avidin/biotin block was performed for 20min each and was followed by protein block for 1 hour at least in humidified chambers. 5% BSA/PBS solution was used to dilute both primary antibody and the appropriate isotype controls. Incubation was maintained at 4°C overnight. After washing, PBS solution was used to dilute the appropriate secondary antibodies. Incubation was maintained at room temperature for 30min. After washing, ABC-AP reagent was added and incubated for 30 min. Followed was the substrate reaction, the staining process was being checked under a microscope, once an acceptable staining intensity was observed, the reaction was stopped immediately by removing the reagent and washing them in PBS for 5min. Counterstaining was performed in fresh hematoxylin for 1 second. After the slides dried at room temperature, coverslips were mounted on the slides with 1-2 drops of the aqueous mounting agent.

Quality control after immunohistochemistry was implemented according to Maxwell et al.[94] Only stained slides with an excellent quality were permissible for further analysis.

2.2.4. Digital Imaging

The slides were visualized under the microscope. Images of hotspots were captured with 200x enlargement using the ZEN software.

2.2.5. Methodological Considerations Regarding the Development of the Quantification Algorithm

For the development of a reliable and accurate algorithm to quantify the tumor stroma (QTS), ten slides stained with anti-CD3 and CD8 were chosen in different tumor entities. These markers represent the most often chosen markers for tumor infiltrating leukocytes. Additionally, these markers were common in all collaborating working groups to ensure comparability. These tumor entities were: PDAC, mCRC, and OvCa from the respective working groups in an interdisciplinary collaboration. Eventually, in total, 80 slides were obtained. These experiments were approved and registered by the Human Tissue and Cell Research (HTCR) foundation and institutional review board of the Ludwig-Maximilians University in Munich (PDAC: 807-16, OvCa: 278-04, mCRC: 252-04).

The staining of PDAC slides was performed by Miksch (Working group: D'Haese). The staining procedures of frozen sections (OvCa and mCRC) were done by Dötzer and Schlüter (Working group: Mayer). Formal consent by the working group leaders has been obtained to use data and imagery in this dissertation.

Reliability and accuracy of computed quantification were tested to develop a general algorithm:

First, the reliability of identification of hotspots was investigated using two blinded observers (HCC: Hao and Schoenberg, PDAC: Hao and Miksch, OvCa and mCRC: Dötzer and Schlüter). The absolute amounts of cells were compared with the intraclass correlation coefficient (ICC) to identify differences between two blinded observers.

Second, accuracy was tested. The following computerized counting methods: ZEN2 software automated counting (ZAC), ImageJ software with a subjective threshold (IST) and ImageJ with color deconvolution (ICD) were compared to a manual counting (regarded as golden standard) using a linear regression analysis.

Furthermore, duration to count one hotspot and the economic costs were compared for every method.

2.2.6. Counting Methods

Manual Counting

Manual counting was defined as the gold standard. Using the ImageJ Software, the cells were manually counted by the functions of “Analyze” and “Cell Counter” (Figure. 3).

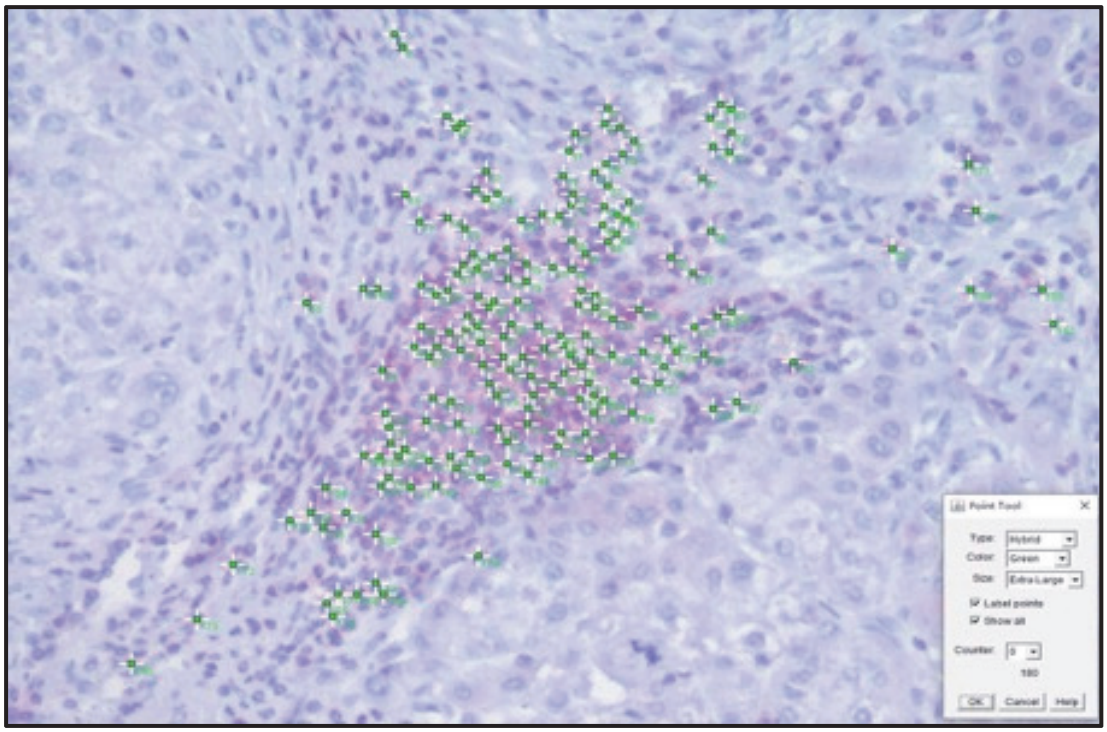

Figure 3. Manual Counting Method Shown Representatively of Infiltrating $CD3^+$ Cells in Hepatocellular Carcinoma

Zen 2 Software Automated Counting (ZAC)

The image analysis was configured by defining the measuring frame. Then, automatic segmentation by the specification of the color spectrum was included. “Interactive segmentation” was the next function.

Finally, I defined the measurement features (scope, area, color spectrum, density) and measured the stained cells. These steps were standardized for each antibody and each tumor entity (Figure. 4).

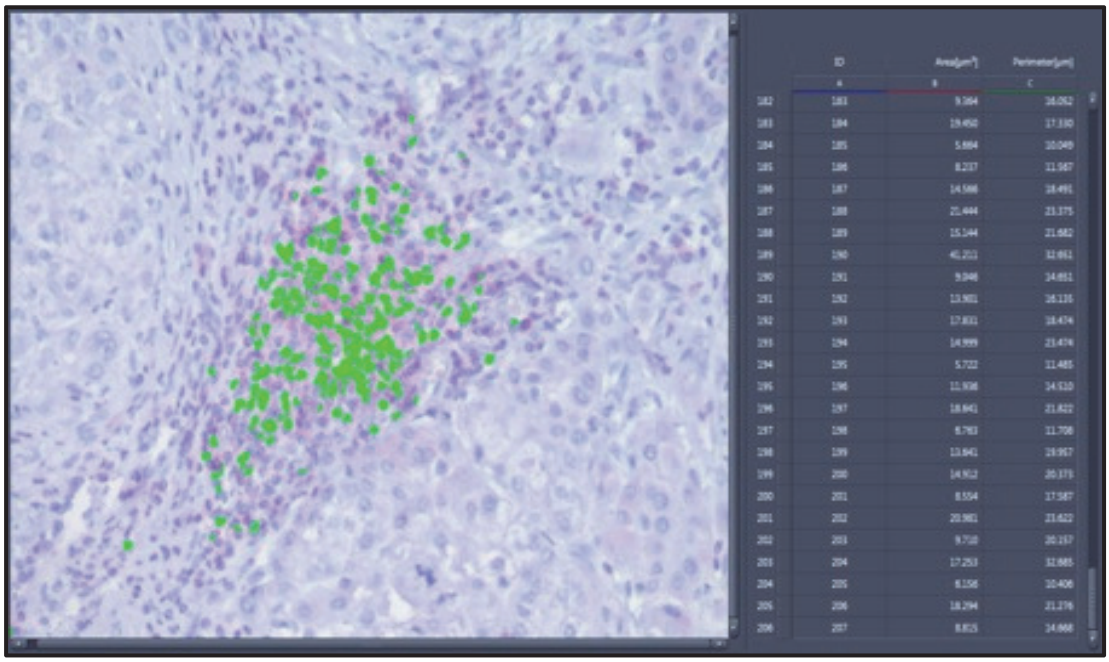

Figure 4. Automated ZEN2 Software Counting Method Shown Representatively of Infiltrating $CD3^+$ Cells in Hepatocellular Carcinoma

ImageJ with Subjective Threshold (IST)

First, the original picture was changed to a 32-bit format and the subjective staining threshold defined using the standard ImageJ software. With the so-called watershed function of the ImageJ software, a separation of larger particles was performed. These particles were then automatically counted using the software function “analyze particles” for quantification (Figure. 5).

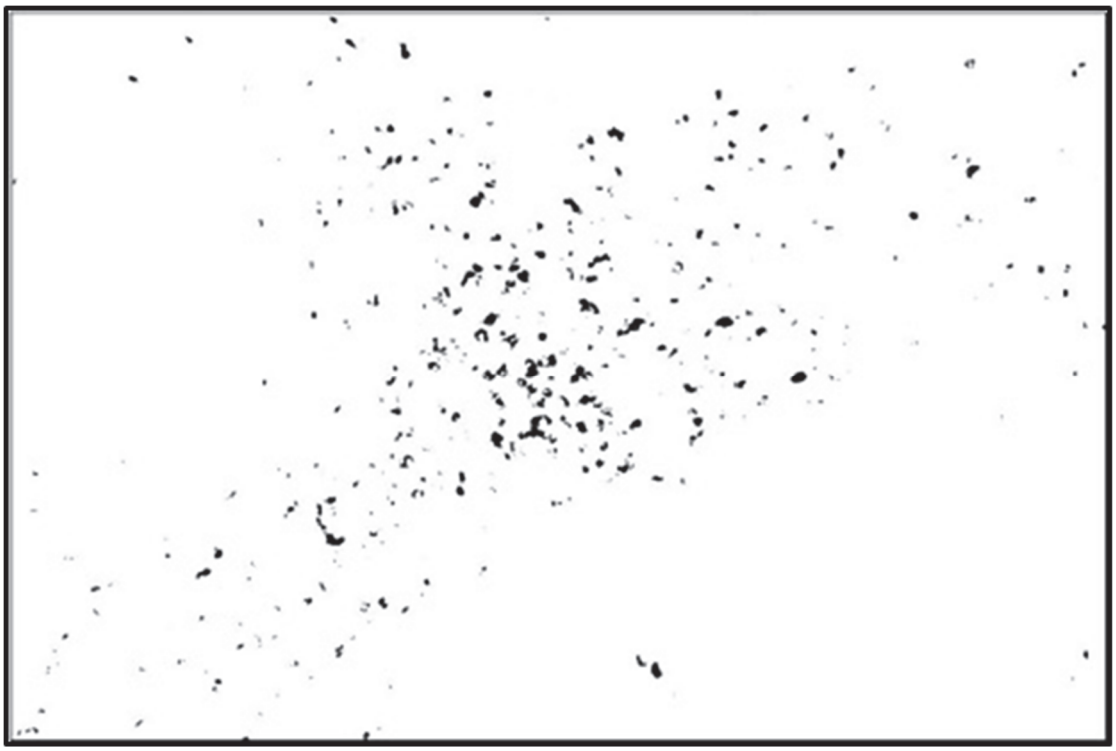

Figure 5. ImageJ with Subjective Threshold Method Shown Representatively of Infiltrating $CD3^+$ Cells in Hepatocellular Carcinoma

ImageJ with Color Deconvolution (ICD)

The color deconvolution application for ImageJ is freely available as an add-on tool to the standard software. The original picture of a hot spot was split into three color spectra, and quantification of red particles is performed (Figure. 6).

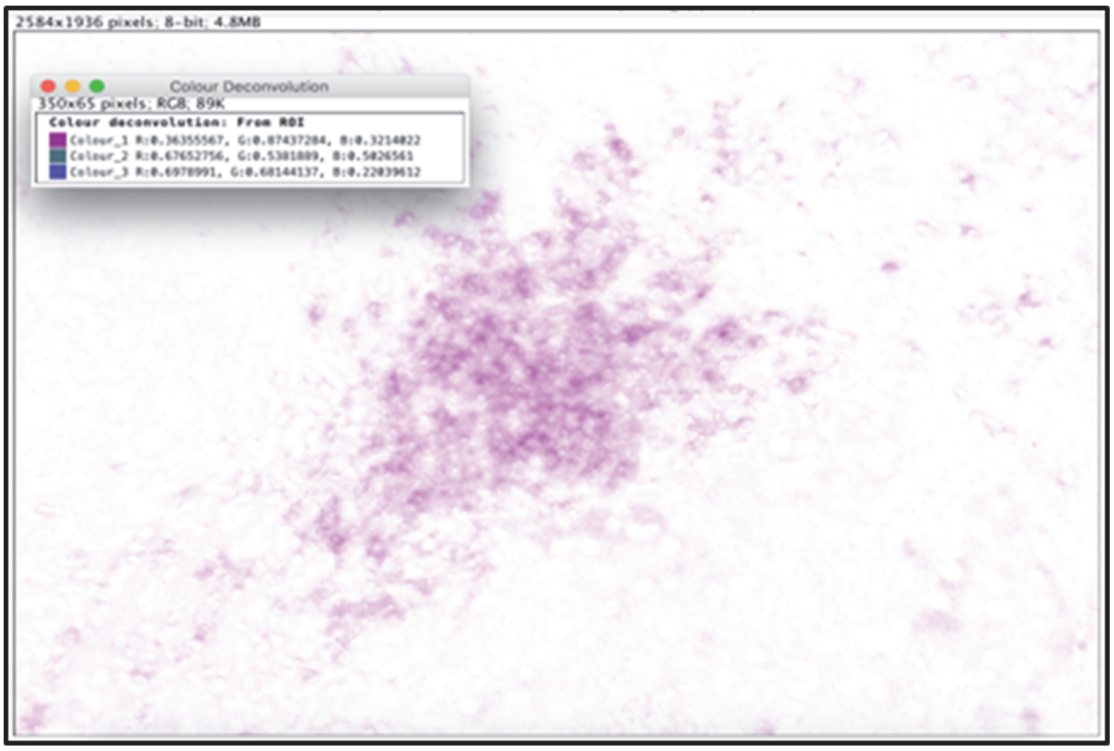

Figure 6. ImageJ with Color Deconvolution Method Shown Representatively of Infiltrating $CD3^+$ Cells in Hepatocellular Carcinoma

2.2.7. Statistical Analysis

Continuous numbers are presented as mean ± SD or as median ± SD appropriately. They were compared using the independent t-test if in normal distribution or using the Mann-Whitney u test if they were non-normally distributed. The χ-square test or Fisher's exact test was adopted to compare contingency variables. Pearson's r values were calculated for evaluation of correlation. The values of intraclass correlation (ICC) and regression coefficient B were adopted for the reliability and accuracy in the methodological development. Median overall and disease-free survival time were calculated to classify the patients into good (>medians) or bad (≤medians) survivor groups for receiver operating characteristic (ROC) analysis. The median values of each kind of immune cells were calculated to classify the patients into high (>medians) or low (≤medians) groups for survival analysis. The log-rank test was used in univariate survival analysis. The Cox model was used in multivariate survival analysis. The Collett's model selection approach was adopted for covariate screening. It is a

selection process that employs a stepwise evaluation of covariates with $p_1<0.200$ in the univariate analysis. After inclusion in the multivariate analysis, these covariates are eliminated if p_2 is greater than 0.100. To double check all other covariates a forward selection multivariate analysis with the non-predictive variables is performed (p_3). If the variables achieve $p_3<0.100$ they too can be included in the final multivariate analysis. For the final multivariate analysis, covariates are independently predictive if $p_4<0.050$.[95] DFS was defined as the period after resection in which there was no sign of HCC recurrence. OS was defined as the time from resection to death. Reporting on these biomarkers for DFS and OS was conducted according to the REMARK statements. All statistics were performed using Prism and SPSS statistics software. $P<0.050$ was considered statistically significant.

3. Results

3.1. Literature Review

As is shown in Figure 7, 467 related publications were obtained following the searching strategy, 435 articles were excluded per the exclusion criteria. The reasons for exclusion were listed in the flow-chart depicted in Figure 7. One paper had to be excluded because no full-text was available.[96] Finally, 32 studies were included for this literature review.

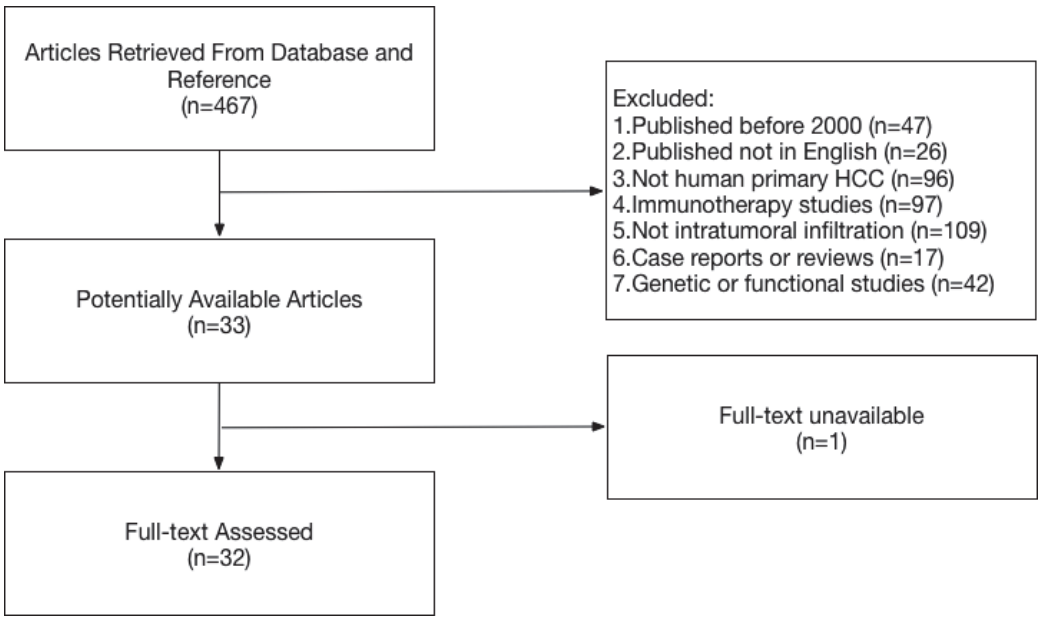

Figure 7. Flowchart of the Study Selection.

Altogether tumor-infiltrating immune cells were examined in 32 studies including 4701 patients. 27 (84.4%) studies were performed in East Asia, especially in China (24, 75.0%), where the highest HCC burden worldwide is recorded due to the endemic HBV infections. In contrast, only four studies were conducted in Europe (12.5%) and one study in North America (3.1%). (Figure 8A) Also, most of the studies (30, 93.8%) emphasized the influence of immunological infiltration on the prognosis of curative resected patients. However, only three studies (9.4%) offered information on patients undergoing liver transplantation.[97-99] (Figure 8B)

In most studies (26, 81.3%) immunohistochemistry (IHC) combined with computer assisted counting methods were used. Tissue microarrays (TMAs) were used in 6 (18.8%) studies, whereas 20 (62.5%) studies examined entire slides conventionally. In four prospective studies, fluorescence-related methods, including immunofluorescence microscopy[100] and flow cytometry[101-103], could characterize antigen expression using fresh tissue with multicolor combinations. (Figure 8C)

The most common grouping method was division by the median value of the cell counts. This was examined in 19 studies (59.4%). The other grouping methods included mean value of cell counts (3 studies, 9.4%),[97, 104, 105] certain ratio of relative cell types (2 studies, 6.3%),[99, 106] an outcome-based cut-point optimization software (X-tile, Yale University, US) (1 study, 3.1%), and undefined or subjective decisions (7 studies, 21.9%).[102, 103, 107-111] (Figure 8D)

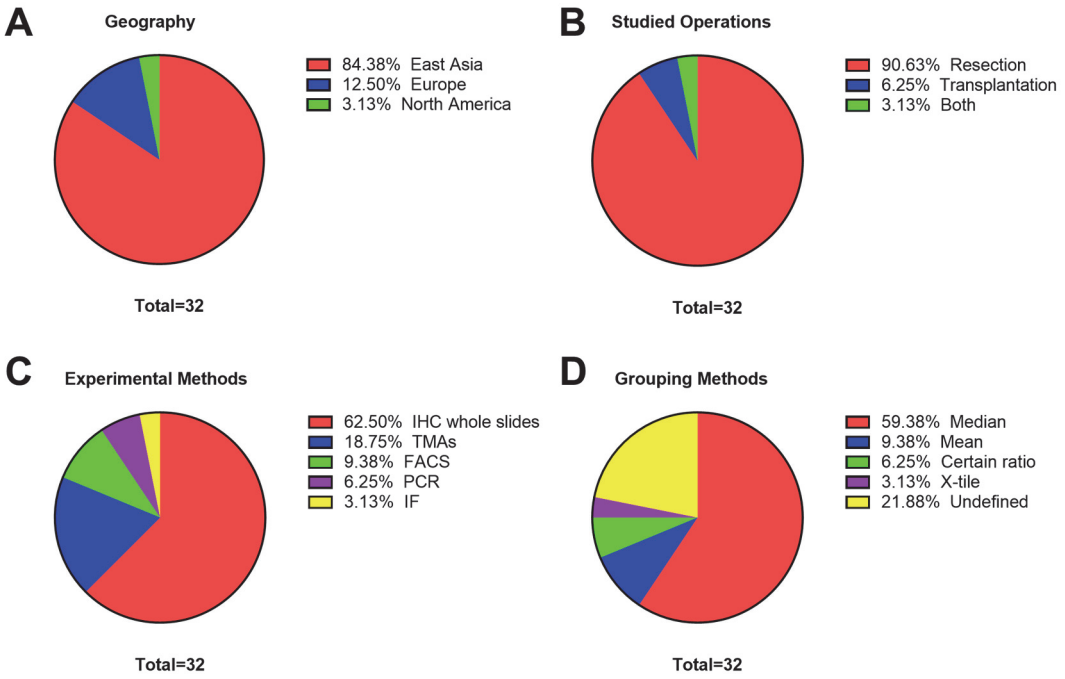

Figure 8. Characteristics of Included Literature. (Abbreviations: IHC: Immunohistochemistry; TMAs: Tissue Micro-Arrays; PCR: Real-time Polymerase Chain Reaction; FACS: Fluorescence-activated Cell Sorting; IF: Immunofluorescence Microscopy.)

In the following paragraphs, the results regarding their correlation with clinical characteristics are listed divided by the adaptive and innate immunology as well as by cell subsets.

As shown in Table 1, higher intratumoral infiltration of $CD3^+$ cells (CD3 commonly expressed on total T cells) predicted better disease-free survival (DFS) or overall survival (OS) after HCC resection in five studies.[69, 71, 100, 107, 112] However in three studies, there was no significant difference.[98, 113, 114] Fourteen articles provided information regarding infiltrating $CD8^+$ cells (CD8 commonly expressed on cytotoxic T cells), eight of them demonstrated that it could predict a better DFS or OS,[69, 71, 97, 105, 107, 110, 115, 116] two of them failed to validate the predictive significance but found it correlated with lower TNM stages and fewer tumor lesions, respectively.[114, 117] $FoxP3^+$ cells (FoxP3 commonly expressed on regulatory T cells) were investigated in 14 papers, ten of them found it could predict worse DFS or OS,[80, 99, 106, 113, 114, 118-122] and nine of them verified that it had closer relationships with more aggressive clinical characteristics including the presence of cirrhosis, tumor size, vascular invasions, tumor differentiation, tumor encapsulations, and staging.[80, 97, 103, 109, 114, 118, 120, 121] However, $CD25^+$ cells (CD25 commonly expressed on regulatory T cells and conventional T cells) had no significance on survival in two cohorts.[97, 102] Furthermore, $CD45RO^+$ cells (CD45RO commonly expressed on memory T cells) also had been proved to correlate with the better outcome by three research groups.[98, 107, 123] The $IL\text{-}17^+$ cells (commonly known as Th17 cells) had a controversial effect on the outcome. Two studies confirmed its protective effect and three studies with contradicting results.[104, 106, 108, 111, 122] Also, Yan, J., et al. revealed that the $IFN\text{-}gamma^+$ cells (commonly known as total Th1 cells) correlated with worse outcome.[104]

Only two studies were found to investigate the intratumoral infiltration of $CD20^+$ cells (CD20 commonly expressed on B cells). One proved its positive correlation with OS after resection; the other one found no significance at all.[98, 100]

As shown in Table 1, there was only one study providing information that intratumoral infiltrating $CD66b^+$ cells (CD66b commonly expressed on neutrophils) could predict

worse DFS and OS.[116] Contradictory prognostic significances regarding the $CD68^+$ cells (CD68 commonly expressed on macrophages) were reported in two studies respectively; another two studies failed to validate them.[98, 110, 123, 124] One research group found $CD16^+$ cells (CD16 commonly expressed on monocytes) correlated with worse DFS and OS.[115] Wu, Y., et al. found that the $CD57^+$ cells (CD57 commonly expressed on NK cells) correlated with better survival and clinical characteristics including vascular invasions and staging which had not been validated in a former study.[98, 125] However, Chew, V., et al. previously had demonstrated the benefits of infiltrating $CD56^+$ cells (CD56 commonly expressed on NK cells).[110] $S\text{-}100^+$ cells (S-100 commonly expressed on DCs) were reported to be associated with better DFS in one study.[107] Mast cells (commonly characterized by mast cell tryptase (MCT)) were also reported by Tu, J.F., et al. to be associated with poor OS.[111]

In light of the published literature, the combination of CD3, CD8, CD20, and CD66b promises to yield an overview of the cellular and humoral adaptive immune system as well as the innate immune system.

Table 1. Summary of Included Studies

Abbreviations: CTLs: Cytotoxic T Lymphocytes; DCs: Dendritic Cells; Tregs: Regulatory T cells; NK: Natural Killer; MCT: mast cell tryptase; IHC: Conventional Immunohistochemistry with whole sildes; TMA: Tissue Micro-Arrays; PCR: Real-time Polymerase Chain Reaction; FACS: Fluorescence-activated Cell Sorting; IF: Immunofluorescence Microscopy; AFP: Preoperative Serum Alpha-fetoprotein; DFS: Disease-free Survival; OS: Overall Survival; N/A: Data not found; N.S.: Data found but no significance.

Reference	**Study Population**			**Study Cell Type**				**Higher Infiltration Association**	
	No. Patients	**Region**	**Surgery**	**Marker**	**Cell Type**	**Experimental Method**	**Grouping Method**	**Clinicopathological Characteristics**	**Survivals**
Ikeguchi, M., et al. 2004[117]	60	Japan	Resection	CD8	CTLs	IHC	Medians	Lower TNM Stages	N.S.
Cai, X. Y., et al. 2006[107]	123	China	Resection	CD3	T cells	IHC	N/A	N/A	Better DFS
				CD8	CTLs			N/A	Better DFS
				CD45RO	Memory T cells			N/A	Better DFS
				S-100	DCs			N/A	Better DFS
Unitt, E., et al. 2006[97]	69	UK	Transplantation	CD4	Helper T cells	IHC	Ratio	N.S.	Worse DFS
				CD8	CTLs		Ratio	N.S.	Better DFS
				CD25	Tregs		Means	N.S.	N.S.

				FoxP3	Tregs		Means	More Vascular Invasions	N.S.
Kobayashi, N., et al. 2007[118]	218	Japan	Resection	CD8	CTLs	IHC	Medians	More Females	N.S.
				FoxP3	Tregs			Poorer Differentiation	Worse DFS and OS
Gao, Q., et al. 2007[113]	302	China	Resection	CD3	T cells	TMA	Medians	N/A	N.S.
				CD4	Helper T cells			N/A	N.S.
				CD8	CTLs			N/A	N.S.
				FOXP3	Tregs			Fewer Tumor Encapsulations	Worse DFS and OS
								More Vascular invasions	
				Gr-B	Activated CTLs			N.S.	Better OS
Sasaki, A., et al. 2008[119]	252	Japan	Resection	FoxP3	Tregs	IHC	Medians	Lower AFP	Worse DFS
Zhou, J., et al. 2009[120]	85	China	Resection	FoxP3	Tregs	IHC	Medians	More Presences of Cirrhosis	Worse DFS and OS

								Higher TNM Stage	
Zhang, J. P., et al. 2009[108]	108	China	Resection	IL-17	Th17 cells	IHC	70%	N.S.	Worse DFS and OS
Ding, T., et at. 2009[124]	137	China	Resection	CD68	Macrophages	IHC	Medians	Larger Tumor Size	Worse DFS and OS
Li, Y.W., et al. 2009[123]	302	China	Resection	CD45RO	Memory T cells	TMA	Medians	Higher AFP	Better DFS
				CD68	Macrophages			N.S.	Better DFS and OS
Shen, X., et al. 2010[109]	31	China	Resection	FoxP3	Tregs	PCR	N/A	Higher TNM Stage	N/A
Yang, Z. Q., et al. 2010[103]	48	China	Resection	FoxP3	Tregs	FACS	N/A	Higher TNM Stage	N/A
Li, Y. W., et al. 2011[116]	281	China	Resection	CD8	CTLs	TMA	Medians	N.S.	Better DFS and OS
				CD66b	Neutrophils			N.S.	Worse DFS and OS
Shen, S. L., et al. 2011[121]	76	China	Resection	FoxP3	Tregs	IHC	Medians	More Vascular Invasions	Worse DFS and OS
Cariani, E., et al. 2012[112]	42	Italy	Resection	CD3/L	T cells	PCR	Medians	N/A	Better DFS

				CD4/3	Helper T cells			N/A	Better DFS and OS
				CD8/3	CTLs			N/A	Worse DFS and OS
				CD4 $RA^{+}127^{-}$				N/A	Worse OS
				CD8 $RA^{-}127^{+}$				N/A	Better DFS
Chen, K. J., et al. 2012[114]	141	China	Resection	CD3	T cells	IHC	Medians	N/A	N.S.
				CD4	Helper T cells			N/A	N.S.
				CD8	CTLs			Fewer Tumor Lesions	N.S.
				FoxP3	Tregs			More Presences of Cirrhosis	Worse OS
								Larger Tumor Size	
Chew, V., et al. 2012[110]	46	Singapore, China, and Switzerland	Resection	CD8	CTLs	IHC	N/A	N/A	Better OS
	36			CD56	NK cells			N/A	Better OS
	33			CD68	Macrophages			N/A	N.S.

Gao, Q., et al. 2012[98]	206	China	Transplantation	CD3	T cells	TMA	Medians	N/A	N.S.
				CD8	CTLs			N/A	N.S.
				Gr-B	Activated CTLs			N/A	N.S.
				FoxP3	Tregs			N/A	N.S.
				CD45RO	Memory T cells			Better Differentiation	Better DFS
				CD20	B cells			N/A	N.S.
				CD1a	DCs			N/A	N.S.
				CD83	DCs			N/A	N.S.
				CD57	NK cells			N/A	N.S.
				CD68	Macrophages			N/A	N.S.
Huang, Y., et al. 2012[80]	54	China	Resection	CD8	CTLs	IHC	Medians	N.S.	N.S.
				FoxP3	Tregs			N.S.	Worse DFS and OS
Lee, W.C., et al. 2012[102]	30	China	Resection	$CD4^+CD25^+$	Tregs	FACS	N.A.	Larger Tumor Size	N/A

Mathai, A. M., et al. 2012[99]	74 17	US	Transplantation Resection	FoxP3	Tregs	IHC	Ratio to CD8	Poorer differentiation	Worse DFS and OS
Lin, S. Z., et al. 2012[106]	245	China	Resection	FoxP3 IL-17	Tregs Th17 cells	IHC	Ratio to CD4 Ratio to CD3	N.S. N.S.	Worse DFS and OS Better DFS and OS
Wu, Y., et al. 2013[125]	256	China	Resection	CD57	NK cells	IHC	Medians	Less Vascular Invasions Lower TNM Stage	Better DFS and OS
Huang, Y., et al. 2014[122]	56	China	Resection	IL-17 FoxP3	Th17 cells Tregs	IHC	Medians	Larger Tumor Size Poorer Differentiation	Worse DFS Worse DFS and OS
Yan, J., et al. 2014[104]	150	China	Resection	IFN-g IL-17	Th1 cells Th17 cells	IHC	Means	More Hepatitis Background More Hepatitis Background	Worse DFS and OS Better DFS and OS
Sun, C., et al. 2015[71]	449	China	Resection	CD3	T cells	TMA	Medians	N/A.	Better DFS and OS

				CD8	CTLs			N/A	Better DFS and OS
Brunner, S. M., et al. 2015[105]	119	Germany	Resection	CD8	CTLs	IHC	Means	N/A.	Better OS
Li, X., et al. 2015[101]	34	China	Resection	$CD3^+CD56^+FoxP3^+$	-	FACS	Medians	N/A	Worse DFS and OS
Gabrielson, A., et al. 2016[69]	65	US	Resection	CD3 CD8	T cells CTLs	IHC	Medians	N/A N/A	Better DFS and OS Better DFS
Garnelo, M., et al. 2015[100]	112	Singapore, China, and Switzerland	Resection	CD3 CD20	T cells B cells	IF	Medians	N/A N/A	Better OS Better OS
Liao, R., et al. 2016[115]	387	China	Resection	CD8 CD16	CTLs Monocytes	TMA	X-tile	N/A.	Better DFS and OS Worse DFS and OS
Tu, J.F., et al. 2016[111]	57	China	Resection	IL-17 MCT	Th17 cells Mast cells	IHC	N/A	N/A. N/A	Worse OS Worse OS

3.2. Hepatocellular Carcinoma Has the Lowest Level of Lymphocyte Infiltration Compared with Other Tumor Entities

Immunohistochemical analysis revealed a positive CD3 and CD8 staining in all cancer tissue sections of mCRC, OvCa, HCC, and PDAC (Figure. 9). Therefore, the cells were counted manually using three hotspots per slide and analyzed using a descriptive statistic (Table 2). The highest level of $CD3^+$ cell infiltration was found in mCRC samples, in PDAC and OvCa this level was intermediate, and HCC samples showed the lowest level of the infiltration (Figure. 10A). These differences were significant. No difference was found of the $CD8^+$ cell infiltration in the tumor samples tested (Figure. 10B). As expected, the amount of $CD3^+$ cells were higher compared to $CD8^+$ ones (Figure. 10).

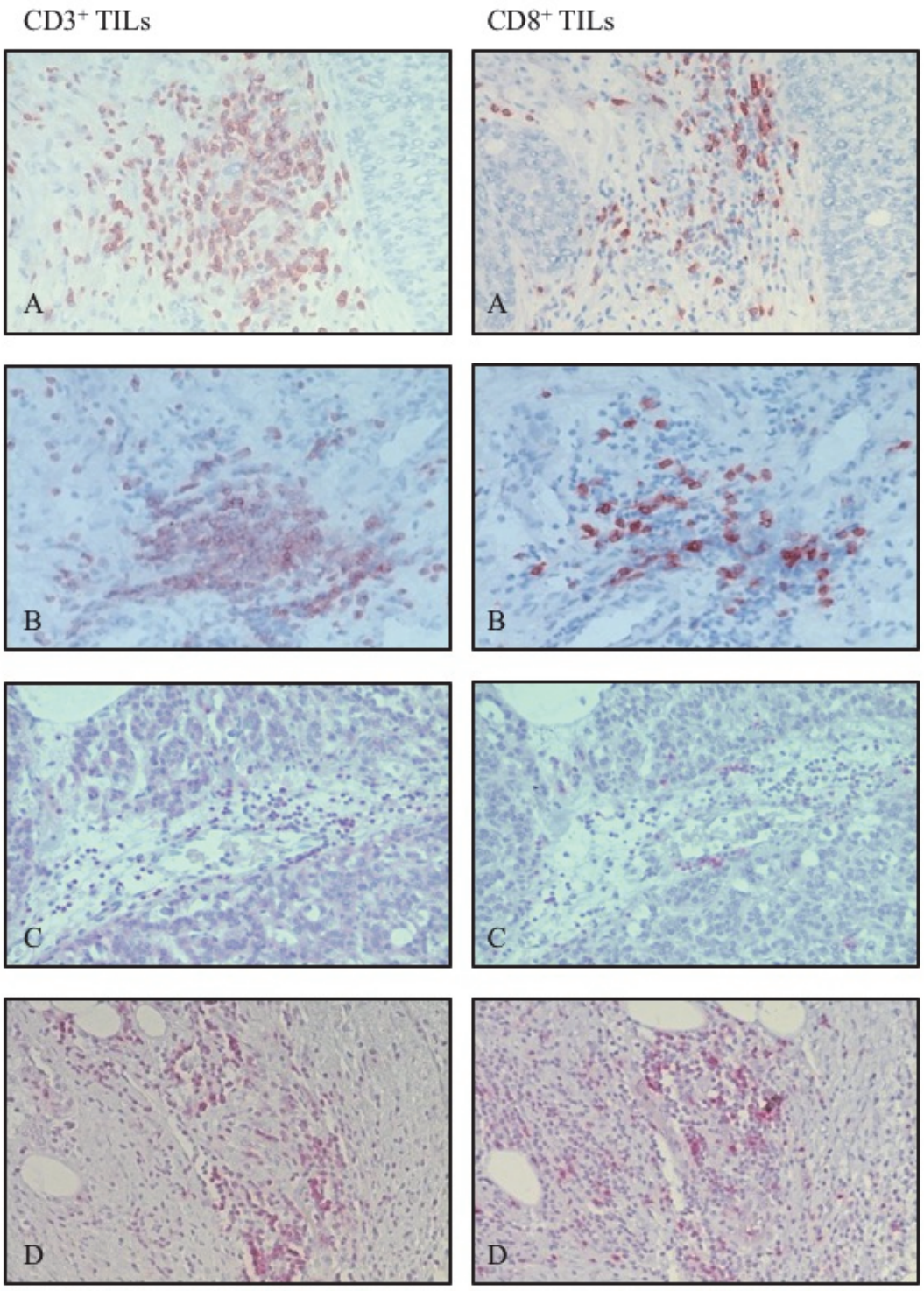

Figure 9. Representative Hotspots of Infiltrating $CD3^+$ and $CD8^+$ Cells out of the Same Area Using a Magnification of 200x (A: metastatic colorectal cancer, B: ovarian cancer, C: hepatocellular carcinoma, D: pancreatic ductal adenocarcinoma).

Table 2. Descriptive Statistic of Immunohistochemistry Analysis of Tumor Samples.

(Abbreviations: mCRC: metastatic colorectal cancer, OvCa: Ovarian cancer, HCC: Hepatocellular carcinoma, PDAC: Pancreatic ductal adenocarcinoma)

Cell amount		mCRC	OvCa	HCC	PDAC
CD3$^+$	Minimum	302	59	0	88
	25% Percentile	354.3	118	8.5	112.3
	Median	453	169.5	59.5	189
	75% Percentile	524.3	216.5	98.75	279
CD8$^+$	Minimum	23	5	0	0
	25% Percentile	90.75	38.5	2.75	77
	Median	127	87	64	115.5
	75% Percentile	146	119.8	116.3	152.8

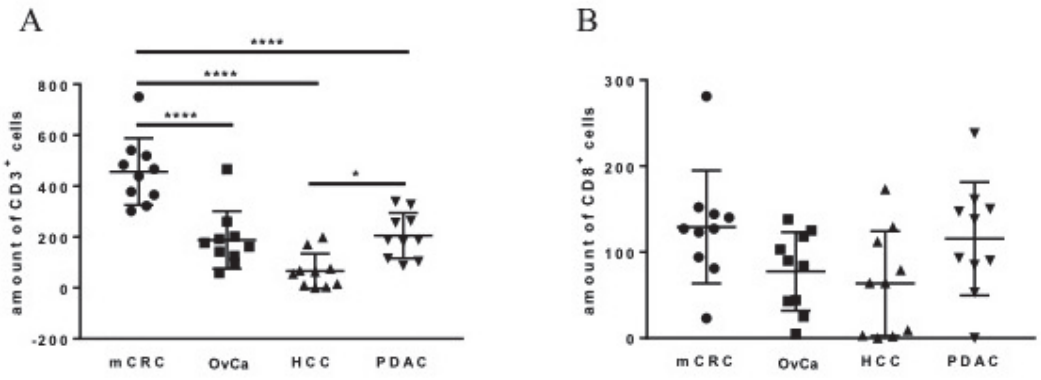

Figure 10. Amount of CD3$^+$ (A) and CD8$^+$ (B) Cells Identified with IHC in Tumor Samples. (The data of staining of 10 patients from each group are presented with SD and analyzed with the ordinary one-way ANOVA with Tukey's multiple comparisons post-test, * $p<0.05$ and **** $p<0.0001$.)

3.3. One Observer for Choosing Hotspots was Reliable

Quantification results with ICC from two blinded observers for reliable detection of hotspots were 0.949 in mCRC, 0.843 in OvCa, 0.805 in HCC, and 0.957 in PDAC. There was no significant difference in finding the largest hotspot in all tumor entities

comparing the two blinded observers. Therefore, one observer showed a high level of internal consistency.

3.4. ImageJ Subjective Threshold Counting Method is Accurate

The absolute cell count in one hotspot compared to the average in three hotspots did differ concerning regression coefficient B values over 1.2 for mCRC, OvCa, and PDAC and not HCC (ICC scores: 0.973 in mCRC, 0.945 in OvCa, 0.963 in HCC, and 0.952 in PDAC). Comparison of the computed methods to the gold standard of manual counting showed mostly excellent accuracy (Table 3). However, ZAC in PDAC yielded inconsistencies with ICC=0.601 and regression coefficient B=1.280. IST reached excellent results in all groups (Table 3). ICD reached excellent accuracy in frozen sections of mCRC, OvCa, and HCC but not in PDAC (Table 3).

Table 3. Different Methods of the Staining Analysis Compared to Manual Counting. (Abbreviations: ZAC: Zen2 Software Automated Counting, IST: Image J with Subjective Threshold, ICD: Image J with Color Deconvolution, mCRC: metastatic colorectal cancer, OvCa: Ovarian cancer, HCC: Hepatocellular carcinoma, PDAC: Pancreatic ductal adenocarcinoma, ICC: Intraclass correlation coefficient, B: Regression coefficient B)

Methods		mCRC	OvCa	HCC	PDAC
ZAC	**ICC**	0.926	0.987	0.869	0.601
	B	0.868	0.968	0.621	1.28
IST	**ICC**	0.973	0.992	0.955	0.934
	B	0.851	1.03	0.723	0.914
ICD	**ICC**	0.986	0.99	0.976	0.932
	B	0.945	1.06	0.791	1.327

3.5. Counting Time and Costs of All Four Counting Methods

Furthermore, the counting time was compared for each tumor entity and software. Manual counting and ImageJ software with subjective threshold took most of the time,

whereas time could be saved using computer assisted automatic counting methods (ZAC and ICD) (Table 4).

Regarding costs for the measurement the presence of a microscope is required and presumed for each laboratory. However, the price for hardware to connect to the microscope and the computer is more 2000€ (Solution used in our laboratory: AxioVision, Carl Zeiss Inc., Germany). While ImageJ can be downloaded for free, the proprietary ZEN 2 blue software costs more than 4000€. This amounts to costs in excess of 6000€ for the proprietary software solution.

Table 4. Counting Time in Minutes for Each Method.

(Abbreviations: ZAC: Zen2 Software Automated Counting, IST: Image J with Subjective Threshold, ICD: Image J with Color Deconvolution, mCRC: metastatic colorectal cancer, OvCa: Ovarian cancer, HCC: Hepatocellular carcinoma, PDAC: Pancreatic ductal adenocarcinoma.)

	MC		ZAC		IST		ICD	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
mCRC	10	1-12	1	1-2	10	5-14	6	4-7
OvCa	10	1-12	1	1-2	10	5-14	6	4-7
HCC	10	1-12	2	1-3	5	1-8	10	1-14
PDAC	8	1-12	1	1-2	4	1-9	2	1-3

3.6. Characteristics of HCC Study Cohort

Tissue samples from 60 resected HCC tumors were assessed. The demographical characteristics are summarized in Table 5. Most (81.67%) of the patients were male. The median age was 66.00±15.43 years old. Only seven patients had hepatitis B in their medical history. No hepatitis C infection was present in the study cohort. According to the preoperative imaging and postoperative pathological reports, nine patients (15.00%) had more than one tumor lesion. 18 cases (30.00%) had microvascular invasions, and six patients (10.00%) showed macrovascular invasions on imaging. 47 (78.33%) were outside the Milan criteria.

Table 5. Demographics of Study Population.

(Abbreviations: SD, standard deviation; HBV, hepatitis B; HCV, hepatitis C; AFP: serum alpha-fetoprotein; ALT: alanine transaminase; AST: aspartate transaminase; APTT: activated partial thromboplastin time; CRP: C-reactive protein)

Variables	Results
Gender(Male/Female)	49 (81.7%)/11 (18.3%)
Age(Year) (Median ± SD)	66.00±15.43
Hepatitis (HBV/HCV)	7 (11.7%)/0 (0.0%)
Cirrhosis	15 (25.0%)
AFP (negative/low/high)	33(55.0%)/13(21.7%)/9 (15.0%)
Tumor multiplicity	9 (15.0%)
Microvascular invasion	18 (30.0%)
Macrovascular invasion	6 (10.0%)
Beyond Milan Criteria	47 (78.3%)
Bilirubin (mg/dl) (Mean ± SD)	0.80±0.64
Albumin (mg/dl) (Mean ± SD)	42.56±4.74
ALT (U/L) (Mean ± SD)	52.09±51.33
AST (U/L) (Mean ± SD)	58.42±47.72
APTT (s) (Mean ± SD)	29.91±7.68
Creatinine (mg/dl) (Mean ± SD)	1.01±0.25
CRP (mg/L) (Mean ± SD)	18.34±30.36
Leukocytes (/mm^3) (Mean ± SD)	7246.67±2094.03
Platelets (/mm^3) (Mean ± SD)	251.32±101.30

The median follow-up after resection was 51.2 months. The estimate cumulative proportion of overall surviving at 1-, 3-, 5-, and 8-year was 96.4%, 84.2%, 68.1%, and 36.5%, disease-free surviving was 75.5%, 66.2%, 48.1%, and 24.8% respectively. (Figure 11) The median overall and disease-free survival time were 83.5 and 59.0 months, respectively.

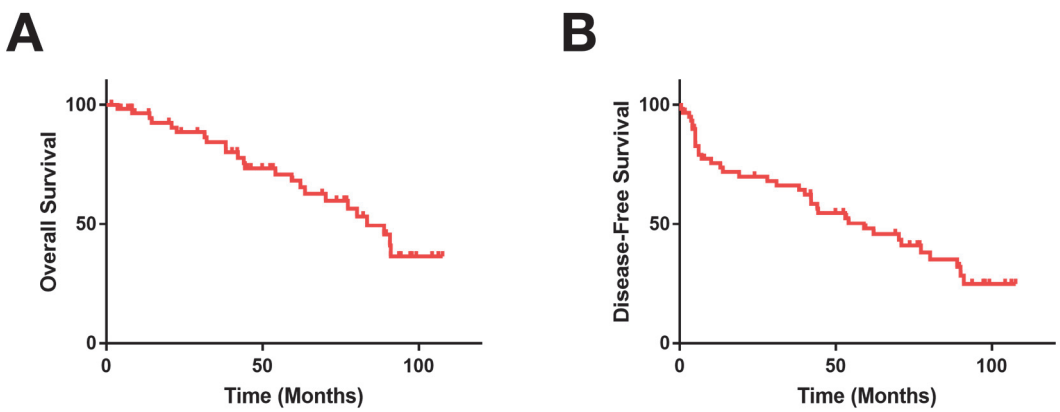

Figure 11. The Overall (A) and Disease-free Survival (B) of All Patients.

During the follow-up period, 23 (38.33%) patients had recorded recurrence. 17 (73.91%) of all patients that suffered recurrence received postoperative treatments for recurrence. The postoperative treatments included trans-arterial chemoembolization (TACE) (8 cases, 47.06%), Sorafenib (7 cases, 41.18%), repeat hepatectomy (4 cases, 23.53%), selective internal radiation therapy (SIRT) (2 cases, 11.76%), radiofrequency ablation (RFA) (2 cases, 11.76%), and transplantation (1 case, 5.88%).

3.7. Intratumoral Infiltration is Predominantly Located Around Vessels

The patterns of the infiltration showed three representative categories: a. Completely negative without any recognized staining, b. Infiltration was evenly distributed (Figure 12), c. Infiltration was found to be located around the intratumoral vessels (Figure 13). As shown in Table 6, despite the complete negative cases, the perivascular distributional patterns were found in most cases among CD3, CD8, CD20, and CD66b. The representative perivascular patterns under different magnification are summarized in Figure 13. As mentioned above, 200x magnification was adopted for picture capturing and further analysis.

Table 6. Number of Cases in Three Different Patterns

	Negative	Evenly Distribution	Perivascular Distribution
CD3	9(15.0%)	16(26.7%)	35(58.3%)
CD8	20(33.3%)	10(16.7%)	30(50.0%)
CD20	23(38.3%)	10(16.7%)	27(45.0%)
CD66b	2(3.3%)	24(40.0%)	34(56.7%)

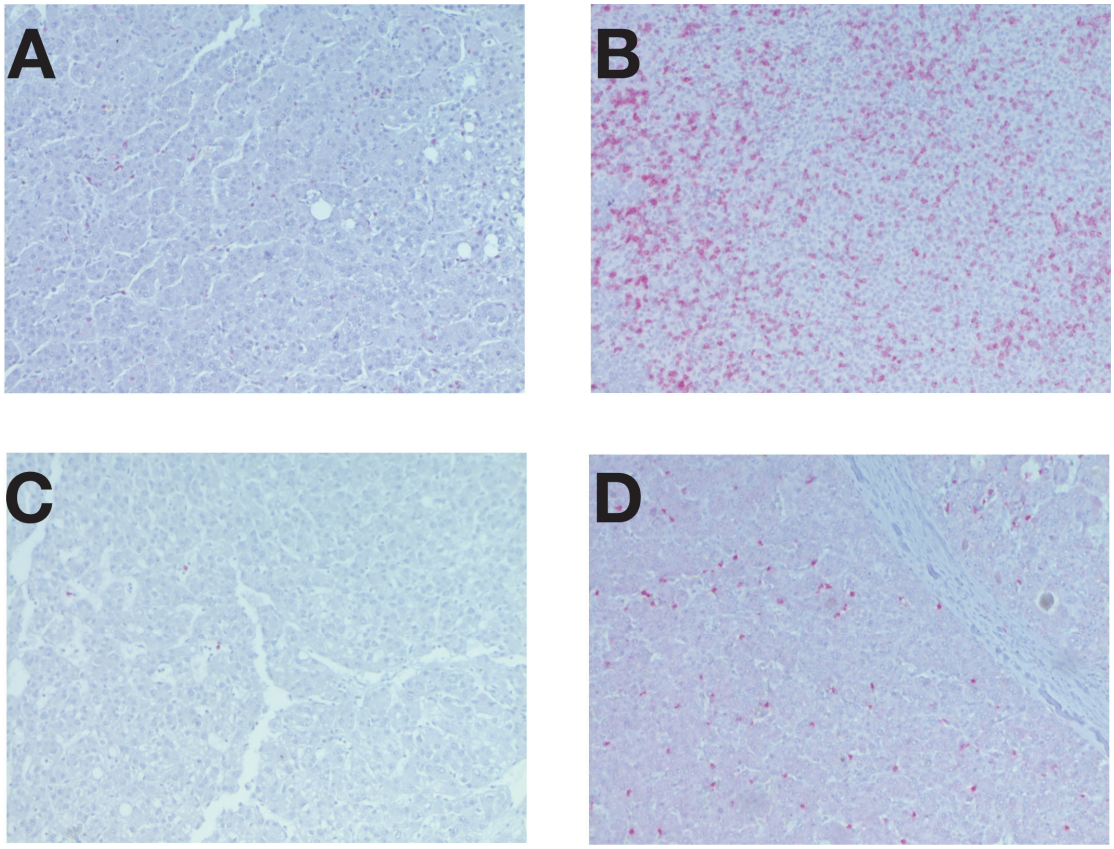

Figure 12. Evenly Distributed Infiltration Patterns of $CD3^+$ (A), $CD8^+$ (B), $CD20^+$ (C), and $CD66b^+$ (D) Cells under 100x Magnifications.

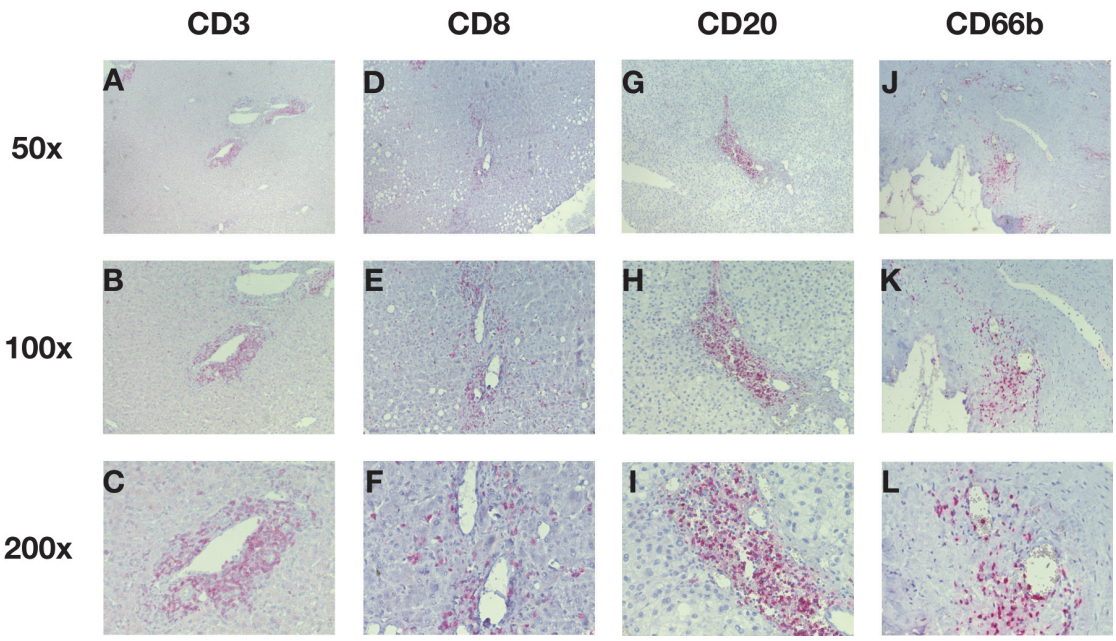

Figure 13. Representative Perivascular Patterns of $CD3^+$, $CD8^+$, $CD20^+$, and $CD66b^+$ Cells under 50x, 100x, and 200x Magnifications.

3.8. Amount of Perivascular Infiltrating $CD8^+$ Cells Positively Correlates with $CD20^+$ Cells

Furthermore, the perivascular regions were examined in detail. The relationships among perivascular infiltrating $CD3^+$, $CD8^+$, $CD20^+$, and $CD66b^+$ cells were analyzed. As shown in Figure 14C, the $CD8^+$ cells densities strongly correlated with $CD20^+$ cells ($r=0.856$, $p<0.001$). Significant correlations were also found between $CD3^+$ cells and $CD8^+$ cells ($r=0.375$, $p=0.003$, Figure 14A), as well as between $CD3^+$ cells and $CD20^+$ cells ($r=0.487$, $p<0.001$, Figure 14B). The $CD66b^+$ cells densities had no correlation with $CD3^+$ ($r=-0.016$, $p=0.905$, Figure 14D), $CD8^+$ ($r=-0.028$, $p=0.833$, Figure 14E), or $CD20^+$ ($r=-0.071$, $p=0.589$, Figure 14F) lymphocytes.

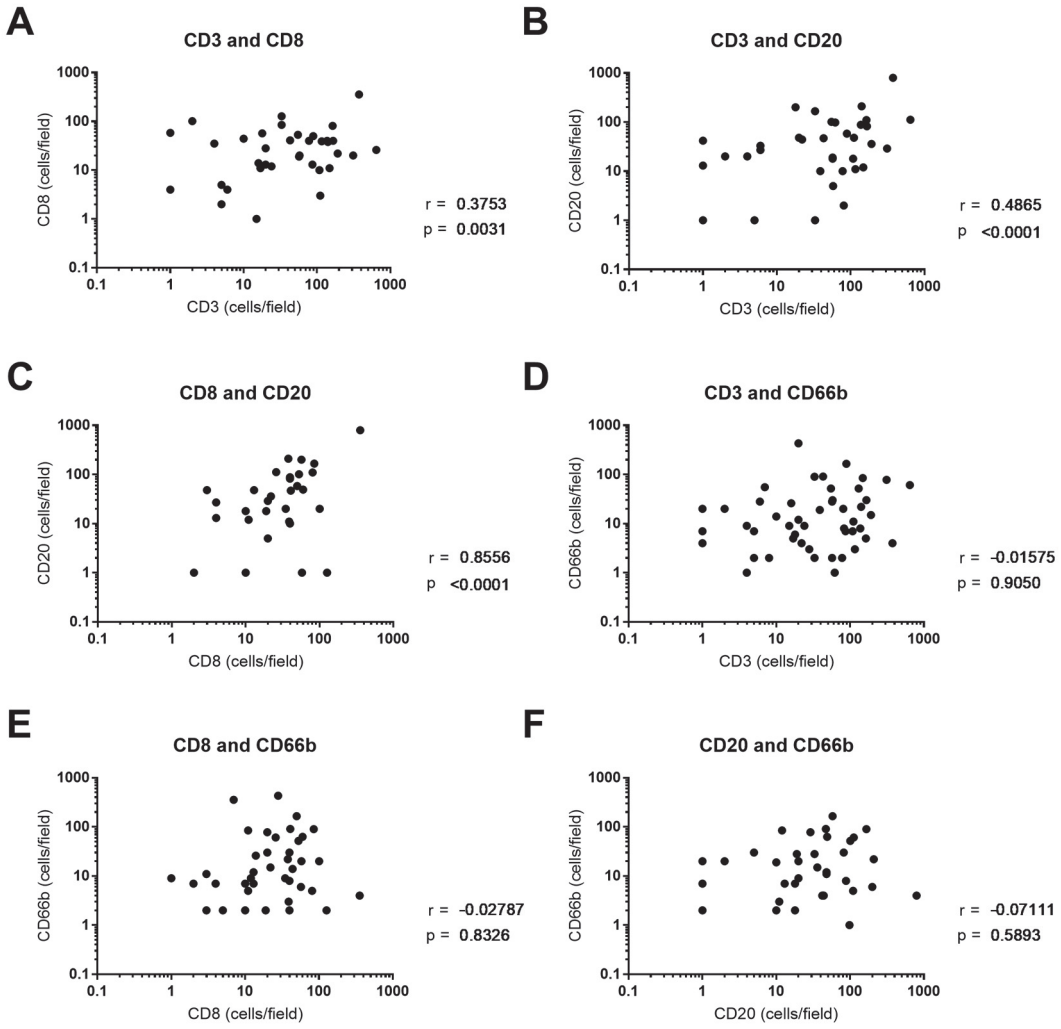

Figure 14. Correlations among perivascular infiltrating $CD3^+$, $CD8^+$, $CD20^+$, and $CD66b^+$ cells

3.9. Amount of Perivascular Infiltrating $CD66b^+$ Cells Positively Correlates with Circulating Leukocytes, Platelets, and C-reactive Protein.

There was no significance in the relationship between immunohistochemistry staining and clinical characteristics as shown in Table 7, except for weak correlations between infiltration of neutrophils and preoperative circulating leukocytes (r=0.343, p=0.007), platelets (r=0.420, p=0.001), and C-reactive protein (r=0.344, p=0.008) (Figure 15).

Table 7. Relationship and Correlation of Perivascular Infiltration and Clinical Parameters.

(Abbreviations: AFP: serum alpha-fetoprotein; ALT: alanine transaminase; AST: aspartate transaminase; APTT: activated partial thromboplastin time; CRP: C-reactive protein. Correlations between two continuous data were examined by Pearson's test and presented as r and p values. Relationships between continuous and contingency data were examined by Mann-Whitney u test and presented as p values only.)

Clinical Parameters	CD3	CD8	CD20	CD66b
Gender	p=0.653	p=0.785	p=0.414	p=0.379
Age	r=0.000 p=0.905	r=0.076 p=0.562	r=0.096 p=0.467	r=-0.200 p=0.125
Hepatitis	p=0.107	p=0.207	p=0.071	p=0.768
Cirrhosis	p=0.839	p=0.491	p=0.705	p=0.555
AFP	p=0.401	p=0.408	p=0.682	p=0.256
Tumor Multiplicity	p=0.166	p=0.277	p=0.853	p=0.792
Microvascular Invasion	p=0.467	p=0.325	p=0.474	p=0.963
Macrovascular Invasion	p=0.755	p=0.287	p=0.521	p=0.169
Beyond Milan	p=0.904	p=0.526	p=0.587	p=0.695
Bilirubin	r=-0.014 p=0.916	r=-0.064 p=0.631	r=-0.051 p=0.699	r=0.027 p=0.841
Albumin	r=0.089 p=0.530	r=0.219 p=0.118	r=0.232 p=0.098	r=0.156 p=0.269
ALT	r=-0.027 p=0.843	r=-0.125 p=0.352	r=-0.048 p=0.720	r=-0.120 p=0.372
AST	r=-0.108 p=0.434	r=-0.140 p=0.309	r=-0.111 p=0.420	r=0.023 p=0.869
APTT	r=-0.012 p=0.927	r=-0.017 p=0.901	r=-0.036 p=0.788	r=0.052 p=0.698
Creatinine	r=-0.023 p=0.863	r=0.081 p=0.537	r=0.052 p=0.695	r=-0.059 p=0.655
CRP	r=-0.035 p=0.793	r=-0.135 p=0.310	r=-0.097 p=0.463	**r=0.344 p=0.008**
Leukocytes	r=0.089 p=0.500	r=0.246 p=0.058	r=0.236 p=0.070	**r=0.342 p=0.007**
Platelets	r=-0.149 p=0.257	r=-0.142 p=0.281	r=-0.110 p=0.402	**r=0.420 p=0.001**

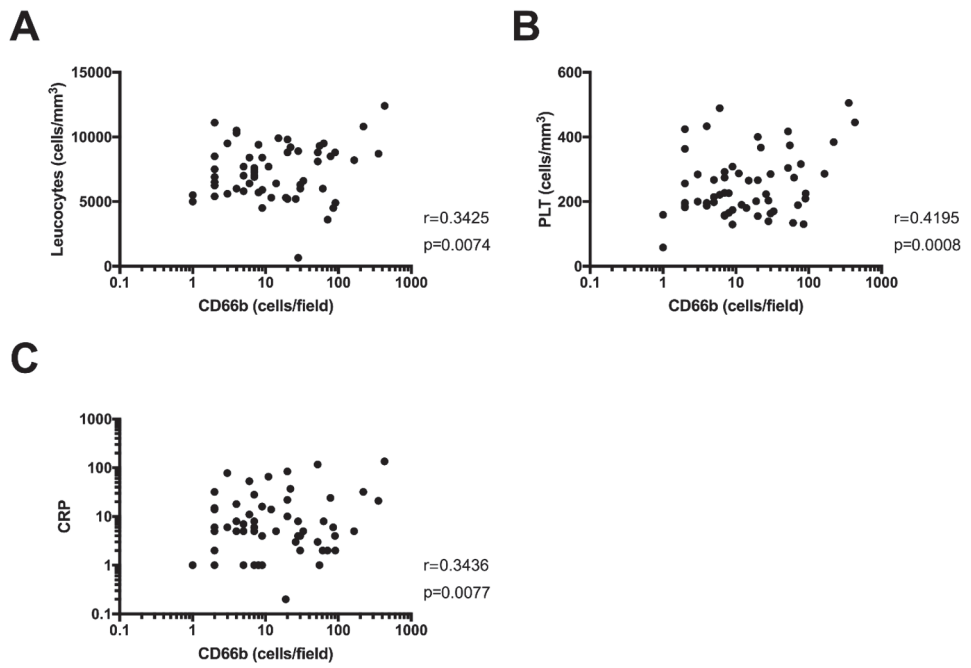

Figure 15. Infiltrating CD66b$^+$ Neutrophils Correlate with Circulating Leukocytes, Platelets, and CRP. (Abbreviations: PLT, platelet; CRP, C-reactive protein.)

3.10. Amount of Perivascular Infiltrating Immune Cells Shows No Significant Ability to Discriminate Better Overall Survivors or Disease-Free Survivors

For receiver operating characteristic (ROC) analysis, patients were divided into good or bad survivor groups depends on the median overall and disease-free survival time, respectively. As shown in Figure 16, none of CD3$^+$ (Area=0.596, p=0.282), CD8$^+$ (Area=0.589, p=0.319), CD20$^+$ (Area=0.551, p=0.564), or CD66b$^+$ (Area=0.509, p=0.923) cells showed significant ability to discriminate the better overall survivors.

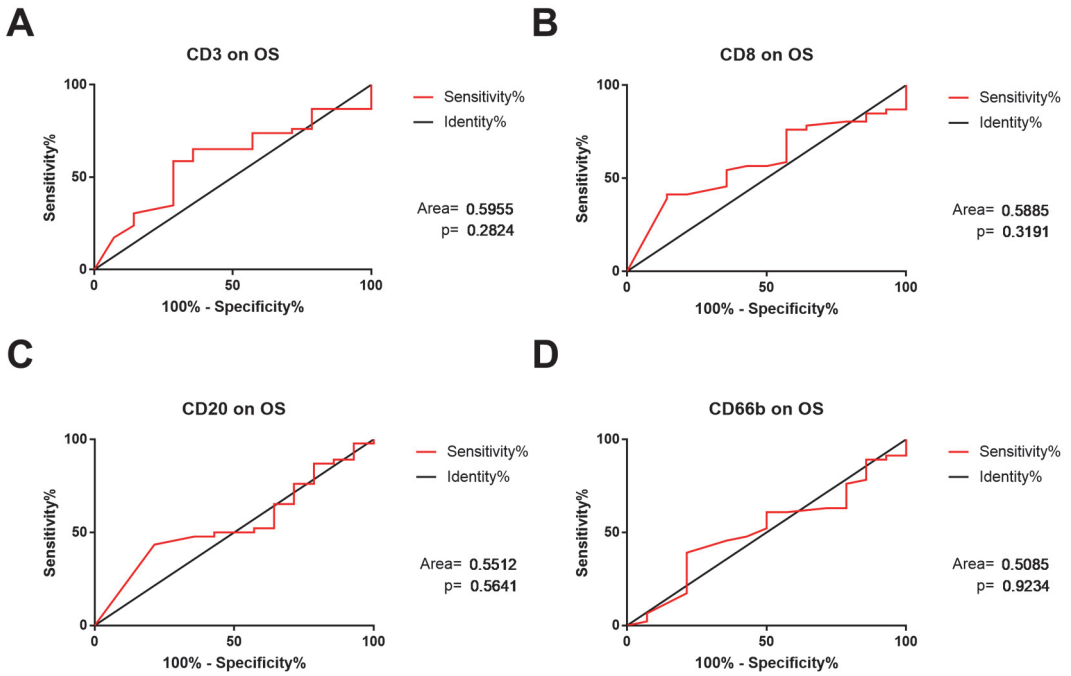

Figure 16. Receiver Operating Characteristic Curves of $CD3^+$(A), $CD8^+$(B), $CD20^+$(C), and $CD66b^+$(D) Cells on Overall Survival.

Regarding DFS, as shown in Figure 17, none of $CD3^+$ (Area=0.559, p=0.456), $CD8^+$ (Area=0.601, p=0.195), $CD20^+$ (Area=0.575, p=0.334), or $CD66b^+$ (Area=0.5, p>0.999) cells showed significant ability to discriminate the better disease-free survivors.

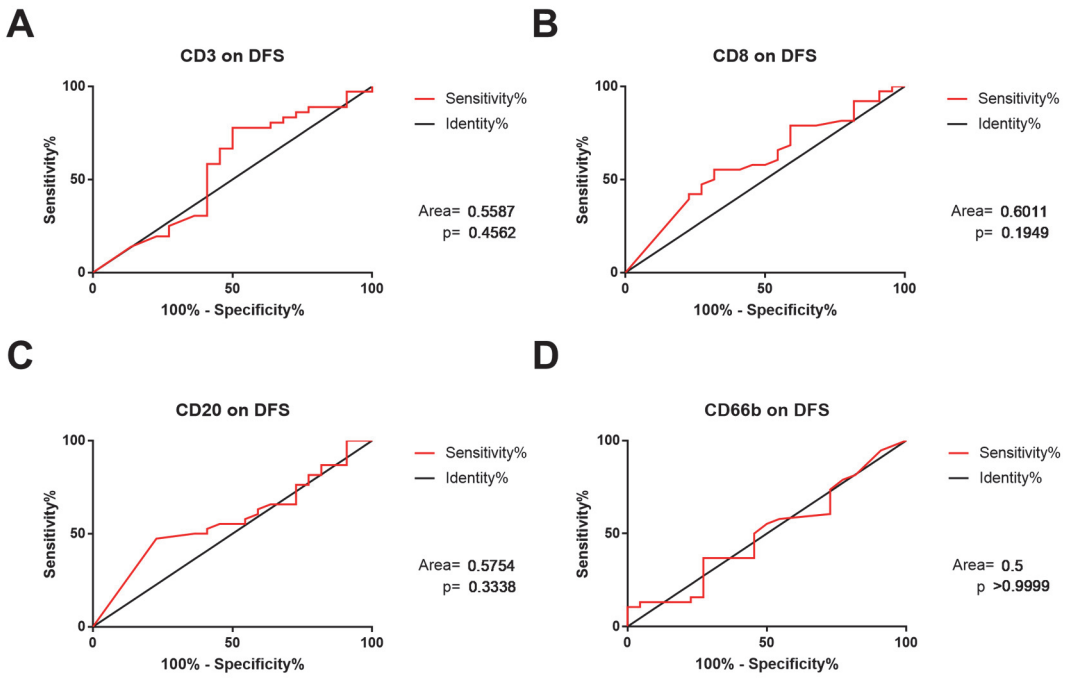

Figure 17. Receiver Operating Characteristic Curves of $CD3^+$(A), $CD8^+$(B), $CD20^+$(C), and $CD66b^+$(D) Cells on Disease-fee Survival.

3.11. Perivascular Infiltrating $CD3^+$, $CD8^+$, $CD20^+$, and $CD66b^+$ Cells Have No Significant Influence on Overall Survival

For Kaplan-Meier survival analysis, patients were divided into high or low infiltration groups according to the median values as described in the material and methods section. As shown in Figure 18, no evidence was found for influence of $CD3^+$ (p=0.058), $CD8^+$ (p=0.297), $CD20^+$ (p=0.535), or $CD66b^+$ (p=0.616) cells on overall survival. The estimate cumulative proportions of overall surviving for each group at 1-, 3-, 5-, and 8-year are listed in Table 8.

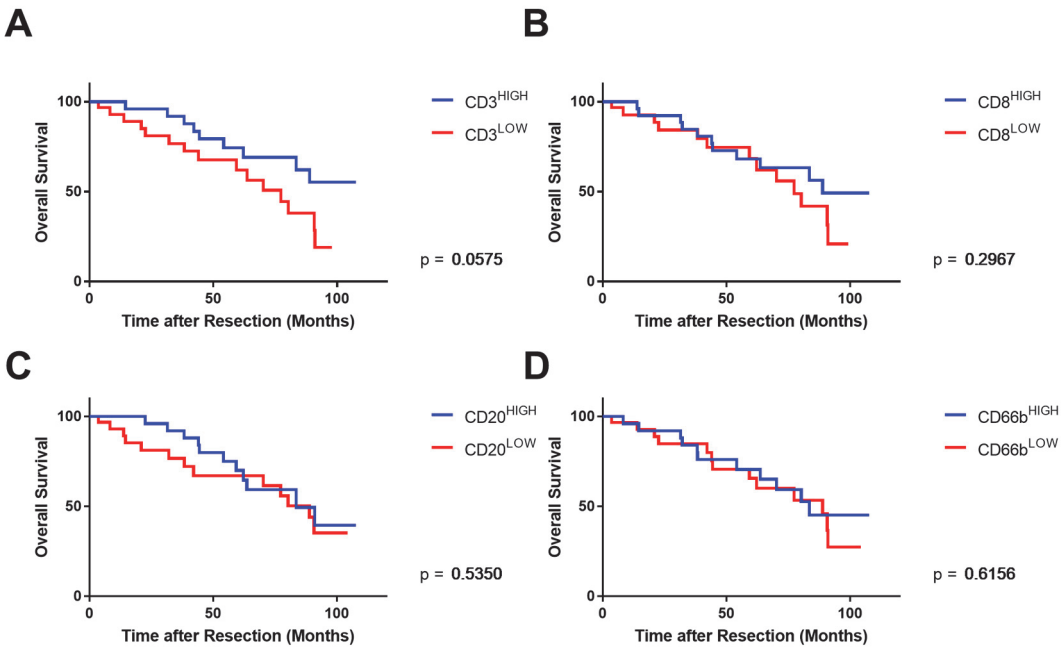

Figure 18. Kaplan-Meier Curves of $CD3^+$(A), $CD8^+$(B), $CD20^+$(C), and $CD66b^+$(D) Cells on Overall Survival.

Table 8. Estimate Cumulative Proportion of Overall Surviving Regarding $CD3^+$, $CD8^+$, $CD20^+$, and $CD66b^+$ cells

Groups	1-year	3-year	5-year	8-year
$CD3^{LOW}$	92.95%	76.72%	61.99%	19.02%
$CD3^{HIGH}$	100%	91.83%	74.35%	55.23%
$CD8^{LOW}$	92.64%	84.22%	68.35%	20.97%
$CD8^{HIGH}$	100%	84.62%	68.15%	49.22%
$CD20^{LOW}$	93.05%	76.72%	67.05%	35.20%
$CD20^{HIGH}$	100%	92.00%	70.00%	39.49%
$CD66b^{LOW}$	96.67%	84.73%	65.57%	27.48%
$CD66b^{HIGH}$	96.00%	84.00%	70.57%	45.12%

3.12. Perivascular Infiltrating CD3$^+$ and CD8$^+$ Cells Significantly Influence Disease-free Survival

Higher perivascular infiltration of CD3$^+$ (p=0.016) and CD8$^+$ (p=0.028) cells predicted better disease-free survival (DFS). Also, a positive tendency forwards better DFS was noted on CD20$^+$ cells (p=0.076). There was no evidence that infiltration of CD66b$^+$ cells influences DFS (p=0.521) (Figure 19). The estimate cumulative proportions of disease-free surviving for each group at 1-, 3-, 5-, and 8-year are listed in Table 9.

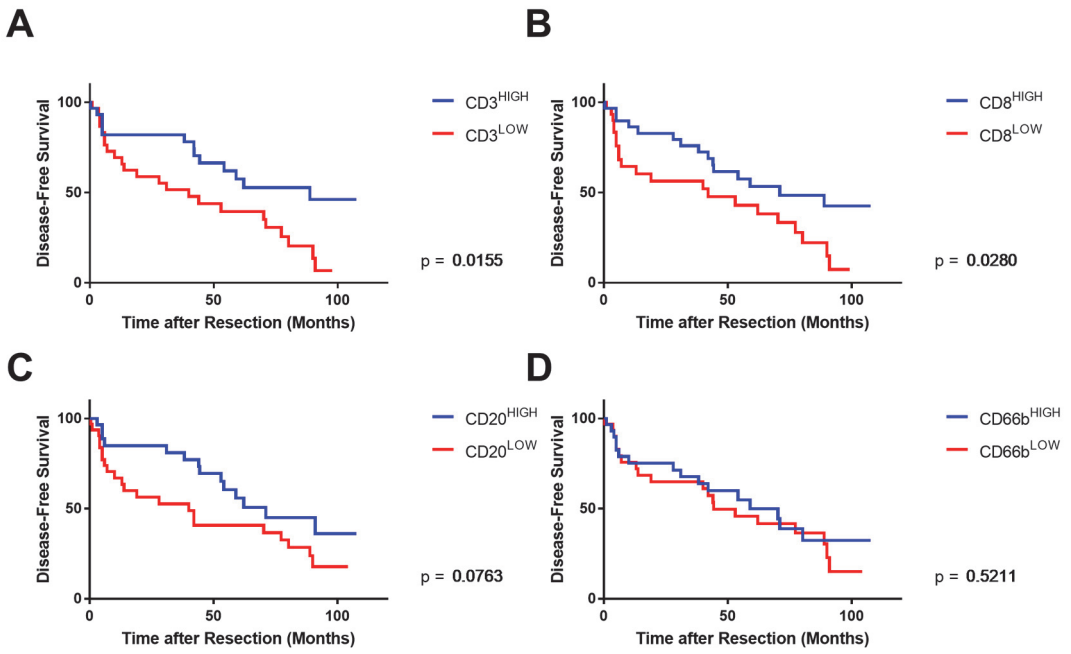

Figure 19. Kaplan-Meier Curves of CD3$^+$(A), CD8$^+$(B), CD20$^+$(C), and CD66$^+$(D) Cells on Disease-free Survival.

Table 9. Estimate Cumulative Proportion of Disease-free Surviving Regarding $CD3^+$, $CD8^+$, $CD20^+$, and $CD66b^+$ cells

Groups	1-year	3-year	5-year	8-year
$CD3^{LOW}$	69.33%	51.57%	39.50%	6.83%
$CD3^{HIGH}$	82.03%	82.03%	57.55%	46.16%
$CD8^{LOW}$	64.39%	56.35%	42.91%	7.42%
$CD8^{HIGH}$	86.31%	75.95%	53.41%	42.48%
$CD20^{LOW}$	66.93%	52.60%	40.71%	17.81%
$CD20^{HIGH}$	84.86%	81.00%	50.72%	36.07%
$CD66b^{LOW}$	75.68%	64.87%	45.79%	15.18%
$CD66b^{HIGH}$	75.14%	71.38%	49.90%	32.34%

3.13. Scoring of $CD3^+$, $CD8^+$, and $CD20^+$ Cells Significantly Predicts Disease-free Survival

Furthermore, based on the median values, each patient was given a binary score (1 as high and 0 as low) for each kind of lymphocytes (CD3, CD8, and CD20). Then I evaluated the prognostic significance of four possible scorings by summation of these binary scores: CD3+CD8, CD3+20, CD8+CD20, and CD3+CD8+CD20. Considering the limited sample size, the scoring of CD3+CD8+CD20 was further divided into two groups. (Low Group: Score 0-1, High Group: Score 2-3) Kaplan-Meier analysis of these scoring on overall survival showed that the scorings of CD3+CD8 (p=0.194), CD3+CD20 (p=0.282), CD8+CD20 (0.580), and CD3+CD8+CD20 (p=0.081) had no significant prognostic effect on OS (Figure 20). The estimate cumulative proportions of overall surviving for each scoring at 1-, 3-, 5-, and 8-year are listed in Table 10. In contrast, the combination of CD3+CD8 (p=0.021), CD3+CD20 (p=0.048), CD8+CD20 (0.048), and CD3+CD8+CD20 (p=0.006) had significant prognostic values on DFS (Figure 21). The estimate cumulative proportions of disease-free surviving for each scoring at 1-, 3-, 5-, and 8-year are listed in Table 11.

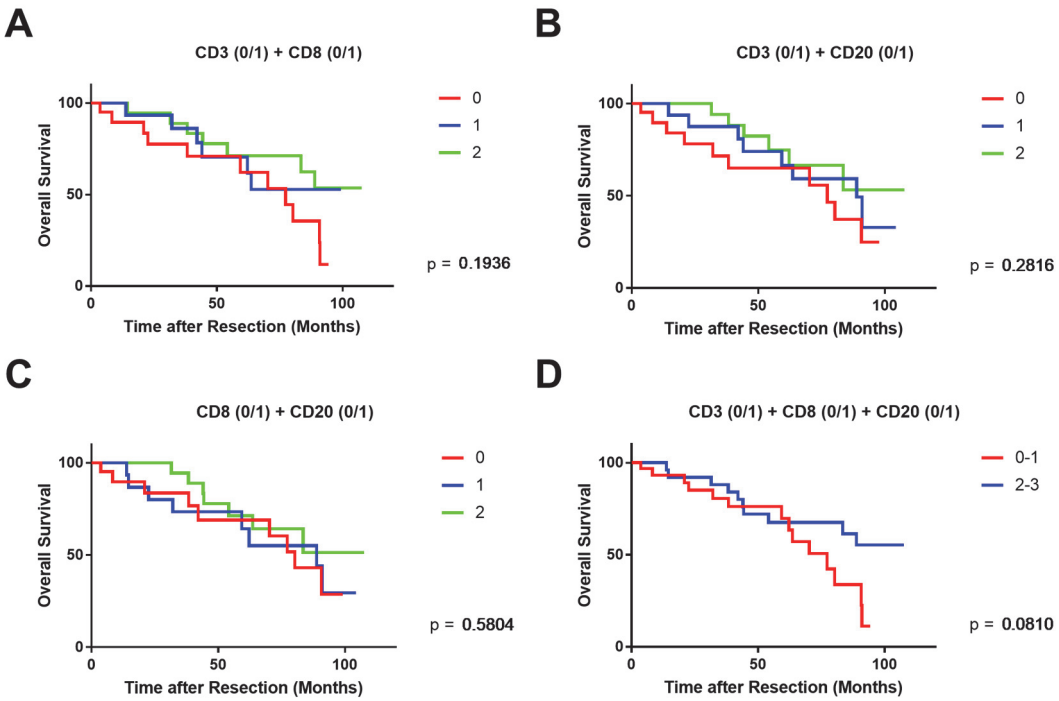

Figure 20. Kaplan-Meier Curves of Scoring of $CD3^+$, $CD8^+$, and $CD20^+$ Cells on Overall Survival.

Table 10. Estimate Cumulative Proportion of Overall Surviving Regarding Scoring of $CD3^+$, $CD8^+$, $CD20^+$, and $CD66b^+$ cells

Groups	1-year	3-year	5-year	8-year
CD3+CD8: 0	89.41%	77.49%	62.15%	11.84%
CD3+CD8: 1	100%	86.15%	70.49%	52.87%
CD3+CD8: 2	100%	88.89%	71.30%	53.47%
CD3+CD20: 0	89.64%	71.53%	65.03%	24.77%
CD3+CD20: 1	100%	87.50%	66.64%	32.91%
CD3+CD20: 2	100%	94.12%	74.87%	53.24%
CD8+CD20: 0	89.64%	83.66%	69.02%	28.76%
CD8+CD20: 1	100%	73.33%	73.33%	29.33%
CD8+CD20: 2	100%	94.44%	71.30%	51.33%
CD3+CD8+CD20: 0-1	93.15%	80.57%	69.76%	11.27%
CD3+CD8+CD20: 2-3	100%	88.00%	67.50%	55.23%

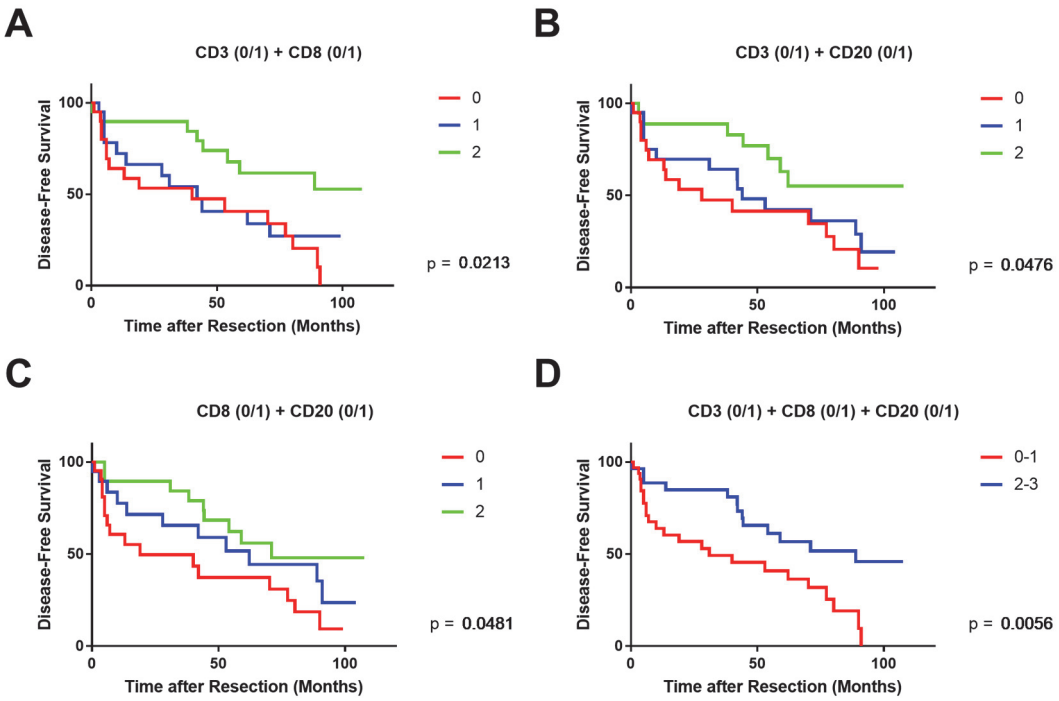

Figure 21. Kaplan-Meier Curves of Scoring of $CD3^+$, $CD8^+$, and $CD20^+$ Cells on Disease-free Survival.

Table 11. Estimate Cumulative Proportion of Disease-free Surviving Regarding Scoring of $CD3^+$, $CD8^+$, $CD20^+$, and $CD66b^+$ cells

Groups	1-year	3-year	5-year	8-year
CD3+CD8: 0	64.00%	53.33%	40.64%	0%
CD3+CD8: 1	72.22%	54.16%	40.62%	27.08%
CD3+CD8: 2	89.72%	89.72%	61.57%	52.78%
CD3+CD20: 0	69.33%	47.41%	41.18%	10.37%
CD3+CD20: 1	69.64%	69.64%	42.19%	19.29%
CD3+CD20: 2	88.82%	88.82%	62.98%	55.11%
CD8+CD20: 0	60.71%	49.68%	37.26%	9.31%
CD8+CD20: 1	83.51%	65.61%	51.67%	23.62%
CD8+CD20: 2	89.47%	84.21%	55.98%	47.98%
CD3+CD8+CD20: 0-1	63.95%	49.26%	40.93%	0%
CD3+CD8+CD20: 2-3	88.71%	84.86%	56.83%	45.92%

3.14. Amount of Perivascular Infiltrating $CD3^+$ Cells is an Independent Predictor for OS, and $CD8^+$ Cell is an Independent Predictor for DFS

Univariate analysis for each covariate was performed first as shown in Table 12. Following the Collett's model selection approach as mentioned in the statistical analysis section, the variables of $CD3^+$ cells and Age (≥60 years) eventually remained in the Cox multivariate model predicting overall survival as shown in Table 13 and Figure 22. These results show that high and low amount of $CD3^+$ cells is an independent predictor of overall survival (p=0.022). Regarding the disease-free survival, the same selection process was used. Following the protocol, the variables gender and $CD8^+$ cells eventually remained after the selection as shown in Table 14 and Figure 23. The results showed that the $CD8^+$ cells are an independent predictor of disease-free survival (p=0.006), as well as gender (p=0.003).

Table 12. Univariate Analysis of All Factors on Overall and Disease-free Survival

(Abbreviations: HR: Hazard ratio; CI: confidence interval; p: p value; AFP: serum alpha-fetoprotein) The p values under 0.200 were bolded.

Variables	Overall Survival				Disease-free Survival			
	HR	95% CI		p	HR	95% CI		p
Gender	1.243	0.781	1.978	0.355	2.312	1.112	4.808	**0.021**
Age (≥60 years)	0.440	0.150	1.291	**0.124**	1.496	0.754	2.971	0.246
Hepatitis	0.237	0.077	0.732	**0.007**	2.451	0.932	6.447	**0.059**
Cirrhosis	0.525	0.219	1.256	**0.141**	0.843	0.399	1.779	0.652
AFP(≥20ng/ml)	0.913	0.389	2.145	0.835	0.638	0.322	1.266	**0.195**
AFP(≥400ng/ml)	1.798	0.420	7.706	0.423	1.364	0.478	3.889	0.560
Tumor Multiplicity	1.521	0.355	6.517	0.570	0.818	0.339	1.973	0.653
Microvascular Invasion	0.677	0.272	1.690	0.401	0.708	0.346	1.447	0.338
Macrovascular Invasion	0.702	0.201	2.457	0.578	0.618	0.232	1.644	0.328
Beyond Milan	0.553	0.073	4.187	0.560	0.272	0.037	2.006	**0.169**
$CD3^+$ Cells	2.196	0.956	5.046	**0.057**	2.277	1.145	4.527	**0.016**
$CD8^+$ Cells	1.531	0.684	3.425	0.297	2.074	1.064	4.043	**0.028**
$CD20^+$ Cells	1.291	0.574	2.904	0.535	0.549	0.279	1.079	**0.076**
$CD66b^+$ Cells	1.228	0.550	2.744	0.616	1.239	0.641	2.394	0.521

Table 13. Cox Multivariate Model for Predicting Overall Survival with Collett´s Model for Selection of Covariates.

(Abbreviations: HR: Hazard ratio; CI: confidence interval)

Variables	HR	95% CI		p
Age (≥60 years)	0.332	0.109	1.012	0.053
$CD3^+$ Cells	2.754	1.158	6.548	0.022

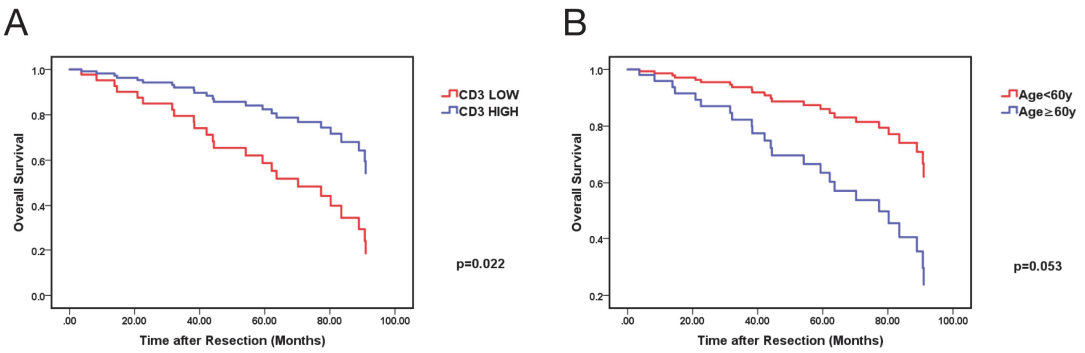

Figure 22. Cox Regression Curves of $CD3^+$ cells (A) and Age (B) on Overall Survival with Collett´s Model for Selection of Covariates

Table 14. Cox Multivariate Model for Predicting Disease-free Survival with Collett´s Model for Selection of Covariates.

(Abbreviations: HR: Hazard ratio; CI: confidence interval)

Variables	HR	95% CI		p
Gender	3.405	1.517	7.645	0.003
$CD8^+$ Cells	2.819	1.353	5.876	0.006

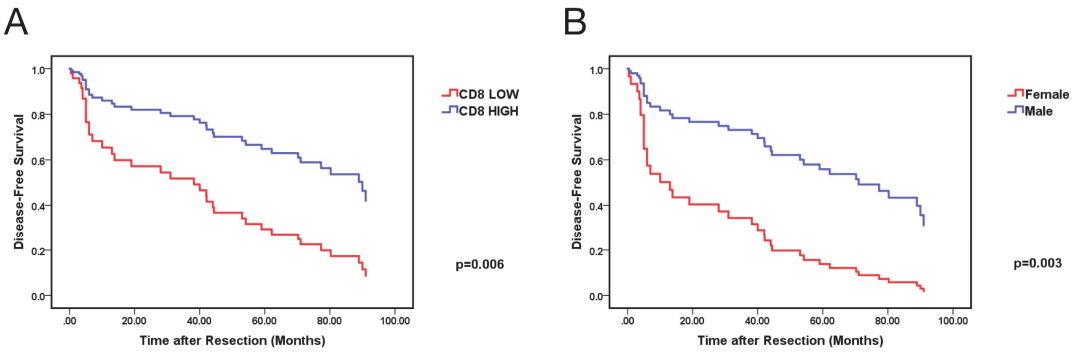

Figure 23. Cox Regression Curves of $CD8^+$ cells (A) and Gender (B) on Disease-free Survival with Collett´s Model for Selection of Covariates

4. Discussion

Evaluation of tumor tissue is mostly based on clinicopathologic staging systems. Nevertheless, tumor burden and further components of the tumor microenvironment help to distinguish subtypes in different tumor entities.[73]

The tumor stroma which constitutes its microenvironment plays a major role in the understanding of tumor biology, progression, therapy, and lastly prognosis.[65] Especially, the immune system and its effector cells are known to influence prognosis.[74] For example, high amount of $CD3^+$ and $CD8^+$ cells have been shown to influence the prognosis of various tumor entities favorably.[69, 71, 73, 126-132] Scorings of infiltrating immune cells in breast cancer[133], lung cancer[134], and colorectal cancer[73] have been published. Because of the high predictability, it has been suggested that these scores showing different groups of immune cell density could amend the traditional TNM system.[73, 75, 133, 134]

4.1. QTS Algorithm

To have scores of quantified tumor-infiltrating leukocytes being added to clinical scoring systems, standardization is critical. However, in the literature counting methods and definitions are not clear, and therefore results differ.[73, 134, 135] It complicates the comparison of studies.[73, 136] For other quantification methods standards do exist and help guide clinicians during the daily routine.[76, 77]

The aim of the development of quantification of tumor stroma (QTS) was to prepare the main study to facilitate a reliable, accurate and affordable algorithm for clinical and pathological practices (Figure. 24). In this development, the immune cell infiltration with $CD3^+$ and $CD8^+$ cells was used as the most widely examined representative of the tumor stroma.[69, 71, 126-128, 137]

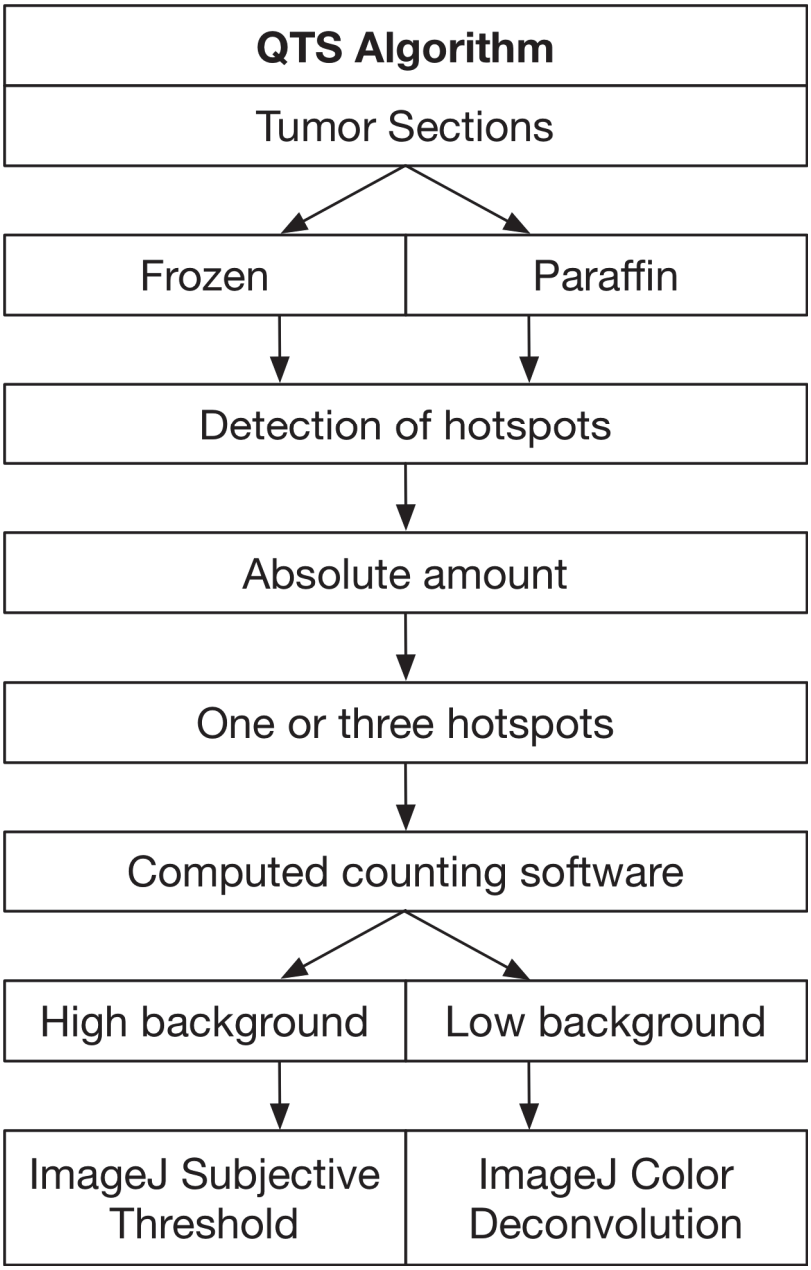

Figure 24. Quantification of the Tumor Stroma (QTS) Algorithm: From the Type of Tumor Sections to the Final Quantification of the Tumor Stroma.

The area with the highest density of $CD3^+$ or $CD8^+$ cells was defined as a hotspot.[138, 139] Manual counting was regarded as the gold standard which was compared to computed software results. This underlying assumption is supported by the literature.[76] Hotspot selection under the microscope was shown to be quicker at a lower cost than whole slide image scanning as reported in the literature.[140, 141] Furthermore, high demands of data processing and storage are needed when the

entire slide is scanned.[140, 142] A typical scanned slide requires approx. 4.6 GB of memory[141], whereas a picture of one hotspot requires between 2.4 and 3 MB.

According to the subjective selection of hotspots, one observer is justified and reliable. This is also used in clinical practice – e.g. counting of Ki67.[76] Regarding the number of hotspots, quantification of the mean of three hotspots is advisable and reliable. Alternatively, if the infiltration is relatively low, one hotspot is also recommended to present diversity which might be decreased by the process of averaging three hotspots which might not include any cells. To give an absolute cell count over an area, many researchers do not reveal their methods. It is not clear if the computed area is representative of the tumor section.[70, 73, 133, 134]

By now, computed counting methods achieve acceptable accuracy when compared to manual counting as the gold standard.[72, 76] ZEN and ImageJ software are by far not the only possible methods.[70, 72, 73, 133-135, 138, 143] As shown in our results background staining should also be factored in: IST is accurate for sections with high background staining because it allows for human adjustments. In this method, the subjective threshold is useful to differentiate the staining reactions. ICD can be used in sections with low background staining.

Furthermore, overlapping of cell layers may be a confounding factor. Therefore, subjective methods like IST and ICD compared to fully automated ZEN 2 software help to distinguish between conglomeration and single cells. Furthermore, ImageJ software is free and therefore is better concerning costs, also counting time is lower compared to the ZEN 2 software. Software used by other authors may differ and are dependent on access and funding.[144]

Accordingly, the IST was adopted in this study to conduct the quantification of perivascular immune infiltration in early HCC that were resected with curative intent.

4.2. Perivascular Infiltration

Prior studies that have noted the importance of the location of the infiltration encouraged the discussions about the origin of immune infiltration. Most of these studies focused on the peritumoral regions with the hypothesis the invasive margin could be the battlefront of the immunoreaction.[78] To our knowledge, the current study is first to explore perivascular infiltration.

HCC displays more angiogenic characteristics than other solid tumors, such as colorectal cancer. [87, 88] In here I introduced perivascular region as a potential battlefront between tumor cells and immune cells. In the initial pattern review of our slides, I observed that high densities of intratumoral infiltration of immune cells were located near the vessels, with the gradually decreasing densities into the tumor parenchyma. Thus I could speculate migration from the vessels to the parenchyma. I have also confirmed that patients with curative resection could benefit from perivascular infiltration of immune cells. In this setting, it suggested that the intratumoral neovascularization might have dual effects. It could allow potential metastatic cells, especially of the cancer stem cells, escape from the primary tumor into circulation that could cause relapse or metastasis postoperatively.[145] Meanwhile, as a potential border to the healthy immune system, it also promotes immune cell infiltration which is logically dependent on the network of blood vessels, mainly in the post-capillary venules. The mechanism of trafficking of immune cells in tumor remains controversial, Freeman MR et al. reported that the peripheral and tumor-infiltrating T-cell could synthesize vascular endothelial growth factor (VEGF) which is a mediator of neovascularization.[146] But Huang H et al. evidenced that VEGF suppresses T-cell infiltration through inhibition of NF-κB-induced endothelial activation,[147] as well the inhibitor of VEGF, Sorafenib was described to enhance the antitumor immunity in a murine model.[148] So, a deeper understanding mechanisms of intratumoral immune cells migration is urgently needed and will be greatly valuable for risk stratification.

4.3. Influence of Infiltrating Leukocytes on Survival

In the literature review, I found that CD3, CD4, CD8, and FoxP3 were the four most common markers used in the examined studies which represent total T-cell, helper T-cell, cytotoxic T-cell, and regulatory T-cell respectively. T-cell is the most important component playing a central role in cell-mediated immunity. After a T-cell has been activated, it can proliferate and differentiates into one of those four subtypes with functions of killing, activation, and regulation, respectively. From the literature review, several studies have shown that higher infiltration of $CD3^+$ or $CD8^+$ cells is associated with favorable prognosis of HCC, and $FoxP3^+$ cells with the opposite effect. In comparison with other tumor types, the favorable prognostic values of $CD3^+$ or $CD8^+$ cells have also been suggested in many kinds of human solid tumors, including colorectal cancer,[149, 150] ovarian cancer,[151, 152] pancreatic cancer,[153] breast cancer[154, 155]. However, the influence of $FoxP3^+$ cells has been reported to have contradictory results in different tumor types. In most solid tumors including cervical, renal, ovarian, melanomas, pancreatic, breast, and gastric cancers, it correlates with shorter survival. But it correlates with longer survival in colorectal, head and neck, and esophageal cancers.[156] In our experimental study, I found that high perivascular infiltration of $CD3^+$ cells was an independent predictor for favorable overall survival after curative resection of HCC. $CD8^+$ cells showed no influence on overall survival. Conversely, although being predictive in the univariate analysis $CD3^+$ cells lost its predictive effects on disease-free survival after multivariate adjustment. For disease-free survival, however $CD8^+$ infiltration could be shown to be an independent predictor. The difference in disease-free survival might be attributed to the fact, that $CD3^+$ cells theoretically contain $CD8^+$ cells in phenotyping. As described before $CD8^+$ cells might be the actual effector cells targeting cancer cells. Therefore, the multivariate procedure with the Collett's model could show independent influence of CD8+ cells on disease-free survival. The inefficiency of $CD8^+$ cells to predict overall survival might be attributed to the prompt postoperative treatments in cases of recurrence as described in the results section. As soon as the recurrences were detected during follow-up, the patients were admitted and evaluated for the postoperative treatments including adjuvant therapies or even re-operations.

The B-cell is a central part of humoral immunity. It has been recently recognized as an important player against tumor progression.[157] The tumor infiltrating B-cell might not only directly kill tumor cells through an antibody-independent mechanism, but also could promote the cell-mediated immunity. Conversely, the B-cell activation, proliferation, and antibody production could also be inhibited by regulatory T-cell.[158] In several kinds of solid tumors, such as ovarian cancer, pancreatic cancer, lung cancer, and cervical cancer, the tumor infiltrating B-cell has been widely proved to be associated better outcome.[153, 159-161] Furthermore, Milne, K. et al. found that, in ovarian cancer, the patients containing both high infiltration of $CD8^+$ and $CD20^+$ lymphocyte had an even higher survival than those with $CD8^+$ or $CD20^+$ alone.[159] This also suggests the cooperative interaction of T- and B-cell immunity in cancer. However, in the literature review, the prognostic significance of intratumoral infiltration of B-cells in HCC has only been validated in one study and failed in another one. [98, 100] In experimental results, the infiltration of $CD20^+$ B lymphocytes was found to be highly correlated with $CD8^+$ T lymphocytes which is in accord with a recent study.[100] The interaction between T and B cells has been wildly considered to be a major event in tumors.[100] Although the prognostic significance of $CD20^+$ B lymphocytes has not been achieved in the present study, I witnessed the high tendency of the influence of $CD20^+$ cells on DFS and its potential in further stratification combined with $CD3^+$ and/or $CD8^+$ cells in several possible scorings. It can thus be suggested that the link between T and B cells in a tumor might also be involved in the progression of HCC, and the adaptive immunity, not only cell-mediated but also humoral immunity might contribute to the prevention of HCC recurrence.

Apart from the adaptive immunity, the innate immunity might also play a vital role in the progression of HCC which frequently occurred in an inflamed liver due to hepatitis. The $CD66b^+$ neutrophils are the most common and abundant subpopulation of the leukocyte and are considered as the battlefront of the defense against the pathogens in innate immunity. In recent years, several studies have demonstrated that the peripheral neutrophil-to-lymphocyte ratio (NLR) negatively correlates with the survival of HCC patients.[162, 163] But, very few studies examined the role of infiltration of

neutrophils in HCC. In HCC, only one study investigated the infiltrating $CD66^+$ cells and found its correlation with worse DFS and OS.[116] In other tumor types, the amount of tumor infiltrating $CD66^+$ cells has correlated with worse outcome in pancreatic cancer,[164] testicular cancer,[165] lung cancer,[166] renal cancer,[167] cervical cancer,[168] and esophageal cancer[169]. But its prognostic effects were reported to be contradictory in two studies in colorectal cancer, respectively.[170, 171] Our results do not support the previously reported probability of prediction outcome regarding the infiltration of $CD66b^+$ cells.[116] Possible explanations for this could be the antibody heterogeneities or different analysis methods. Another possibility could be the etiological difference of the current cohort. There were only seven cases of hepatitis and 15 with cirrhosis, in compared with that over 90% cases had hepatitis in that cohort.[116] The neutrophils in the tumor can polarize to pro- or anti-tumorigenic subtypes depending on the tumor microenvironment which varies hugely reliant on the primary liver disease.[45] However, I found that the densities of infiltrating neutrophils were associated with preoperative lab values concerning the inflammatory status such as leukocyte counts, platelets, and CRP.

The correlation found between circulating leukocyte counts and intratumoral neutrophils hints at the connection between systemic and local immune response in cancer. There have already been studies demonstrating that the constitution of peripheral immune cells could mirror the status of intratumoral infiltration.[115, 172] Additionally, the improvements of flow cytometric immunophenotyping have offered us the opportunity to measure not only conventional immune cell types but also their subtypes and functional status.[173] Therefore, in the foreseeable future, liquid biopsies of circulating immune cells could provide considerable information for treatment allocation.

According to the literature review, limited information was found regarding transplantation unexpectedly. In three studies infiltrating leukocytes could be found, the results of anti-tumor effects of $CD8^+$ and $CD45RO^+$ infiltration, as well as the pro-tumor effects of $CD4^+$ and $FoxP3^+$ infiltration could still be observed in accordance with resected patients.[97-99] Additionally, a retrospective clinical study was first to show that

transplanted HCC patients with a history of rejection had lower tumor recurrence.[59] This might be due to the common features from both allogenic and anti-tumor immunity.[59, 174, 175] To date, the relationship between preoperative infiltrating immune cells and the rejection occurrence remains unclear. The prognostic value of immunological infiltration on LT needs to be further validated.

Traditional clinicopathological prognostic predictors for HCC, including AFP, tumor number, microscopic and macroscopic vascular invasion, have shown inefficiencies on predicting prognosis in recent studies.[176, 177] In current cohort, these variables also show no significance on DFS or OS. This might be due to the different study populations and limited sample size. It also could be contributed by continuous improvements of standardized managements and surgical technology, as well as the developments of adjuvant treatments in recent years.[178] The examination of tumor cellular and molecular behaviors, such as immunological phenotyping, cancer stemness, and gene expression, is being increasingly applicable by taking advantages of the improving technologies in clinical practice. These parameters in some cases have been demonstrated to be more valuable than traditional markers in HCC prognosis and prediction.[179, 180]

4.4. Limitations of the Study

This study has limitations: This algorithm was developed in a limited set of samples. However, 80 different samples n=10 for any marker and tumor entity were used. With this sample size, the QTS Algorithm was statistically consistent. Furthermore, the results showed comprehensible and reproducible differences depending on the selected sections (paraffin (HCC, PDAC) or frozen sections (mCRC, OvCa)). In the experimental part, the sample size was limited because of the relatively low occurrence of HCC in Germany. This could possibly be the reason for the statistical inferiority of infiltrating B cells, as well as ROC results. Also, due to the restraints of retrospective studies and immunohistochemistry technology, I was not able to investigate further cells types or functional status which require multi-color staining.

A project to analyze circulating and infiltrating immune cells by flow-cytometry in a prospective cohort is ongoing in our working group.

4.5. Conclusion

The development of this newly introduced QTS algorithm showed that choosing hotspots by one observer is reliable, computer-assisted counting is accurate, and this solution is cost-effective. Using this novel algorithm, it is shown that HCC has relative low immune infiltration in compared with PDAC, mCRC, and OvCa. The amount of perivascular infiltrating $CD3^+$, $CD8^+$, and $CD20^+$ cells correlate with each other. Among these, perivascular infiltrating $CD66b^+$ cells correlate with circulating leukocytes, CRP, and platelets. The scoring of $CD3^+$, $CD8^+$, and $CD20^+$ infiltration showed significance on predicting disease-free survival. The amount of perivascular infiltrating $CD3^+$ cells is an independent predictor of better overall survival, and $CD8^+$ cells predicted prolonged disease-free survival independently.

5. Summary

Hepatocellular carcinoma (HCC) is a predominant type of primary liver cancer. The most available curative treatment is liver resection. The outcome in some resected patients remains unsatisfying. It has been suggested that a dysregulated immune system contributes to the development of HCC. Also, it is known that immune response might be involved in tumor progression after hepatectomy because of HCC. Therefore, this makes having a profound understanding of the immune system in HCC patients a priority which could enable us to establish a clinical evaluation of the immune status for risk stratification. This for example can be achieved by standardized counting of tumor-infiltrating leukocytes.

Independent from the type and the number of tumor-infiltrating leukocytes, the location needs to be considered when predicting the outcome. HCC has a high degree of angiogenesis, and the surface area of these vessels represents the potential border from malignant tumor to the immune system. I first conducted a literature review based on a comprehensive search in PubMed database up to November 2016 for all available studies to assess the prognostic value of infiltrating immune cells in patients with resected HCC. Based on the inconsistencies in the literature, a quantification algorithm across four different tumor entities was developed (mCRC, OvCa, HCC, and PDAC) by evaluating the reliability and accuracy of counting steps. Finally, slides from 60 patients who underwent curative resection from November 2004 through October 2015 in our institution were obtained. The immunohistochemistry staining was performed against CD3, CD8, CD20, CD66b. I quantified the perivascular infiltrating immune cells by using the newly developed QTS algorithm and analyzed the relationship to clinicopathological characteristics and survival. In the study cohort, the perivascular $CD8^+$ T cells densities strongly correlated with $CD20^+$ B cells ($r=0.856$, $p<0.001$). The perivascular $CD66b^+$ neutrophil-like correlates with preoperative circulating leukocytes ($r=0.343$, $p=0.007$), platelets ($r=0.420$, $p=0.001$), and C-reactive protein ($r=0.344$, $p=0.008$). No evidence was found for the influence of $CD3^+$ ($p=0.058$), $CD8^+$ ($p=0.297$), $CD20^+$ ($p=0.535$), or $CD66b^+$ ($p=0.616$) cells on OS. Higher perivascular infiltration of $CD3^+$ ($p=0.016$) and $CD8^+$ ($p=0.028$) cells significantly predicted better

DFS. The $CD20^+$ (p=0.076) and $CD66b^+$ (p=0.521) cells had no significant influence on DFS. In multivariate analysis, the amount of perivascular infiltrating $CD3^+$ cells is an independent predictor of better overall survival (p=0.022), and $CD8^+$ cells predict prolonged disease-free survival independently (p=0.006).

In conclusion, the development of this newly introduced QTS algorithm showed that choosing hotspots by one observer is reliable, computer-assisted counting is accurate, and this solution is cost-effective. Using this novel algorithm, it is shown that HCC has relative low immune infiltration in compared with PDAC, mCRC, and OvCa. The amount of perivascular infiltrating $CD3^+$, $CD8^+$, and $CD20^+$ cells correlate with each other. Among of perivascular infiltrating $CD66b^+$ cells correlates circulating leukocytes, CRP, and platelets. The scorings of $CD3^+$, $CD8^+$, and $CD20^+$ infiltrations show significances on predicting disease-free survival. The amount of perivascular infiltrating $CD3^+$ cells is an independent predictor of better overall survival, and $CD8^+$ cells predict prolonged disease-free survival independently.

6. Zusammenfassung

Das Hepatozelluläre Karzinom (HCC) ist der häufigste primäre maligne Lebertumor. Sofern verfügbar ist die häufigste Therapie die Leberesektion (LR). Jedoch ist das Überleben der meisten Patienten unbefriedigend. In einigen Studien konnte gezeigt werden, dass das Immunsystem zur Entwicklung des HCC beiträgt. Zudem scheint die Prognose nach LR eines HCC auch zum Teil von der lokalen Immunreaktion abhängig zu sein. Daher ist ein profundes Verständnis des Immunsystems und der Immunreaktion gegen den Tumor bei HCC-Patienten essentiell. Dies erlaubt unter Umständen eine klinische Bewertung des Immunstatus für die Risikostratifizierung der an HCC leidenden Patienten. So eine Risikostratifikation könnte möglicherweise durch das standardisierte Zählen von Tumor infiltrierenden Leukozyten erreicht werden. Zudem muss unabhängig von der Art und der Anzahl der Tumor-infiltrierenden Lymphozyten, muss die Lokalisation bei der Prädiktion des Überlebens berücksichtigt werden. HCC hat einen hohen Grad an Neoangiogenese und die Oberfläche dieser Gefäße stellt die größte Grenze von malignen Tumor zum Immunsystem dar. Zunächst wurde eine Literaturrecherche durchgeführt, die auf einer umfassenden Suche in der PubMed-Datenbank bis November 2016 für alle verfügbaren Studien basierte. Diese diente der Identifikation des prädiktiven Werts der TILS bei Patienten mit HCC nach einer Operation. In der Literatur wurde eine sehr große Heterogenität bezüglich der Zählweisen der TILs evident. Somit wurde ein einheitlicher Quantifizierungsalgorithmus an verschiedenen Tumorentitäten entwickelt (mCRC, OvCa, HCC und PDAC), für den die Zuverlässigkeit und Genauigkeit der Zählschritte gezeigt werden konnten.

Schließlich wurden Proben von 60 Patienten, die von November 2004 bis Oktober 2015 in unserer Institution einer kurativer Resektion unterzogen wurden, analysiert. Die Immunhistochemie-Färbung wurde gegen CD3, CD8, CD20, CD66b durchgeführt. Es wurden die perivaskulären infiltrierenden Immunzellen unter Verwendung des neu entwickelten QTS-Algorithmus quantifiziert. Korrelationsanalysen mit klinikopathologischen Eigenschaften und Überleben wurde durchgeführt. In der Studienkohorte korrelierten die perivaskulären $CD8^+$ T-Zellen stark mit $CD20^+$ B-Zellen

(r=0,856, p<0,001). Die perivaskulären CD66b$^+$ Leukozyten korreliert mit präoperativen zirkulierenden Leukozyten (r=0,343, p=0,007), Plättchen (r=0,420, p=0,001) und C-reaktives Protein (r=0,344, p=0,008). Es zeigte sich kein signifikanter Einfluss von CD3$^+$ (p=0,058), CD8$^+$ (p=0,297), CD20$^+$ (p=0,535) oder CD66b$^+$ (p=0,616) Zellen auf das OS. Eine höhere perivaskuläre Infiltration von CD3$^+$ (p=0,016) und CD8$^+$ (p=0,028) Zellen prognostizierte ein besseres DFS. Die CD20$^+$ (p=0,076) und CD66b$^+$ (p=0,521) gefärbten Zellen hatten keinen signifikanten Einfluss auf die DFS. In der multivariaten Analyse konnte gezeigt werden, dass CD3$^+$ infiltrierende Leukozyten ein unabhängiger Prädiktor für das OS waren (p=0,022). CD8$^+$ infiltrierende Leukozyten waren prädiktiv für das DFS (p=0,006). Als Scoring zeigte sich die Kombination aus CD3$^+$, CD8$^+$ und CD20$^+$ infiltrierenden Leukozyten prädiktiv für das DFS jedoch nicht für das OS.

Abschließend ist unser neu eingeführter QTS-Algorithmus zuverlässig, präzise und kostengünstig. Unter Verwendung dieses neuartigen Algorithmus' konnte gezeigt werden, dass perivaskulär infiltrierende Leukozyten das tumorspezifische Überleben von Patienten, die sich einer kurativen Resektion unterziehen, unabhängig voraussagen können.

III. Reference

1. World Health Organization. *International Agency for Research on Cancer*. 2016 http://www.iarc.fr/.
2. El-Serag, H.B. and K.L. Rudolph, *Hepatocellular carcinoma: epidemiology and molecular carcinogenesis.* Gastroenterology, 2007. **132**(7): p. 2557-76.
3. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
4. European Association For The Study Of The, L., R. European Organisation For, and C. Treatment Of, *EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma.* J Hepatol, 2012. **56**(4): p. 908-43.
5. Aragon, R.J. and N.L. Solomon, *Techniques of hepatic resection.* J Gastrointest Oncol, 2012. **3**(1): p. 28-40.
6. Wakai, T., et al., *Anatomic resection independently improves long-term survival in patients with T1-T2 hepatocellular carcinoma.* Ann Surg Oncol, 2007. **14**(4): p. 1356-65.
7. Chen, J., et al., *The safety and efficacy of laparoscopic and open hepatectomy in hepatocellular carcinoma patients with liver cirrhosis: a systematic review.* Int J Clin Exp Med, 2015. **8**(11): p. 20679-89.
8. Levi Sandri, G.B., et al., *Laparoscopic and robotic approach for hepatocellular carcinoma-state of the art.* Hepatobiliary Surg Nutr, 2016. **5**(6): p. 478-484.
9. Khandoga, A., et al., *Differential significance of early surgical complications for acute and long-term recurrence-free survival following surgical resection of hepatocellular carcinoma: do comorbidities play a role?* Eur J Gastroenterol Hepatol, 2017.
10. Akamatsu, N. and N. Kokudo, *Liver transplantation for hepatocellular carcinoma from living-donor vs. deceased donor.* Hepatobiliary Surg Nutr, 2016. **5**(5): p. 422-428.
11. Bruix, J., M. Sherman, and D. American Association for the Study of Liver, *Management of hepatocellular carcinoma: an update.* Hepatology, 2011. **53**(3): p. 1020-2.

12. Belghiti, J. and R. Kianmanesh, *Surgical treatment of hepatocellular carcinoma.* HPB (Oxford), 2005. **7**(1): p. 42-9.
13. Llovet, J.M., A. Burroughs, and J. Bruix, *Hepatocellular carcinoma.* Lancet, 2003. **362**(9399): p. 1907-17.
14. Duffy, J.P., et al., *Liver transplantation criteria for hepatocellular carcinoma should be expanded: a 22-year experience with 467 patients at UCLA.* Ann Surg, 2007. **246**(3): p. 502-9; discussion 509-11.
15. Llovet, J.M., et al., *Sorafenib in advanced hepatocellular carcinoma.* N Engl J Med, 2008. **359**(4): p. 378-90.
16. Arii, S., et al., *Predictive factors for intrahepatic recurrence of hepatocellular carcinoma after partial hepatectomy.* Cancer, 1992. **69**(4): p. 913-9.
17. Ikeda, K., et al., *Risk factors for tumor recurrence and prognosis after curative resection of hepatocellular carcinoma.* Cancer, 1993. **71**(1): p. 19-25.
18. Cha, C., et al., *Predictors and patterns of recurrence after resection of hepatocellular carcinoma.* J Am Coll Surg, 2003. **197**(5): p. 753-8.
19. Ko, S., et al., *Significant influence of accompanying chronic hepatitis status on recurrence of hepatocellular carcinoma after hepatectomy. Result of multivariate analysis.* Ann Surg, 1996. **224**(5): p. 591-5.
20. Tsai, T.J., et al., *Clinical significance of microscopic tumor venous invasion in patients with resectable hepatocellular carcinoma.* Surgery, 2000. **127**(6): p. 603-8.
21. Sumie, S., et al., *Microvascular invasion in patients with hepatocellular carcinoma and its predictable clinicopathological factors.* Ann Surg Oncol, 2008. **15**(5): p. 1375-82.
22. Ng, I.O., et al., *Prognostic significance of pathologic features of hepatocellular carcinoma. A multivariate analysis of 278 patients.* Cancer, 1995. **76**(12): p. 2443-8.
23. Nagasue, N., et al., *Incidence and factors associated with intrahepatic recurrence following resection of hepatocellular carcinoma.* Gastroenterology, 1993. **105**(2): p. 488-94.
24. Hasegawa, K., et al., *Prognostic impact of anatomic resection for hepatocellular carcinoma.* Ann Surg, 2005. **242**(2): p. 252-9.

25. Eguchi, S., et al., *Comparison of the outcomes between an anatomical subsegmentectomy and a non-anatomical minor hepatectomy for single hepatocellular carcinomas based on a Japanese nationwide survey.* Surgery, 2008. **143**(4): p. 469-75.
26. Kim, B.W., et al., *Risk factors for immediate post-operative fatal recurrence after curative resection of hepatocellular carcinoma.* World J Gastroenterol, 2006. **12**(1): p. 99-104.
27. Fu, S.J., et al., *Glypican-3 is a potential prognostic biomarker for hepatocellular carcinoma after curative resection.* Surgery, 2013. **154**(3): p. 536-44.
28. Fan, S.T., et al., *Prediction of posthepatectomy recurrence of hepatocellular carcinoma by circulating cancer stem cells: a prospective study.* Ann Surg, 2011. **254**(4): p. 569-76.
29. Sun, Y.F., et al., *Circulating stem cell-like epithelial cell adhesion molecule-positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection.* Hepatology, 2013. **57**(4): p. 1458-68.
30. Chen, P.J., et al., *Clonal origin of recurrent hepatocellular carcinomas.* Gastroenterology, 1989. **96**(2 Pt 1): p. 527-9.
31. Poon, R.T., et al., *Different risk factors and prognosis for early and late intrahepatic recurrence after resection of hepatocellular carcinoma.* Cancer, 2000. **89**(3): p. 500-7.
32. Imamura, H., et al., *Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy.* J Hepatol, 2003. **38**(2): p. 200-7.
33. Portolani, N., et al., *Early and late recurrence after liver resection for hepatocellular carcinoma: prognostic and therapeutic implications.* Ann Surg, 2006. **243**(2): p. 229-35.
34. Gerunda, G.E., et al., *Role of transarterial chemoembolization before liver resection for hepatocarcinoma.* Liver Transpl, 2000. **6**(5): p. 619-26.
35. Mazzaferro, V., et al., *Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis.* Hepatology, 2006. **44**(6): p. 1543-54.

36. Bruix, J., et al., *Adjuvant sorafenib for hepatocellular carcinoma after resection or ablation (STORM): a phase 3, randomised, double-blind, placebo-controlled trial.* Lancet Oncol, 2015. **16**(13): p. 1344-54.
37. Chan, A.C., et al., *Treatment strategy for recurrent hepatocellular carcinoma: salvage transplantation, repeated resection, or radiofrequency ablation?* Liver Transpl, 2013. **19**(4): p. 411-9.
38. Faber, W., et al., *Repeated liver resection for recurrent hepatocellular carcinoma.* J Gastroenterol Hepatol, 2011. **26**(7): p. 1189-94.
39. Rossi, S., et al., *Repeated radiofrequency ablation for management of patients with cirrhosis with small hepatocellular carcinomas: a long-term cohort study.* Hepatology, 2011. **53**(1): p. 136-47.
40. Tabrizian, P., et al., *Recurrence of hepatocellular cancer after resection: patterns, treatments, and prognosis.* Ann Surg, 2015. **261**(5): p. 947-55.
41. Murphy, K. and C. Weaver, *Janeway's immunobiology*. 2016: Garland Science.
42. Schenk, W.G., Jr., et al., *Direct measurement of hepatic blood flow in surgical patients: with related observations on hepatic flow dynamics in experimental animals.* Ann Surg, 1962. **156**: p. 463-71.
43. Jarnagin, W.R. and L. Blumgart, *Blumgart's surgery of the liver, biliary tract, and pancreas*. 2012: Elsevier Saunders.
44. Novobrantseva, T.I., et al., *Attenuated liver fibrosis in the absence of B cells.* J Clin Invest, 2005. **115**(11): p. 3072-82.
45. Xu, R., et al., *The role of neutrophils in the development of liver diseases.* Cell Mol Immunol, 2014. **11**(3): p. 224-31.
46. Crispe, I.N., *Liver antigen-presenting cells.* J Hepatol, 2011. **54**(2): p. 357-65.
47. Crispe, I.N., *Immune tolerance in liver disease.* Hepatology, 2014. **60**(6): p. 2109-17.
48. Sachdeva, M., Y.K. Chawla, and S.K. Arora, *Immunology of hepatocellular carcinoma.* World J Hepatol, 2015. **7**(17): p. 2080-90.
49. Sauzay, C., et al., *Alpha-foetoprotein (AFP): A multi-purpose marker in hepatocellular carcinoma.* Clin Chim Acta, 2016. **463**: p. 39-44.
50. El-Serag, H.B., *Epidemiology of viral hepatitis and hepatocellular carcinoma.* Gastroenterology, 2012. **142**(6): p. 1264-1273 e1.

51. Guidotti, L.G., et al., *Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes.* Immunity, 1996. **4**(1): p. 25-36.
52. Chang, M.H., et al., *Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. Taiwan Childhood Hepatoma Study Group.* N Engl J Med, 1997. **336**(26): p. 1855-9.
53. Davila, J.A., et al., *Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study.* Gut, 2005. **54**(4): p. 533-9.
54. Wolf, M.J., et al., *Metabolic activation of intrahepatic CD8+ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes.* Cancer Cell, 2014. **26**(4): p. 549-64.
55. McMillan, D.C., *The systemic inflammation-based Glasgow Prognostic Score: a decade of experience in patients with cancer.* Cancer Treat Rev, 2013. **39**(5): p. 534-40.
56. Xiao, W.K., et al., *Prognostic significance of neutrophil-lymphocyte ratio in hepatocellular carcinoma: a meta-analysis.* BMC Cancer, 2014. **14**: p. 117.
57. Aravalli, R.N. and C.J. Steer, *Immune-Mediated Therapies for Liver Cancer.* Genes (Basel), 2017. **8**(2).
58. El-Khoueiry, A.B., et al., *Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial.* Lancet, 2017.
59. Toso, C., et al., *Factors predicting survival after post-transplant hepatocellular carcinoma recurrence.* J Hepatobiliary Pancreat Sci, 2013. **20**(3): p. 342-7.
60. Burra, P. and K.I. Rodriguez-Castro, *Neoplastic disease after liver transplantation: Focus on de novo neoplasms.* World J Gastroenterol, 2015. **21**(29): p. 8753-68.
61. Liu, Z.N., et al., *De Novo Malignancies After Liver Transplantation With 14 Cases at a Single Center.* Transplant Proc, 2015. **47**(8): p. 2483-7.
62. Hao, J.C., et al., *Effect of low-dose tacrolimus with mycophenolate mofetil on renal function following liver transplantation.* World J Gastroenterol, 2014. **20**(32): p. 11356-62.

63. Song, J.L., et al., *Minimizing tacrolimus decreases the risk of new-onset diabetes mellitus after liver transplantation.* World J Gastroenterol, 2016. **22**(6): p. 2133-41.
64. Sood, S. and A.G. Testro, *Immune monitoring post liver transplant.* World J Transplant, 2014. **4**(1): p. 30-9.
65. Fridman, W.H., et al., *The immune contexture in human tumours: impact on clinical outcome.* Nat Rev Cancer, 2012. **12**(4): p. 298-306.
66. Bindea, G., et al., *Natural immunity to cancer in humans.* Curr Opin Immunol, 2010. **22**(2): p. 215-22.
67. Bindea, G., et al., *The prognostic impact of anti-cancer immune response: a novel classification of cancer patients.* Semin Immunopathol, 2011. **33**(4): p. 335-40.
68. Yang, J.J., et al., *Prognostic significance of neutrophil to lymphocyte ratio in pancreatic cancer: a meta-analysis.* World J Gastroenterol, 2015. **21**(9): p. 2807-15.
69. Gabrielson, A., et al., *Intratumoral CD3 and CD8 T-cell Densities Associated with Relapse-Free Survival in HCC.* Cancer Immunol Res, 2016. **4**(5): p. 419-30.
70. De Monte, L., et al., *Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer.* J Exp Med, 2011. **208**(3): p. 469-78.
71. Sun, C., et al., *The predictive value of centre tumour CD8(+) T cells in patients with hepatocellular carcinoma: comparison with Immunoscore.* Oncotarget, 2015. **6**(34): p. 35602-15.
72. Karakhanova, S., et al., *Prognostic and predictive value of immunological parameters for chemoradioimmunotherapy in patients with pancreatic adenocarcinoma.* Br J Cancer, 2015. **112**(6): p. 1027-36.
73. Anitei, M.G., et al., *Prognostic and predictive values of the immunoscore in patients with rectal cancer.* Clin Cancer Res, 2014. **20**(7): p. 1891-9.
74. Mlecnik, B., et al., *The tumor microenvironment and Immunoscore are critical determinants of dissemination to distant metastasis.* Sci Transl Med, 2016. **8**(327): p. 327ra26.

75. Galon, J., et al., *The immune score as a new possible approach for the classification of cancer.* J Transl Med, 2012. **10**: p. 1.
76. Reid, M.D., et al., *Calculation of the Ki67 index in pancreatic neuroendocrine tumors: a comparative analysis of four counting methodologies.* Mod Pathol, 2016. **29**(1): p. 93.
77. Kroneman, T.N., et al., *Comparison of Three Ki-67 Index Quantification Methods and Clinical Significance in Pancreatic Neuroendocrine Tumors.* Endocr Pathol, 2015. **26**(3): p. 255-62.
78. Galon, J., et al., *Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.* Science, 2006. **313**(5795): p. 1960-4.
79. Koelzer, V.H. and A. Lugli, *The tumor border configuration of colorectal cancer as a histomorphological prognostic indicator.* Front Oncol, 2014. **4**: p. 29.
80. Huang, Y., et al., *Tumor-infiltrating FoxP3+ Tregs and CD8+ T cells affect the prognosis of hepatocellular carcinoma patients.* Digestion, 2012. **86**(4): p. 329-37.
81. Nakashima, T. and M. Kojiro, *Histological Growth Patterns of Hepatocellular Carcinoma*, in *Hepatocellular Carcinoma: An Atlas of Its Pathology*. 1987, Springer Japan: Tokyo. p. 81-104.
82. Wu, T.H., et al., *Encapsulation is a significant prognostic factor for better outcome in large hepatocellular carcinoma.* J Surg Oncol, 2012. **105**(1): p. 85-90.
83. Crispe, I.N., *Hepatic T cells and liver tolerance.* Nat Rev Immunol, 2003. **3**(1): p. 51-62.
84. Jiang, B., et al., *Tumor-infiltrating immune cells: triggers for tumor capsule disruption and tumor progression?* Int J Med Sci, 2013. **10**(5): p. 475-97.
85. Matsui, O., *Imaging of multistep human hepatocarcinogenesis by CT during intra-arterial contrast injection.* Intervirology, 2004. **47**(3-5): p. 271-6.
86. Martinet, L., et al., *Human solid tumors contain high endothelial venules: association with T- and B-lymphocyte infiltration and favorable prognosis in breast cancer.* Cancer Res, 2011. **71**(17): p. 5678-87.

87. Bellone, M. and A. Calcinotto, *Ways to enhance lymphocyte trafficking into tumors and fitness of tumor infiltrating lymphocytes.* Front Oncol, 2013. **3**: p. 231.

88. Muto, J., et al., *Review of angiogenesis in hepatocellular carcinoma.* Hepatol Res, 2015. **45**(1): p. 1-9.

89. Oelkrug, C. and J.M. Ramage, *Enhancement of T cell recruitment and infiltration into tumours.* Clin Exp Immunol, 2014. **178**(1): p. 1-8.

90. Mazzaferro, V., et al., *Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis.* N Engl J Med, 1996. **334**(11): p. 693-9.

91. Nathan, H., et al., *National trends in surgical procedures for hepatocellular carcinoma: 1998-2008.* Cancer, 2012. **118**(7): p. 1838-44.

92. Zheng, Z., et al., *Liver transplantation versus liver resection in the treatment of hepatocellular carcinoma: a meta-analysis of observational studies.* Transplantation, 2014. **97**(2): p. 227-34.

93. Thasler, W.E., et al., *Charitable State-Controlled Foundation Human Tissue and Cell Research: Ethic and Legal Aspects in the Supply of Surgically Removed Human Tissue For Research in the Academic and Commercial Sector in Germany.* Cell Tissue Bank, 2003. **4**(1): p. 49-56.

94. Maxwell, P. and W.G. McCluggage, *Audit and internal quality control in immunohistochemistry.* J Clin Pathol, 2000. **53**(12): p. 929-32.

95. Collett, D., *Modelling survival data in medical research*. 2015: CRC press.

96. Yin, X.Y., et al., *Prognostic significances of tumor-infiltrating S-100 positive dendritic cells and lymphocytes in patients with hepatocellular carcinoma.* Hepatogastroenterology, 2003. **50**(53): p. 1281-4.

97. Unitt, E., et al., *Tumour lymphocytic infiltrate and recurrence of hepatocellular carcinoma following liver transplantation.* J Hepatol, 2006. **45**(2): p. 246-53.

98. Gao, Q., et al., *Infiltrating memory/senescent T cell ratio predicts extrahepatic metastasis of hepatocellular carcinoma.* Ann Surg Oncol, 2012. **19**(2): p. 455-66.

99. Mathai, A.M., et al., *Role of Foxp3-positive tumor-infiltrating lymphocytes in the histologic features and clinical outcomes of hepatocellular carcinoma.* Am J Surg Pathol, 2012. **36**(7): p. 980-6.

100. Garnelo, M., et al., *Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma.* Gut, 2015.

101. Li, X., et al., *Identification of a FOXP3(+)CD3(+)CD56(+) population with immunosuppressive function in cancer tissues of human hepatocellular carcinoma.* Sci Rep, 2015. **5**: p. 14757.

102. Lee, W.C., et al., *The impact of CD4+ CD25+ T cells in the tumor microenvironment of hepatocellular carcinoma.* Surgery, 2012. **151**(2): p. 213-22.

103. Yang, Z.Q., et al., *Increased liver-infiltrating CD8+FoxP3+ regulatory T cells are associated with tumor stage in hepatocellular carcinoma patients.* Hum Immunol, 2010. **71**(12): p. 1180-6.

104. Yan, J., et al., *Prevalence and clinical relevance of T-helper cells, Th17 and Th1, in hepatitis B virus-related hepatocellular carcinoma.* PLoS One, 2014. **9**(5): p. e96080.

105. Brunner, S.M., et al., *Tumor-infiltrating, interleukin-33-producing effector-memory CD8(+) T cells in resected hepatocellular carcinoma prolong patient survival.* Hepatology, 2015. **61**(6): p. 1957-67.

106. Lin, S.Z., et al., *Prediction of recurrence and survival in hepatocellular carcinoma based on two Cox models mainly determined by FoxP3+ regulatory T cells.* Cancer Prev Res (Phila), 2013. **6**(6): p. 594-602.

107. Cai, X.Y., et al., *Dendritic cell infiltration and prognosis of human hepatocellular carcinoma.* J Cancer Res Clin Oncol, 2006. **132**(5): p. 293-301.

108. Zhang, J.P., et al., *Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients.* J Hepatol, 2009. **50**(5): p. 980-9.

109. Shen, X., et al., *Increased prevalence of regulatory T cells in the tumor microenvironment and its correlation with TNM stage of hepatocellular carcinoma.* J Cancer Res Clin Oncol, 2010. **136**(11): p. 1745-54.

110. Chew, V., et al., *Chemokine-driven lymphocyte infiltration: an early intratumoural event determining long-term survival in resectable hepatocellular carcinoma.* Gut, 2012. **61**(3): p. 427-38.

111. Tu, J.F., et al., *Mast Cells Comprise the Major of Interleukin 17-Producing Cells and Predict a Poor Prognosis in Hepatocellular Carcinoma.* Medicine (Baltimore), 2016. **95**(13): p. e3220.

112. Cariani, E., et al., *Immunological and molecular correlates of disease recurrence after liver resection for hepatocellular carcinoma.* PLoS One, 2012. **7**(3): p. e32493.

113. Gao, Q., et al., *Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection.* J Clin Oncol, 2007. **25**(18): p. 2586-93.

114. Chen, K.J., et al., *Intratumoral regulatory T cells alone or in combination with cytotoxic T cells predict prognosis of hepatocellular carcinoma after resection.* Med Oncol, 2012. **29**(3): p. 1817-26.

115. Liao, R., et al., *Systemic and intratumoral balances between monocytes/macrophages and lymphocytes predict prognosis in hepatocellular carcinoma patients after surgery.* Oncotarget, 2016. **7**(21): p. 30951-61.

116. Li, Y.W., et al., *Intratumoral neutrophils: a poor prognostic factor for hepatocellular carcinoma following resection.* J Hepatol, 2011. **54**(3): p. 497-505.

117. Ikeguchi, M., et al., *CD8+ lymphocyte infiltration and apoptosis in hepatocellular carcinoma.* Eur J Surg Oncol, 2004. **30**(1): p. 53-7.

118. Kobayashi, N., et al., *FOXP3+ regulatory T cells affect the development and progression of hepatocarcinogenesis.* Clin Cancer Res, 2007. **13**(3): p. 902-11.

119. Sasaki, A., et al., *Prognostic value of tumor-infiltrating FOXP3+ regulatory T cells in patients with hepatocellular carcinoma.* Eur J Surg Oncol, 2008. **34**(2): p. 173-9.

120. Zhou, J., et al., *Increased intratumoral regulatory T cells are related to intratumoral macrophages and poor prognosis in hepatocellular carcinoma patients.* Int J Cancer, 2009. **125**(7): p. 1640-8.

121. Shen, S.L., et al., *Foxp3+ regulatory T cells and the formation of portal vein tumour thrombus in patients with hepatocellular carcinoma.* Can J Surg, 2011. **54**(2): p. 89-94.

122. Huang, Y., et al., *Intrahepatic interleukin-17+ T cells and FoxP3+ regulatory T cells cooperate to promote development and affect the prognosis of hepatocellular carcinoma.* J Gastroenterol Hepatol, 2014. **29**(4): p. 851-9.

123. Li, Y.W., et al., *Tumor-infiltrating macrophages can predict favorable prognosis in hepatocellular carcinoma after resection.* J Cancer Res Clin Oncol, 2009. **135**(3): p. 439-49.

124. Ding, T., et al., *High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection.* Hum Pathol, 2009. **40**(3): p. 381-9.

125. Wu, Y., et al., *Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions.* Hepatology, 2013. **57**(3): p. 1107-16.

126. Kwak, Y., et al., *Immunoscore encompassing CD3+ and CD8+ T cell densities in distant metastasis is a robust prognostic marker for advanced colorectal cancer.* Oncotarget, 2016.

127. Fukunaga, A., et al., *CD8+ tumor-infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma.* Pancreas, 2004. **28**(1): p. e26-31.

128. Ene-Obong, A., et al., *Activated pancreatic stellate cells sequester CD8+ T cells to reduce their infiltration of the juxtatumoral compartment of pancreatic ductal adenocarcinoma.* Gastroenterology, 2013. **145**(5): p. 1121-32.

129. Ademmer, K., et al., *Effector T lymphocyte subsets in human pancreatic cancer: detection of CD8+CD18+ cells and CD8+CD103+ cells by multi-epitope imaging.* Clin Exp Immunol, 1998. **112**(1): p. 21-6.

130. Santoiemma, P.P., et al., *Systematic evaluation of multiple immune markers reveals prognostic factors in ovarian cancer.* Gynecol Oncol, 2016. **143**(1): p. 120-7.

131. Okabe, M., et al., *Predictive factors of tumor immune microenvironment for long-term follow-up in early stage breast cancer.* Cancer Sci, 2016.

132. Rodriguez-Vida, A., M. Strijbos, and T. Hutson, *Predictive and prognostic biomarkers of targeted agents and modern immunotherapy in renal cell carcinoma.* ESMO Open, 2016. **1**(3): p. e000013.

133. Curigliano, G. and E.A. Perez, *Immunoscoring breast cancer: TILs remember what they target.* Ann Oncol, 2014. **25**(8): p. 1455-6.

134. Donnem, T., et al., *Strategies for clinical implementation of TNM-Immunoscore in resected nonsmall-cell lung cancer.* Ann Oncol, 2016. **27**(2): p. 225-32.

135. Jiang, Y., et al., *ImmunoScore Signature: A Prognostic and Predictive Tool in Gastric Cancer.* Ann Surg, 2016.

136. Kwak, Y., et al., *Immunoscore encompassing CD3+ and CD8+ T cell densities in distant metastasis is a robust prognostic marker for advanced colorectal cancer.* Oncotarget, 2016. **7**(49): p. 81778-81790.

137. Li, J., et al., *The prognostic value of tumor-infiltrating T lymphocytes in ovarian cancer.* Oncotarget, 2017.

138. Kmiecik, J., et al., *Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level.* J Neuroimmunol, 2013. **264**(1-2): p. 71-83.

139. Zidlik, V., et al., *The changes of angiogenesis and immune cell infiltration in the intra- and peri-tumoral melanoma microenvironment.* Int J Mol Sci, 2015. **16**(4): p. 7876-89.

140. Pantanowitz, L., et al., *Review of the current state of whole slide imaging in pathology.* J Pathol Inform, 2011. **2**: p. 36.

141. Park, S., L. Pantanowitz, and A.V. Parwani, *Digital imaging in pathology.* Clin Lab Med, 2012. **32**(4): p. 557-84.

142. Indu, M., R. Rathy, and M.P. Binu, *"Slide less pathology": Fairy tale or reality?* J Oral Maxillofac Pathol, 2016. **20**(2): p. 284-8.

143. Kai, K., M. Masuda, and S. Aishima, *Inverse correlation between CD8+ inflammatory cells and E-cadherin expression in gallbladder cancer: Tissue microarray and imaging analysis.* World J Clin Cases, 2017. **5**(1): p. 1-8.

144. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis.* Nature methods, 2012. **9**(7): p. 671-675.

145. Yang, Z.F., et al., *Identification of local and circulating cancer stem cells in human liver cancer.* Hepatology, 2008. **47**(3): p. 919-28.

146. Freeman, M.R., et al., *Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis.* Cancer Res, 1995. **55**(18): p. 4140-5.

147. Huang, H., et al., *VEGF suppresses T-lymphocyte infiltration in the tumor microenvironment through inhibition of NF-kappaB-induced endothelial activation.* FASEB J, 2015. **29**(1): p. 227-38.

148. Cao, M., et al., *Kinase inhibitor Sorafenib modulates immunosuppressive cell populations in a murine liver cancer model.* Lab Invest, 2011. **91**(4): p. 598-608.

149. Mei, Z., et al., *Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis.* Br J Cancer, 2014. **110**(6): p. 1595-605.

150. Liu, Z., et al., *Presence of FOXP3(+)Treg cells is correlated with colorectal cancer progression.* Int J Clin Exp Med, 2014. **7**(7): p. 1781-5.

151. Zhang, L., et al., *Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.* N Engl J Med, 2003. **348**(3): p. 203-13.

152. Preston, C.C., et al., *The ratios of CD8+ T cells to CD4+CD25+ FOXP3+ and FOXP3- T cells correlate with poor clinical outcome in human serous ovarian cancer.* PLoS One, 2013. **8**(11): p. e80063.

153. Tewari, N., et al., *The presence of tumour-associated lymphocytes confers a good prognosis in pancreatic ductal adenocarcinoma: an immunohistochemical study of tissue microarrays.* BMC Cancer, 2013. **13**: p. 436.

154. Ibrahim, E.M., et al., *The prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancer: a meta-analysis.* Breast Cancer Res Treat, 2014. **148**(3): p. 467-76.

155. Mella, M., et al., *Tumor infiltrating CD8+ T lymphocyte count is independent of tumor TLR9 status in treatment naive triple negative breast cancer and renal cell carcinoma.* Oncoimmunology, 2015. **4**(6): p. e1002726.

156. Shang, B., et al., *Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis.* Sci Rep, 2015. **5**: p. 15179.

157. Reuschenbach, M., M. von Knebel Doeberitz, and N. Wentzensen, *A systematic review of humoral immune responses against tumor antigens.* Cancer Immunol Immunother, 2009. **58**(10): p. 1535-44.

158. Nelson, B.H., *CD20+ B cells: the other tumor-infiltrating lymphocytes.* J Immunol, 2010. **185**(9): p. 4977-82.

159. Milne, K., et al., *Systematic analysis of immune infiltrates in high-grade serous ovarian cancer reveals CD20, FoxP3 and TIA-1 as positive prognostic factors.* PLoS One, 2009. **4**(7): p. e6412.

160. Al-Shibli, K.I., et al., *Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer.* Clin Cancer Res, 2008. **14**(16): p. 5220-7.

161. Nedergaard, B.S., et al., *A comparative study of the cellular immune response in patients with stage IB cervical squamous cell carcinoma. Low numbers of several immune cell subtypes are strongly associated with relapse of disease within 5 years.* Gynecol Oncol, 2008. **108**(1): p. 106-11.

162. Gomez, D., et al., *Preoperative neutrophil-to-lymphocyte ratio as a prognostic predictor after curative resection for hepatocellular carcinoma.* World J Surg, 2008. **32**(8): p. 1757-62.

163. Halazun, K.J., et al., *Negative impact of neutrophil-lymphocyte ratio on outcome after liver transplantation for hepatocellular carcinoma.* Ann Surg, 2009. **250**(1): p. 141-51.

164. Ino, Y., et al., *Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer.* Br J Cancer, 2013. **108**(4): p. 914-23.

165. Yamada, Y., et al., *Prognostic value of CD66b positive tumor-infiltrating neutrophils in testicular germ cell tumor.* BMC Cancer, 2016. **16**(1): p. 898.

166. Ilie, M., et al., *Predictive clinical outcome of the intratumoral CD66b-positive neutrophil-to-CD8-positive T-cell ratio in patients with resectable nonsmall cell lung cancer.* Cancer, 2012. **118**(6): p. 1726-37.

167. Jensen, H.K., et al., *Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma.* J Clin Oncol, 2009. **27**(28): p. 4709-17.

168. Carus, A., et al., *Tumour-associated CD66b+ neutrophil count is an independent prognostic factor for recurrence in localised cervical cancer.* Br J Cancer, 2013. **108**(10): p. 2116-22.

169. Zhang, J., et al., *Vasculogenic mimicry is associated with increased tumor-infiltrating neutrophil and poor outcome in esophageal squamous cell carcinoma.* Onco Targets Ther, 2017. **10**: p. 2923-2930.

170. Rao, H.L., et al., *Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis.* PLoS One, 2012. **7**(1): p. e30806.

171. Governa, V., et al., *The Interplay Between Neutrophils and CD8+ T Cells Improves Survival in Human Colorectal Cancer.* Clin Cancer Res, 2017. **23**(14): p. 3847-3858.

172. Le Gal, F.A., et al., *Tissue homing and persistence of defined antigen-specific CD8+ tumor-reactive T-cell clones in long-term melanoma survivors.* J Invest Dermatol, 2007. **127**(3): p. 622-9.

173. Finak, G., et al., *Standardizing Flow Cytometry Immunophenotyping Analysis from the Human ImmunoPhenotyping Consortium.* Sci Rep, 2016. **6**: p. 20686.

174. Guba, M., et al., *Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor.* Nat Med, 2002. **8**(2): p. 128-35.

175. Cheng, J.W., et al., *An immune function assay predicts post-transplant recurrence in patients with hepatocellular carcinoma.* J Cancer Res Clin Oncol, 2011. **137**(10): p. 1445-53.

176. Asrih, M., et al., *Alpha-fetoprotein: a controversial prognostic biomarker for small hepatocellular carcinoma.* World J Gastroenterol, 2013. **19**(3): p. 328-30.

177. Yip, V.S., et al., *Tumour size and differentiation predict survival after liver resection for hepatocellular carcinoma arising from non-cirrhotic and non-fibrotic liver: a case-controlled study.* Int J Surg, 2013. **11**(10): p. 1078-82.

178. Torzilli, G., et al., *A snapshot of the effective indications and results of surgery for hepatocellular carcinoma in tertiary referral centers: is it adherent to the EASL/AASLD recommendations?: an observational study of the HCC East-West study group.* Ann Surg, 2013. **257**(5): p. 929-37.

179. Villanueva, A., et al., *Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma.* Gastroenterology, 2011. **140**(5): p. 1501-12 e2.

180. Vilchez, V., et al., *Cancer stem cell marker expression alone and in combination with microvascular invasion predicts poor prognosis in patients undergoing transplantation for hepatocellular carcinoma.* Am J Surg, 2016. **212**(2): p. 238-45.

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