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Valtteri Kairaluoma

THE EVOLVING TREATMENT AND BIOMARKERS OF HEPATOCELLULAR CARCINOMA

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Abstract

Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related death worldwide. Overall prognosis for survival in HCC is poor. Risk factors for HCC include hepatitis viruses, especially in Eastern countries. In Western countries, heavy alcohol consumption and metabolic liver disease are major causes of liver cirrhosis and cancer. The detection of HCC is challenging because the symptoms often appear at later stages of the cancer. The tumoral microenvironment plays an important role in tumor progression, growth and metastasis. Tumor budding, defined as a single tumor cell or a cell cluster of up to four tumor cells at the invasive front of carcinomas, is associated with poor prognosis in several cancer types. Tumor-Stroma Ratio (TSR) is defined as the percentage of tumor cell component relative to the surrounding stroma. Low TSR (high proportion of stroma) in tumor tissue has been recognized as an important factor for tumor prognosis in gastric cancer, breast cancer, lung cancer and esophageal cancer, for example. Toll-like receptors (TLRs) are innate immunity receptors that are involved in bacterial and viral recognition but also have a role in diverse vital functions, adaptive immunity, and carcinogenesis.

This thesis examined the treatment trends and results of HCC patients treated in Oulu University hospital in 1983–2018. Tumor-Stroma ratio and tumor budding were examined from Hematoxylin and Eosin-stained (HE) histological samples that were originally used for diagnostic purposes. The prognostic value of tumor budding, TSR, TLR2, TLR4, TLR5 and TLR8 staining in HCC was evaluated. Based on our study on the Northern Finland population, the rate of surgical treatment has decreased significantly since the introduction of local treatments and TACE. Compared to other treatments, the surgical resection of HCC has an acceptable complication rate and best long-term survival. We observed that tumor budding, TLR4 and TLR5 are independent factors of poor prognosis in HCC. According to our study, tumor budding is a prognostic factor that can be reliably replicated and routinely analyzed from HE-stained slides. TLR4 and TLR5 are possible therapeutic targets in the future.

Keywords: hepatocellular carcinoma, surgery, Toll-like receptors, tumor budding, Tumor-Stroma Ratio

Kairaluoma, Valtteri, Hepatosellulaarisen karsinooman kehittyvä hoito ja biomerkkiaineet.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala

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Tiivistelmä

Hepatosellulaarinen karsinooma (HCC) on maailmanlaajuisesti neljänneksi yleisin syöpäkuolemien syy. HCC-potilaiden ennuste on huono. Suurimpiin riskitekijöihin kuuluvat etenkin itäisissä maissa hepatiittivirukset, kun taas länsimaissa suurimpina riskeinä ovat runsas alkoholin käyttö ja maksan rasvoittuminen, jotka voivat johtaa maksakirroosiin ja syöpään. HCC:n havaitseminen on haasteellista, koska syöpä ei aiheuta juurikaan oireita, kuin vasta taudin myöhäisvaiheissa.

Syövän mikroympäristö ja sitä ympäröivä sidekudos (strooma) ovat tärkeässä roolissa syövän koon kehityksessä, kasvussa ja metastasoinnissa. Kasvaimen silmuilu (tumor budding) määritellään laskemalla tuumorisilmut (tumor buds). Tuumorisilmu on yhdestä neljään syöpäsolua kattava solurypäs syövän invasiivisella alueella. Tuumorisilmujen esiintymisen on havaittu olevan huonon ennusteen merkki useissa eri syövissä. Kasvain-stroomasuhde (Tumor-Stroma Ratio, TSR) on tuumorin syöpäsolukomponentin prosenttiosuus verrattuna ympäröivän strooman määrään. Matalan kasvain-stroomasuhteen (korkean stroomaosuuden) on havaittu olevan huonon ennusteen merkki esimerkiksi maha-, rinta-, keuhko- ja ruokatorvisyövässä. Tollin kaltaiset reseptorit (TLR:t) toimivat osana luontaista immuniteettia tunnistaen virusten ja bakteerien osia, mutta niillä on havaittu olevan rooli myös muissa elimistön tapahtumissa, kuten hankinnaisessa immuniteetissa ja karsinogeneesissä.

Tämä väitöstutkimus kuvaa OYS:ssa hoidettujen HCC-potilaiden hoidon muuttumisen ja tulokset vuosilta 1983–2018. Väitöstutkimuksessa tutkitaan alun perin diagnostisiin tarkoituksiin kerätyistä hematoksyliini-eosiini- värjätyistä näytteistä tuumorisilmujen määrä ja kasvain-stroomasuhde sekä niiden merkitys HCC-potilaan ennusteelle. Väitöstutkimuksessa analysoidaan myös TLR2:n, TLR4:n, TLR5:n ja TLR8:n esiintyminen ja värjäytyminen maksasyöpäsoluissa ja niiden merkitys HCC-potilaiden ennusteelle. Tuloksemme osoittavat, että kirurgiset hoidot ovat vähentyneet paikallisten hoitojen ja transarteriaalisen kemoembolisaation (TACE) käyttöönoton myötä. Maksaresektio on HCC-potilaan pitkäaikaisen ennusteen kannalta paras hoitomuoto muihin hoitoihin verrattuna, ja komplikaatioiden määrä on hyväksyttävällä tasolla. Lisäksi osoitamme, että hepatosellulaarisessa karsinoomassa kasvaimen silmuilu, TLR4 ja TLR5 ovat itsenäisesti ennusteellisia tekijöitä ja niiden esiintyminen korreloi potilaiden huonompaan ennusteeseen. Tutkimustulostemme mukaan kasvaimen silmuilu on luotettavasti kudosnäytteistä havaittava ennustetekijä ja TLR4 ja TLR5 ovat mahdollisia terapeuttisia kohteita hepatosellulaarisessa karsinoomassa.

Asiasanat: kasvaimen silmuilu, kasvain-stroomasuhde, kirurgia, maksasolusyöpä, Tollin kaltaiset reseptorit, tumor budding

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Päivämäärä 22.4.2021. Valtteri Kairaluoma

Abbreviations

AFB1	Aflatoxin B1
AFP	Alpha-fetoprotein
ALPPS	Associating liver partition and portal vein ligation for staged hepatectomy
APHE	Arterial phase hyperenhancement
ARF	Alternate reading protein
BCLC	Barcelona Clinic Liver Cancer system
CAF	Cancer-associated fibroblast
CC	Cholangiocarcinoma
CD8+	Cluster of differentiation 8+
CEA	Carcinoembryonic antigen
CEUS	Contrast-enhanced ultrasound
CK19	Cytokeratin 19
CLR	C-type lectin receptor
CNA	Copy number alterations
CSC	Cancer stem cell
CT	Computer tomography
CTC	General stress protein
DAMP	Damage-associated molecular patterns
DEAF-1	Deformed epidermal autoregulatory factor-1
DCP	Des- γ -carboxyprothrombin
DDR	DNA damage response
DN	Dysplastic nodule
DNA	Deoxyribonucleic acid
EASL	European Association for the Study of Liver
ECM	Extracellular matrix
ECOG	Eastern Cooperative Oncology Group
eHCC	Early HCC
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular-regulated kinase
ESMO	European Society for Medical Oncology
EZH2	Enhancer of zeste homolog 2
FDG PET	Fluorodeoxyglucose-positron emission tomography
FGF19	Fibroblast growth factor 19
FLR	Future liver remnant

HE	Hematoxylin and eosin
HCC	Hepatocellular carcinoma
HBV	Hepatitis virus B
HCV	Hepatitis virus C
HGF	Hepatic growth factor
HSC	Hepatic stellate cell
HSP70	Heat shock protein 70
hTERT	Human telomerase reverse transcription
ICG	Indocyanine green
IFN	Interferone
IGF	Insulin-like growth factor
IHC	Immunohistochemical
IKK	IκB kinase-complex-NF-κB
IKKi	I kappa B kinase i
IκBα	Inhibitory protein I kappa B alpha
IL-1	Interleukin-1
IRAK1	IL-1 receptor-associated kinase 1
IRAK4	IL-1 receptor-associated kinase 4
IRF3	Interferon regulatory factor 3
JAK	Janus kinase
LI-RADS	Liver Imaging Reporting and Data System
LOX	Lysyl oxidase
LPS	Lipopolysaccharide
LR	Liver resection
LRR	Leucine-rich repeat
LT	Liver transplantation
MAPK	Mitogen-activated protein kinase
MD2	Myeloid differentiation factor-2
MDM2	Murine double minute 2
MDM4	Murine double minute 4
MELD	Model for end-stage liver disease
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MTA2	Metastasis-associated tumor gene 2
MyD88	Myeloid differentiation primary response protein 88
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor-kappa B

NK	Natural killer cells
NLRS	Nucleotide oligomerization domain (NOD)-like receptors
NOD	Nucleotide oligomerization domain
PAMP	Pathogen-associated molecular pattern
PDGF	Platelet-derived growth factor
PEI	Percutaneous ethanol injection
pHCC	Progressed HCC
PI3K	Phosphatidylinositol 3-kinase
PRR	Pattern recognition receptor
PVE	Portal vein embolization
PVL	Portal vein ligation
RF	Radiofrequency ablation
RIP-1	Receptor interacting serine/threonine 1
RLR	Retinoic acid-inducible gene-I like receptor
RNA	Ribonucleic acid
SIRT	Selective internal radiotherapy
STAT	Signal transducers and activators of transcription
TAB	TAK/TGF- β -activated kinase
TACE	Transarterial chemoembolization
TAE	Transarterial embolization
TAK1	Transforming growth factor- β -activated kinase 1
TAM	Tumor-associated macrophages
TBK1	TANK-binding kinase-1
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGFβ	Transforming growth factor-beta
TIR	Toll-/IL-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptors
TLR 1-10	Toll-like receptor 1-10
TMA	Tissue micro array
TNF-α	Tumor-necrosis factor α
TNM	Tumor, node, metastasis
TP53	Tumor protein 53
TRAF6	Tumor necrosis factor (TNF) receptor associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing-adaptor inducing interferon-beta

TSR	Tumor-Stroma ratio
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
HIF1 and 2	Hypoxia-inducible factors 1 and 2

List of original publications

I Kairaluoma, V., Karjalainen, M., Pohjanen, V-M., Saarnio, J., Niemelä, J., Huhta, H., & Helminen, O. (2021).
Treatment trends and outcomes of hepatocellular carcinoma in a single center during 35 years. *Minerva Surgery*, *76*(3), 252-263. doi: 10.23736/S2724-5691.21.08426-1.

II Kairaluoma, V., Kemi, N., Pohjanen, V-M., Saarnio, J., & Helminen, O. (2020). Tumour budding and tumour-stroma ratio in hepatocellular carcinoma. *British Journal of Cancer, 123*(1), 38-45. doi: 10.1038/s41416-020-0847-1

III Kairaluoma, V., Kemi, N., Huhta, H., Pohjanen, V-M., & Helminen, O. (2021). Prognostic role of TLR4 and TLR2 in hepatocellular carcinoma. *Acta Oncologica, 60*(4), 554-558. doi: 10.1080/0284186X.2021.1877346

IV Kairaluoma, V., Kemi, N., Huhta, H., Pohjanen, V-M., & Helminen, O. Toll-like receptor 5 and 8 in hepatocellular carcinoma. (2021) *APMIS*, doi: 10.1111/apm.13142. *Online ahead of print.*

The publications have not been used and will not be used in anyone else's thesis.

Contents

1 Introduction

Hepatocellular carcinoma is one of the leading causes of cancer mortality, with patients often presenting at an inoperable stage. The known prognostic factors of HCC include clinical and imaging features, tumor size and number, invasion, serum AFP and comorbidity. Morphological prognostic features include tumor grade, subtype, stage, cirrhosis, immunohistochemical expression of CK19, vascular invasion and intrahepatic metastasis. Known risk factors for the development of HCC include inflammation related to hepatitis viruses, alcohol, smoking and dietary factors. Surgical resection is the first-line therapy for single HCC of any size when hepatic function is preserved, sufficient remnant liver volume is maintained and the patient's physical condition is permissive for surgery. Only 20–30% of the HCC are resectable.

Tumor budding is an acknowledged prognostic factor in colorectal cancer and is associated with poor prognosis in several cancer types. Tumor budding is defined as a single tumor cell or a cell cluster of up to four tumor cells at the invasive front of carcinomas and has been postulated to represent epithelial-to-mesenchymal transition. In HCC, the prognostic value of tumor budding is poorly known.

The tumoral microenvironment plays an important role in tumor progression, growth, and metastasis. As a major part of the tumor microenvironment, the stromal component is crucial for tumor development and support. Tumor-Stroma Ratio (TSR) is defined as the percentage of tumor cell component relative to the surrounding stroma. Low TSR (high proportion of stroma) in tumor tissue has been recognized as an important factor for tumor prognosis in various cancer types. The significance of TSR in HCC is unclear.

Toll-like receptors (TLR) are innate immunity receptors that recognize pathogen-associated molecular patterns and enable rapid responses against invading pathogens. TLRs have a role in diverse vital functions, tissue repair, regeneration, and regulation of sterile inflammation, but also in carcinogenesis. TLRs have their specific ligands, the recognition of which leads to immunologic and carcinogenic responses.

The aim of this thesis was to examine the treatment trends and outcomes of HCC in Northern Finland and evaluate the prognostic significance of TSR, tumor budding, TLR2, TLR4, TLR5 and TLR8 in hepatocellular carcinoma.

2 Review of the literature

2.1 Hepatocellular carcinoma

Hepatocellular carcinoma covers > 80% of primary liver cancers and is the fourth most lethal cancer (Bray et al., 2018). Surveillance and early detection would increase the chance of potentially curative treatment; however, even in countries with sufficient medical resources, HCC surveillance is underutilized (Yang et al., 2019). In Europe, the median age of diagnosis is above 60 years; in contrast, in parts of Asia and Africa, HCC is commonly diagnosed in the range of 30–60 years (Park et al., 2015). The overall survival of patients with HCC varies around the globe, from 2.5 months in sub-Saharan Africa (Yang et al., 2017) to 60 months in Japan (Park et al., 2015). In Europe, the median survival is approximately 24 months (Park et al., 2015). The risk factors for HCC are mentioned in Chapter 2.1.4 (Yang et al., 2019). Clinical signs and symptoms of HCC include right upper quadrant abdominal pain, weight loss, rapid deterioration in the setting of liver cirrhosis, hepatomegaly, splenomegaly, jaundice and rapid increase of ascites (Lokuhetty et al., 2019).

2.1.1 History of liver surgery

Before anesthesia and antiseptic products, liver surgery was usually a very high-risk procedure (Arish et al., 2011). In 1886, the first hepatectomy was performed by Dr. Luis; unfortunately, the patient died 6 hours later due to postoperative hemorrhage (Lius, 1887). The first successful hepatectomy was performed by the German surgeon Carl Johann August Langenbuch in 1888 (Langenbuch, 1888). He performed surgery in which he resected part of the left lobe after ligating the vascular pedicles (Langenbuch, 1888). In 1908, Dr. Pringle presented a technique where he controlled liver bleeding during surgery by obstructing the portal vein and hepatic artery (Pringle, 1908). Pringle's method is still widely used to reduce blood loss during liver resections (Kokudo et al., 2020). Bigger steps towards modern liver surgery were taken in the 1950s when Claude Couinaud published his book where he described the segmental anatomy of the liver (Couinaud, 1954). Later, this concept became the basis of systematic subsegmental hepatectomy (Arish et al., 2011). In the 1950s, the Taiwanese surgeon Lin developed the "finger-fracture method" in which the fingertips are used to crush the liver parenchyma in order to

reduce blood loss and expose vessels needing to be ligated and divided (Lin et al., 1958). The first liver transplantation from a brain-dead donor was performed by Starzl et al. in 1963 (Starzl et al., 1963). The clinical outcomes began to improve and become acceptable in the 1970s and 1980s thanks to new technological approaches and the development of new immunosuppressants (Calne, 1979). Laparoscopic and robotic surgery has decreased the number of open liver surgery (Kokudo et al., 2020). The introduction into practice of ultrasound, also used intraoperatively, in the 1980s allowed clinicians to diagnose small liver tumors and boosted the rapid development of liver surgery (Arish et al., 2011; Stellamor, 1976). Cancer imaging techniques of vascular exclusion of the liver, laparoscopic and robotic techniques, new devices for dissection, Indocyanine green fluorescence imaging and new techniques to grow future liver remnant volume that allow the resection of previously unresectable tumors have allowed longer patient survival and less complications in the field of liver surgery (Arish et al., 2011).

2.1.2 Risk factors

Approximately 90% of HCCs are associated with a known underlying etiology (Fitzmaurice et al., 2017). Globally, chronic hepatitis virus B (HBV) and hepatitis virus C (HCV) are the most important causes of HCC (Fitzmaurice et al., 2017). Chronic HBV infection is the leading cause of HCC in Eastern Asia and Africa (Fitzmaurice et al., 2017). It is estimated that 57 million people have chronic HCV infection; of them, 10-20% will develop liver complications, including cirrhosis and HCC (Hajarizadeh et al., 2013; Heffernan et al., 2019). Cirrhosis, an important risk factor for HCC, may be caused by various factors, for example HBV (Iloeje et al., 2006), HCV (Kanwal, et al., 2014; Raimondi et al., 2009), heavy alcohol consumption (Mancebo et al., 2013; Nahon et al., 2009), dietary factors, such as non-alcoholic fatty liver disease (NAFLD) (Calle et al., 2003), aflatoxins (Hsu et al., 1991) as well as genetic hemochromatosis (Deugnier et al., 1993), or alpha-1-antitrypsin deficiency (Eriksson et al., 1986). Approximately 1-8% of patients with cirrhosis will develop HCC (Ioannou et al., 2007). HCV is the leading cause for HCC in North America, Europe, Japan and Northern Africa (Fitzmaurice et al., 2017). NAFLD is an increasing risk factor for HCC in developed countries (Dyson et al., 2014). Heavy alcohol consumption is the second most common risk factor for HCC in the USA and Europe (Park et al., 2015). Aflatoxins are dietary toxins that have strong hepatocarcinogenic effects (Weng et al., 2017). Aflatoxins are mycotoxins that contaminate staple cereals and oilseeds (Yang et al., 2019).

Exposure to aflatoxins is more common in low-resource or middle-resource countries, such as in West Africa, where inappropriate postharvest processing occurs more often than in more developed countries (Gouas et al., 2009). The main form of aflatoxin is B1 and is produced by *Aspergillus* (Yang et al., 2019). Several sociodemographic characteristics have been associated with HCC, for example ageing, male sex, Hispanic race and smoking (Llovet et al., 2021). Coffee (Bravi et al., 2017), statins (Singh et al., 2013), metformin (Zhou et al., 2016) and aspirin (Sahasrabuddhe et al., 2012) have shown protective effects against the development of HCC. However, there are no existing randomized trials of these associations (Yang et al., 2019).

2.1.3 Incidence of hepatocellular carcinoma

The worldwide incidence of HCC is heterogeneous due to different underlying risk factors behind the disease. It is estimated that most cases occur in Asia (72.5%), 9.7% in Europe, 7.8% in Africa, 5.1% in North America, and the rest in Latin America and Oceania. The highest age-standardized incidence rates per 100,000 occur in Eastern Asia (17.8), followed by Northern Africa (15.2), Micronesia (14.6) and South-Eastern Asia (13.7) (Globocan, 2020). In Northern Europe, the rates are 4.7 per 100,000 (Globocan, 2020). Despite a slowly decreasing trend in global age-standardized incidence rates, the total number of HCC cases globally is rising due to aging and population growth (Yang et al., 2019). In Finland, the incidence of liver cancer in 2018 was 4.9/100,000 and 13.44/100,000 persons per year among females and males, respectively (Finnish Cancer Registry, 2018). Altogether, there were 503 liver cancer patients diagnosed in Finland in 2018.

2.1.4 Pathogenesis

HCC can be induced by multiple etiologies and has a complex pathogenesis (Dhanasekaran et al., 2016). Most causes of HCC mediate liver injury through the development of liver inflammation and fibrosis, which leads to the disordered liver architecture of liver cirrhosis (Balkwill & Mantovani, 2001). Typically, cirrhotic livers exhibit focal areas of abnormal, immature hepatocytes and dysplastic foci or dysplastic nodules, which are considered precancerous lesions and can be classified as low grade or high grade based on histological features (Dhanasekaran et al., 2016). The stromal invasion is considered as the hallmark feature that differentiates early HCC from dysplastic nodules (Kojiro et al., 2009). HCC is subclassified as

early HCC (<2 cm, grade I) and progressed HCC (<2 cm (gradus II or III) or >2 cm, or visible distinct nodules or vascular invasion or metastases)) (Dhanasekaran et al., 2016).The development of HCC is caused by intrinsic factors, which are genetic mutations, either inherited or acquired, and extrinsic risk factors, which are described in Chapter 2.1.4 (Ghouri et al., 2017). In the tumor environment, hepatocytes undergo a malignant transformation through mechanisms that promote tumor progression, prevent tumor destruction, and increase tumor proliferation and neovascularization (Ghouri et al., 2017; Poon et al., 2007). Cirrhosis is a common factor that induces carcinogenic changes and is found in 90% of patients with HCC (Tiribelli et al., 1989).The microenvironment in the cirrhotic liver is changed (Liu et al., 2012). Metabolic and oxidative injury cause inflammation, necrosis and repeated compensatory regeneration and increased turnover of hepatocytes, which leads to accumulation of genetic errors and mutations, such as point mutations and deletions, chromosomal gains, telomere erosion and telomerase reactivation (Ghouri et al., 2017; Jhunjhunwala et al., 2014).

Genetic mechanisms

The most mutated genes in HCC are *CTNNB1* (Guichard et al., 2012; Totoki et al., 2014) and *TP53* (Ahn et al., 2014; Totoki et al., 2014). *CTNNB1* is part of Wnt/ β -catenin signaling, one of the most common disrupted pathways in HCC, which is implicated in embryogenesis, differentiation, cell proliferation and tumorigenesis (De La Coste et al., 1998; Dhanasekaran et al., 2016; Li et al., 2014). Deletion of *AXIN1* mediates Wnt/ β -catenin signaling in HCC (Guichard et al., 2012). *TP53* is part of DNA-repair and surveillance and a well-known tumor suppressor. Chapter 2.5.7 contains more information about TP53.

Genome instability is an important factor in cancer progression, including HCC, and may result in copy number alterations (CNA) with gain or loss in chromosomes of different extents or somatic mutations in genomes (Ho et al., 2016; Kan et al., 2013; Totoki et al., 2014). Dysregulated epigenetic mechanisms contribute to hepatocarcinogenesis by influencing gene transcription, chromosomal stability and cell differentiation (Dhanasekaran et al., 2016; Wang et al., 2015). These mechanisms include changes in methylation, hydroxymethylation, histone modification, chromatin remodeling, microRNAs, long non-coding RNAs and acetylation (Wang et al., 2015). Ethanol metabolites, acetaldehyde and reactive oxygen species induce oxidative stress, chronic inflammation and disrupt interactions of DNA, RNA, lipids and proteins, leading to genomic instability and

insufficient repair pathways (Bautista, 2002; Obe et al., 1986; Seitz & Stickel, 2007).

Telomere-associated pathogenesis

Telomerase, which is usually suppressed in mature cells, is an enzymatic protein complex made up of the telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) that maintains telomere length by synthesizing telomeric DNA sequences and adding them to the end of the chromosomes (Dhanasekaran et al., 2016). Mutations in the TERT promotion region are the most common mutation in HCC and the most frequent mechanism for telomerase activation (Dhanasekaran et al., 2016; Killela et al., 2013; Nault et al., 2013). Chronic liver injury emphasizes telomere shortening; beyond a certain point, this leads to activation of a DNA damage program that leads to apoptosis and disordered liver cell architecture triggering liver fibrosis and eventually cirrhosis, which is a precursor of HCC and can drive chromosomal instability and cancer initiation (Calado et al., 2011; Dhanasekaran et al., 2016).

Tumoral microenvironment-related pathways

Activation of various pathways has been shown to push hepatocarcinogenesis (Dhanasekaran et al., 2016; Yang et al., 2011). Activation of receptor tyrosine kinases induces the Ras-mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways in about 50% of HCCs (Villanueva et al., 2007). Ligand binding and phosphorylation in several different growth factor tyrosine kinase receptors lead to activation of the MAPK and PI3K pathways (Roberts & Gores, 2005). MAPK pathway activation induces proto-oncogene factors and transcription factors that induce cell proliferation (Roberts & Gores, 2005). PI3K pathway activation results in cell growth, proliferation, metabolism and survival (Dhanasekaran et al., 2016).The transforming growth factor-beta (TGF β) pathway has the ability to suppress cellular growth in the early stages of cancer initiation, paradoxically, it can promote invasiveness and angiogenesis in later stages. (Roberts & Wakefield, 2003). TGF β induction in genes has been associated with increased tumor recurrence and metastatic ability of the tumor (Coulouarn et al., 2008). TGF β is known to promote epithelial-to-mesenchymal transition (EMT), which plays a critical role in promoting tumor metastases (Giannelli et al., 2005). Cancer stem cells (CSCs) are responsible for

tumor initiation, but also for tumor persistence, relapse, metastasis, and they also have an influence on tumor chemo- and radioresistance (Yamashita & Wang, 2013).Vascular endothelial growth factor (VEGF) and angiopoetins play an important role in promoting and sustaining neoangiogenesis in HCC (Park et al., 2000; Yoshiji et al., 2005). Hypoxia in HCC induces hypoxia-inducible factors (HIF1 and HIF2) and insulin-like growth factors (IGFs) that promote tumor angiogenesis (Cannito et al., 2015; Kim et al., 1998). HIFs also have an influence on tumor chemo- and radio-resistance (Luo et al., 2014). Activated hepatic stellate cells (HSCs) (see Chapter 2.2) promote HCC growth via activation of nuclear factor-kappa B (NF-κB) and extracellular-regulated kinase (ERK) pathway (Amann et al., 2009). ERK is part of a complex pathway that includes co-operation with proteins of Ras, Raf and MAPK (Birgani & Carloni, 2017). ERK triggers the expression of proliferative genes in the nucleus. JAK/STAT signaling is one of the key activators of HSCs (Birgani & Carloni, 2017). JAK activates tyrosine phosphorylation which activates the STAT pathway, and a variety of cytokines, hormones and growth factors are activated. Activated STATs lead to cell proliferation, migration, differentiation, and apoptosis (Dhanasekaran et al., 2016). Cancer-associated fibroblasts (CAFs) (see Chapter 2.2) are activated by transforming TGF β and remodel the extracellular matrix and modulate the biological activity of HCC. Reciprocally, HCC tumor cells stimulate the activation of CAFs (Fransvea et al., 2009). Tumor-associated macrophages (TAMs) (see Chapter 2.2) are involved in many tumor-initiating cascades, such as neoangiogenesis, metastasis and suppression of adaptive immunity, in inflammation-related HCC (Qian & Pollard, 2010). One reason for this may be the TAMs' capability to enhance tumor progression by impairing cytotoxic CD8+T cell immune response (Barajas et al., 2001). TAMs are usually accumulated in the hypoxic region of the tumors where they take part in pro-angiogenic production, such as VEGF, TNF-α and matrix metalloproteinases (Capece et al., 2013).

Hepatitis B virus

HBV is an enveloped partially double-stranded DNA virus that initiates hepatocarcinogenesis by integrating into the host genome (Imai et al., 1987). HBV infection is associated with a high frequency of mutations, as it replicates through RNA-mediated reverse transcription (Liang, 2009). HBV can induce carcinogenesis by modulating the expression of liver-specific micro-RNAs (Wang et al., 2009). The most frequent site of HBV mediated insertional mutagenesis is

located witin the TERT promoter (Llovet et al., 2021). Integration of HBV affects the TERT and oncogenic proteins such as HBX, which activates a wide range of targets, such as RAS/MAPK1 and PI3K/AKT pathways (Toh et al., 2013; Wang, Hullinger, & Andrisani, 2008).The vaccine against HBV has been available since 1982 (Szmuness et al., 1981)

Hepatitis C virus

HCV is a single-stranded non-retroviral RNA virus that does not integrate into the host genome (Dhanasekaran et al., 2016). Still, HCV can cause double-stranded DNA breaks and increase the mutation frequency. HCV-infected cells exhibit an increased mutation frequency in genes such as immunoglobulin genes, *BCL-6*, *TP53* and *CTNNB1* (Machida et al., 2004). HCV tends to cause chronic infection much more often compared to HBV (Rehermann & Nascimbeni, 2005). The carcinogenesis of HCV is mediated by viral-induced factors and host-induced immunologic response (Ghouri et al., 2017). HCV tends to harbor in the endoplasmic reticulum of the hepatocytes, replicating its RNA and inducing protein synthesis without killing the host (Hino et al., 2002; Macdonald et al., 2003). HCV alters the MAPK signaling pathway, affecting cellular proliferation. NS5A, a protein produced by HCV, inhibits the p53 pathway, which impairs cell antitumoral mechanisms. HCV induces immunologic host responses, such as tumor-necrosis factor (TNF-α), resulting in cell injury, death and regeneration, leading to scarring and fibrosis in hepatocytes (Majumder et al., 2001). The management of HCV has evolved since the introduction of highly active direct-acting antivirals (Sofia et al., 2010).

Screening

Surveillance of HCC involves the use of screening tools in patients at risk for HCC. Cost-effectiveness studies suggest surveillance of HCC in all cirrhotic patients if liver function and comorbidities allow treatment (Sarasin et al., 1996). Surveillance of non-cirrhotic, chronic HBV carriers and HCV patients with bridging fibrosis should be considered (Vogel et al., 2018). Surveillance of patients at risk for HCC should be carried out by abdominal ultrasound every 6 months with or without AFP (Vogel et al., 2018).

2.1.5 Diagnosis

Imaging-based diagnosis

Diagnosis of hepatocellular carcinoma is based on non-invasive criteria and pathology (Galle et al., 2018). Imaging is a crucial part of diagnosis of HCC, contributing primary liver tumor typing and HCC staging. In cirrhotic patients, the European Association for the Study of Liver (EASL) recommends non-invasive criteria and/or pathology while in non-cirrhotic patients, diagnosis should be confirmed by pathology (Galle et al., 2018). Imaging-based diagnosis relies on the vascular derangement occurring during hepatic carcinogenesis and on the high pre-test probability of HCC in the setting of cirrhosis (Bolondi et al., 2001; Burrel et al., 2003; Forner et al., 2008; Matsui et al., 2011; Sangiovanni et al., 2010). The higher pre-test probability is the reason why non-invasive diagnosis is accepted only in cirrhotic patients (Galle et al., 2018). Non-invasive criteria can only be applied to cirrhotic patients for nodule(s) $\geqslant$1 cm, in the light of pre-test probability, and are based on multiphasic CT, dynamic contrast-enhanced MRI or CEUS (Contrast-enhanced ultrasound) (Galle et al., 2018). CEUS can be used for non-invasive diagnosis if CT scan or MRI is not possible, but it is not considered appropriate for tumor staging (Vogel et al., 2018). Diagnosis is based on the identification of typical hallmarks of HCC. The hallmarks differ according to the imaging techniques and contrast agents (arterial phase hyperenhancement (APHE) with washout in the portal venous or delayed phases on CT and MRI using extracellular contrast agents or gadobenate dimeglumine, APHE with washout in the portal venous phase on MRI using gadoxetic acid, APHE with late-onset (>60s) washout of mild intensity on CEUS). Fluorodeoxyglucose-positron emission tomography (FDG PET)-scan is not recommended for early diagnosis due to a high rate of false negative cases (Galle et al., 2018). A typical hallmark is the combination of hypervascularity in late arterial phase and washout in portal venous and/or delayed phases (Matsui et al., 2011). A new set of imaging criteria for HCC diagnosis called CT/MRI LI-RADS® v2018 (Liver Imaging Reporting and Data System) include arterial phase enhancement, tumor size, washout, enhancing capsule and threshold growth (*CT/MRI Diagnostic Table*, 2018.; Mitchell et al., 2015). LI-RADS® categorizes nodules recognized at CT or MRI, in patients at high risk (cirrhosis, chronic HBV even in absence of cirrhosis, current or prior HCC) of HCC, as definitively benign, probably benign, intermediate probability of being HCC, probably HCC, and definitively HCC (Table 1.) (Mitchell et al., 2015) These

imaging criteria are proposed especially to improve the diagnosis of small HCC (Vogel et al., 2018).

Table 1. LI-RADS® categorization of nodules recognized at CT or MRI, in patients at high risk of HCC.

Diagnostic category (LI-RADS)	Conceptual definition	Suggested management
LR-1	Definitely benign, 100% certainty that observation is nonmalignant	Return to surveillance in 6 months
LR-2	Probably benign, high probability but not 100% certainty observation is nonmalignant	Return to surveillance in 6 months. Consider repeat diagnostic imaging in ≤ 6 months
LR-3	Intermediate probability of malignancy, nonmalignant & malignant entities each have moderate probability	Repeat or alternative diagnostic imaging in 3-6 months
LR-4	Probably HCC, high probability but not 100% certainty observation is HCC	Multidisciplinary discussion for tailored workup. May include biopsy
LR-5	Definitely HCC, 100% certainty observation is HCC	HCC confirmed. Multidisciplinary discussion for consensus management

Pathological diagnosis

Pathological diagnosis of HCC is based on morphological parameters; histopathological diagnosis is the gold standard in defining HCC and is based on WHO (World Health Organization) classification and the International Consensus Group for Hepatocellular Neoplasia. Morphological staging of HCC relies on macroscopic and histological assessment of the lesions (Amin et al., 2017). Assessment of resection follows the TNM (tumor, node, metastasis) classification including resection margin evaluation (Vogel et al., 2018) Pathologic differential

diagnosis includes distinction of HCC from other primary and secondary malignancies. Usually, differential diagnosis can be made based on Hematoxylin and Eosin (HE) staining and special histological stains (Amin et al., 2017). Immunohistochemical (IHC) markers are needed especially in cases of poorly differentiated, solid growing carcinomas or tumors of presumed mixed/intermediate/precursor cell differentiation (Galle et al., 2018). IHC assessment is essential for the distinction of benign and premalignant precursor lesions (highly differentiated HCC) and poorly differentiated HCC from intrahepatic cholangiocarcinoma (CC), combined HCC/CC and metastases (Galle et al., 2018). In highly differentiated HCC, definitive signs of malignancy include interstitial or vascular invasion (Kojiro et al., 2009). Often, they are absent from the biopsy. Further consented histological and cytological criteria to support histological diagnosis include trabecular alterations – more than two cell broad trabeculae, pseudoglands, reticulin loss, capsule formation, increased nuclear/cytoplasmic ratio and cytoplasmic basophilia (Kojiro et al., 2009). IHC should be carried out in cases with unclear diagnosis. CD34 can be used to assess capillarization of sinusoids (Kojiro et al., 2009). Other IHC markers, such as glutamine synthetase, glypican 3, general stress protein (CTC), enhancer of zeste homolog 2 (EZH2) and heat shock protein 70 (HSP70) have shown improvement in the diagnosis of highly differentiated HCC (Sciarra et al., 2016). A combination of glutamine synthetase, glypican 3 and HSP70 has shown 70% sensitivity and 100% specificity to HCC when 2/3 of the markers show positivity on a diagnostic panel (Sciarra et al., 2016). Several histological subtypes of HCC have been defined (Chapter 2.1.8) which correlate with clinical and molecular features (Calderaro et al., 2017; Yeh et al., 2015). They may have clinical impact in the future, but to date, HCC subtyping has no major impact on clinical decision-making (Vogel et al., 2018). In addition, cytokeratin 19 (CK19) is one of the clinically relevant IHC markers that identifies HCC with poorer prognosis (Durnez et al., 2006). Beside morphological parameters of malignancy, analysis of human telomerase reverse transcription (hTERT) mutations may help to diagnose malignant transformation (Colombo et al., 2016). It must also be noted that liver tumor biopsy involves risk of bleeding and needle track seeding. In meta-analysis (Rockey et al., 2009) mild bleeding complications occurred within 3-4% of biopsies, while severe bleeding was reported in 0.5% of cases. In meta-analysis (Silva et al., 2008) the risk of needle track seeding was 2.7% with a median time interval between biopsy and seeding of 17 months.

2.1.6 Staging

Staging of HCC is important in order to plan the optimal therapy. Tumor staging includes assessment of tumor extent, AFP level, liver function, portal pressure and clinical performance status (Vogel et al., 2018). Tumor extent is evaluated with contrast-enhanced MRI or CT, and the number and size of nodules are analyzed (Vogel et al., 2018). Extrahepatic spread should be ruled out with CT scan (Vogel et al., 2018). Liver function is assessed by the Child-Pugh classification (A, B and C), which includes serum bilirubin, serum albumin, ascites, prothrombin time and hepatic encephalopathy (Vogel et al., 2018). Esophageal varices and/or splenomegaly suggest clinically important portal hypertension (Vogel et al., 2018). Several different staging systems have been developed, including TNM (TNM Classification of Malignant Tumours, 8th Edition, 2017.) and the Barcelona Clinic Liver Cancer (BCLC) system (Llovet et al., 1999). The TNM system includes microvascular invasion that can only be assessed with pathology (TNM Classification of Malignant Tumours, 8th Edition, 2017). The 8th edition of the TNM-classification criteria of hepatocellular carcinoma is presented in Tables 2 and 3. The most commonly used staging system is BCLC that links tumor stage, liver function, cancer-related symptoms and performance status to an evidence-based algorithm (Llovet et al., 1999). The system identifies patients with early HCC who may benefit from surgical and ablative treatment (very early stage (0) and early stage (A)), those with intermediate stage (B) or advanced stage (C) who may benefit from intra-arterial or systemic treatments, and those with poor survival expectancy (stage D) (Forner et al., 2018). Survival without treatment is >5 years for stage 0 and A, >2.5 years for stage B, >1 year for stage C, and approximately 3 months for stage D (Forner et al., 2018). Figure 1 presents a modified treatment strategy based on BCLC staging (Vogel et al., 2018).

Table 2. TNM categories in hepatocellular carcinoma based on the 8th edition of the TNM classification.

TMA class	Definition
Primary tumor	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1a	Solitary tumor ≤2cm with/without vascular invasion
T1b	Solitary tumor >2cm without vascular invasion
T2	Solitary tumor >2cm with vascular invasion or multifocal tumors, none >5cm
T3	Multiple tumors, at least one of which is >5cm
T4	Single tumor or multifocal tumors of any size involving a major branch of the portal vein or hepatic vein or tumor (s) directly invades adjacent organs other than the gallbladder or tumor perforates the visceral peritoneum
Regional lymph nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant metastases	
M0	No distant metastasis
M1	Distant metastasis

Table 3. TNM-stage grouping in hepatocellular carcinoma based on the 8th edition of the TNM classification.

Stage	Primary tumor (T)	Regional lymph nodes (N)	Distant metastasis (M)
Stage IA	T1A	N0	M0
Stage IB	T1b	N0	M0
Stage II	T2	N0	M0
Stage IIIA	T3	N0	M0
Stage IIIB	T4	N0	M0
Stage IVA	Any T	N1	M0
Stage IVB	Any T	Any N	M1

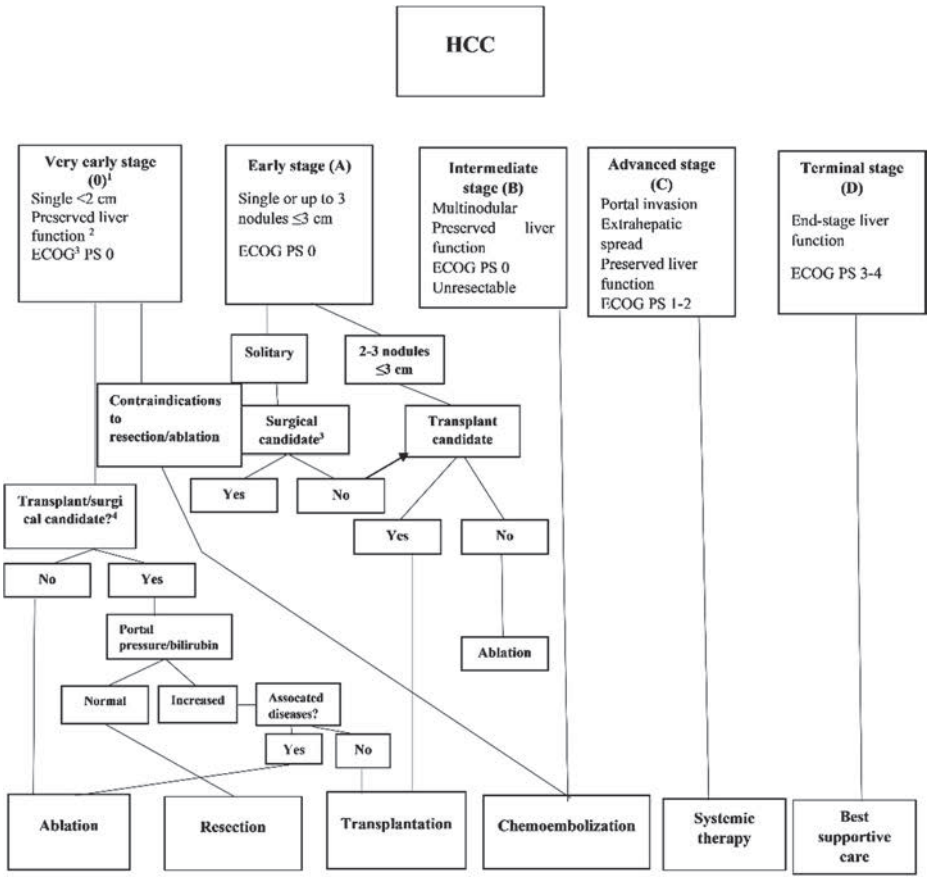

Fig. 1. BCLC staging system and treatment strategy. Modified from EASL (Galle et al., 2018) and ESMO (Vogel et al., 2018) guidelines (Vitale et al., 2017). [1] There is a lack of existing evidence on comparing curative options in very early HCC (Vitale et al., 2017). Radiofrequency ablation in favorable locations can be first-line therapy, even in surgical patients (Galle et al., 2018). [2] Preserved liver function refers to Child-Pugh class A without any ascites. Eastern Cooperative Oncology Group (ECOG) performance status (PS): 0 = fully active, able to carry on all pre-disease performance without restriction, 1= Restricted physical activity, but able to carry out for example light house work, 2= Capable of self-care, but unable to carry out work or activities, 3= Capable of only limited self-care, confined to bed or chair more than 50% of waking hours, 4= completely disabled, cannot carry on any self-care, totally confined to bed or chair (Sørensen et al., 1993). [3] Surgical candidacy is based on multiparametric evaluation, including compensated Child-Pugh class A, acceptable amount of remaining parenchyma and possibility to adopt a laparoscopic/minimally invasive approach (Galle et al., 2018). [4]

Transplant candidate in this meaning is a HCC patient that would be suitable for liver transplantation based on age and comorbidities (Vitale et al., 2017).

2.1.7 Treatment

The treatment modality of HCC depends on patient's physical status, comorbidities, liver function, tumor size, location, invasiveness and the number of nodules. Liver resection, liver transplantation and local ablation offer potentially curative treatment for HCC patients (Vogel et al., 2018). Median survival of patients with early HCC ranges from 50% to 70% at five years after resection, transplantation or local ablation in selected patients (Seshadri et al., 2014; Zhang et al., 2017).

Liver resection

Liver resection is recommended as a treatment of BCLC stage 0 and A patients and is the mainstay of HCC, leading to the best outcomes of any treatment available (Galle et al., 2018). Figure 1 presents a modified BCLC treatment algorithm. Liver resection is recommended as a treatment of choice for non-cirrhotic liver; the indications for resection in cirrhotic patients should be carefully examined (Galle et al., 2018). Liver function, extent of hepatectomy, expected volume of the remaining liver parenchyma, performance status and patients' comorbidities should be assessed (Galle et al., 2018). The most widely practiced method for measuring liver reserve is the Child-Pugh classification (Child & Turcotte, 1964; Pugh et al., 1973) (Chapter 2.1.6); other parameters, such as model for end-stage liver disease (MELD) score (Forman & Lucey, 2001), indocyanine green (ICG) kinetics, liver stiffness measurement and cholinesterase/bilirubin ratio, have shown a significant role in improving patient selection (Cescon et al., 2012; De Gasperi, Mazza, & Prosperi, 2016; Donadon et al., 2015; Imamura et al., 2003; Lisotti et al., 2014). As well, aspartate aminotransferase - to - platelet ratio index (APRI) (Wai et al., 2003), albumin – bilirubin score (ALBI) (Johnson et al., 2015) and combination of both scores (APRI/ALBI) (Mai et al., 2019) has been used for assessing liver function in patients with HCC. The perioperative mortality of liver resection in cirrhotic patients should be under 3% (Galle et al., 2018). Liver resection is recommended for single HCC of any size, particularly in tumor >2 cm, when hepatic function is preserved and the remnant liver volume is sufficient (Galle et al., 2018). HCC patients within Milan criteria (Mazzaferro et al., 1996) (single tumors $\leqslant$5 cm in diameter or no more than three tumors $\leqslant$3 cm in diameter, no

extra-hepatic involvement or major vessel involvement) belong to the early-stage category and could thus be approached with liver resection if eligibility for ablation and liver transplant is suboptimal (Galle et al., 2018). A tumor sized >5 cm can be treated with liver resection but it is worth noting that even if a single tumor beyond 5 cm is considered BCLC (A) early stage, it appears to bear worse prognosis than BCLC stage A <5 cm (Dai et al., 2018; Pawlik et al., 2005). HCC-related macrovascular invasion is a contraindication for liver resection (Galle et al., 2018). Patients with Child-Pugh score A without significant portal hypertension are considered good candidates for liver resection. Child-Pugh B is not an absolute contraindication for surgery, but patient selection among these patients should be evaluated critically. Patients with Child-Pugh C score are not suitable for liver resection. Neoadjuvant or adjuvant therapies are not recommended (Vogel et al., 2018). Liver resection should be performed laparoscopically, if possible (Galle et al., 2018). Compared with open liver resection, laparoscopic liver resection results in reduced intraoperative bleeding, faster postoperative recovery, and does not impair oncological outcome (Fancellu et al., 2011). Especially in cirrhotic HCC patients, liver resection should be performed via laparoscopic approaches (Vogel et al., 2018). Tumor size, location and the number of tumor satellites as well as liver function and patient's physical condition have an influence on the chosen surgical approach (Galle et al., 2018). Liver resection can be anatomic, during which systematic removal of the tumor-bearing portal territories is provided and landmark veins framing the segmental territory are exposed (Shindoh et al., 2015). Non-anatomic liver resection is a resection without regard to segmental, sectional or lobar anatomy (Shindoh et al., 2015). Liver resection can be divided into minor and major resections: minor resection contains <3 liver segments and major resection ≥3 liver segments (Aragon & Solomon, 2012). The extent of resection can be accurately planned with CT/MRI volume calculation (Galle et al., 2018). The extent of the surgical resection (anatomical vs. non-anatomical) is a subject of ongoing debate (Cucchetti et al., 2012; Vogel et al., 2018; Zhou et al., 2011). Theoretically, systematic removal of a hepatic segment through anatomical resection is considered to be more effective in terms of tumor clearance and eradication of micro-metastases (Yuki et al., 1990). This, however, is rarely possible in a cirrhotic liver (Clavien et al., 2007). Anatomic resections should possibly be preferred for HCC nodules at least 2 cm in size (Shindoh et al., 2015). For a single tumor nodule which is ≤2 cm in size and is deeply/centrally located, radiofrequency ablation (RF) also offers competitive results because of its higher cost-effectiveness compared to liver resection (Galle et al., 2018). In tumors up to

4-5 cm, the difference in results of liver resection vs. radiofrequency ablation is indefinite, three randomized controlled trials (RCTs) (Chen et al., 2006; Feng et al., 2012; Ng et al., 2017) have shown no superiority of liver resection over radiofrequency ablation, but meta-analyses (Wang et al., 2014; Xin et al., 2016) and one other RCT (Huang et al., 2010) have emphasized better results with liver resection. However, there is not just one specific way that works best in the field of liver surgery; tumors in different lobes of the liver require a tailored approach on each patient (Galle et al., 2018). The most widely accepted anatomic definition used in the context of liver resections is the division of the liver into eight different segments (Bismuth, 1982; Couinaud, 1999). The anatomical division into right and left side of the liver is divided by ligamentum teres and umbilical fissure, where the main vascular and biliary structures to the functional left liver run (Aragon & Solomon, 2012). The functional division into the right and left sides of the liver is divided by the middle hepatic vein. Right hepatectomy (hemihepatectomy) involves resection of segments V-VIII while left hepatectomy involves resection of segments II-IV. Right lobectomy (extended right hepatectomy) involves resection of IV-VIII, and sometimes I (Aragon & Solomon, 2012). Left lobectomy involves resection of segments II and III, whereas extended left lobectomy includes segments II-IV, V and VIII (Strasberg, 2005).

Liver transplantation

Liver transplantation offers the possibility to cure both the tumor and the underlying liver disease. The Milan criteria (single tumors $\leqslant$5 cm in diameter or no more than three tumors $\leqslant$3 cm in diameter, no extra-hepatic involvement or major vessel involvement) are the benchmark for the selection of HCC patients for liver transplantation (Mazzaferro et al., 1996). Liver transplantation is recommended as the first-line therapy for tumors that are within Milan criteria but unsuitable for resection (Galle et al., 2018). Patients beyond Milan criteria can be considered for liver transplantation after successful downstaging to within Milan criteria (Galle et al., 2018). The low availability of liver allografts is a major problem within liver transplantation and patients waiting for transplantation often confront tumor progression beyond the Milan criteria (Llovet & Bruix, 2008; Mazzaferro et al., 2018; Mehta et al., 2013; Rogiers et al., 1996; Trotter, 2000). When a waiting time (>3 months) is anticipated, patients may be offered other treatment approaches, such as liver resection, radiofrequency ablation or transarterial chemoembolization (TACE) in order to minimize the risk of tumor

progression and to offer a bridge to transplant (Vogel et al., 2018). Several studies on loco-regional treatment have demonstrated significant advantages of neoadjuvant therapies in reducing the dropout risk due to tumor progression, good response to downstaging is frequently related to better prognosis (Ibrahim et al., 2012; Mehta et al., 2013; Parikh et al., 2015; Tsochatzis et al., 2013; Yao et al., 2015). Extrahepatic tumor spread and macrovascular tumor invasion are absolute contraindications for liver transplantation (Galle et al., 2018). The most common transplant is from a deceased organ donor. In Europe, living liver transplantation represents a debated second-line opinion (Galle et al., 2018).

Thermal ablation

Thermal ablation with radiofrequency is a standard treatment for BCLC 0 and A when the tumor is not suitable for resection (Galle et al., 2018). The mechanism behind radiofrequency ablation is based on the frictional heat that causes cell death and coagulative necrosis. Thermal ablation in single tumors up to 3 cm in size may be an alternative for liver resection when the tumor location is challenging for liver resection (Galle et al., 2018). Radiofrequency ablation is also an alternative first-line option in patients with early stage HCC (up to three lesions ≤ 3 cm) irrespective of liver function (Vogel et al., 2018). In patients with very early HCC (BCLC-0), radiofrequency ablation in favorable locations can be used as a first-line therapy, even in surgical patients (Galle et al., 2018). In a recent Cochrane review (Majumdar et al., 2017) comparing radiofrequency ablation vs. liver resection, no significant difference between groups was found in mortality, but the proportion of patients with recurrence was higher in the radiofrequency ablation group. However, the complication rate was significantly higher in the liver resection group (Majumdar et al., 2017). Other local ablation modalities – percutaneous ethanol injection, microwave ablation, laser ablation and cryoablation – have been used over the years. Historical percutaneous ethanol injection induces coagulative necrosis of the lesion. However, percutaneous ethanol injection is associated with incomplete necrosis and suffers a high recurrence rate (Pompili et al., 2015). Microwave ablation uses electromagnetic energy to heat the tumor tissue and has shown promising results (Facciorusso et al., 2016). Laser ablation and cryoablation have not shown any superiority compared to radiofrequency ablation (Di Costanzo et al., 2015; Wang et al., 2015). Follow-up of patients who have undergone radical treatments (liver resection or radiofrequency ablation) should be arranged with dynamic CT or MRI studies every 3 months during the first year and with

surveillance 6 months thereafter to clinically evaluate liver decompensation and for the early detection of tumor recurrence (Vogel et al., 2018).

Non-curative treatments

Non-curative treatments of HCC include trans-arterial therapies, such as transarterial chemoembolization (TACE), transarterial embolization (TAE) and intra-arterial infusion of chemotherapy alone, selective internal radiotherapy (SIRT) and systemic therapies (Vogel et al., 2018). TACE is widely used as primary treatment for unresectable HCC, such as BCLC stage B (Llovet et al., 2002; Lo et al., 2002; Vogel et al., 2018). It is also used as a "bridge treatment" to liver transplantation or when liver resection and radiofrequency ablation are not possible and the patient is on a waiting list for transplantation (Vogel et al., 2018). HCC commonly exhibits vast neoangiogenesis during its progression (Zhu et al., 2011). The principle for TACE is the intra-arterial infusion of a cytotoxic agent followed by embolization of the tumor-feeding blood vessels (Lencioni, 2010). This results in cytotoxic and ischemic effect on the tumor (Kan & Wallace, 1994). TACE should not be used in decompensated liver disease, advanced liver/kidney dysfunction, macroscopic vascular invasion or extrahepatic spread (Galle et al., 2018). TACE should be separated from transarterial embolization (TAE), where no chemotherapeutic agent is used and from intra-arterial infusion, where a chemotherapeutic agent, such as doxorubicin, is used (Galle et al., 2018). SIRT, also called radioembolization, is defined as the infusion of radioactive agents, such as yttrium-90 or similar agents into the hepatic artery. One of the most common indications of SIRT is treatment of patients with locally advanced HCC (Chow & Gandhi, 2017). SIRT seems to have a good safety profile but fails to show overall survival benefit compared to sorafenib in BCLC stage B and C patients (Chow & Gandhi, 2017; Vilgrain et al., 2017). During follow-up, patients with more advanced stages of HCC treated with TACE or systemic agents (sorafenib) are evaluated for signs of liver decompensation and for tumor progression by dynamic CT or MRI every 3 months to guide therapy decisions (Vogel et al., 2018).

Systematic therapies

In the field of systematic therapies, sorafenib is the standard first-line treatment for HCC in advanced disease that prolonges survival approximately 3 months compared to placebo (Cheng et al., 2009; Llovet et al., 2008). Median overall

survival of 10.7 months was observed in patients treated with sorafenib (Llovet et al., 2008). It is used with well-preserved liver function and with advanced tumors such as BCLC-C (Galle et al., 2018). Lenvatinib has also shown similar results in similar patient material (Kudo et al., 2018). Immunotherapy with nivolumab and pembrolizumab can be an option for patients who are intolerant or have progressed under approved tyrosine kinase inhibitors (El-Khoueiry et al., 2017; Zhu et al., 2018). Response rate of 20% and median overall survival of 15.0 months has been reported in patients treated with nivolumab (El-Khoueiry et al., 2017), whereas respective results in patients treated with pembrolizumab were 17% and 12.9 months (Zhu et al., 2018). For a definitive recommendation, the results of randomized trials are awaited. In the field of HCC treatment, research for personalized HCC therapy is ongoing. Investigation of the TERT promoter (Nault et al., 2013; Totoki et al., 2014), CTNNB1 (De La Coste et al., 1998), TP53 (Bressac et al., 1991; Guichard et al., 2012; Hsu et al., 1991; Schulze et al., 2015), ARID1A (Guichard et al., 2012) and ARID2 (Li et al., 2011) has gained interest. Currently, molecular profiling is not recommended as standard practice (Vogel et al., 2018). Patients at BCLC-D stage who are not candidates for LT should receive palliative treatment, including management of pain, nutrition and physical support (Galle et al., 2018).

Portal vein embolization and portal vein ligation

Portal vein embolization (PVE) is a radiological technique performed in local anesthesia, under guiding ultrasound and is used to embolize blood flow of the veins of the selected liver segments to induce hypertrophy of nonembolized liver segments before resection. After occlusion, only arterial circulation of these segments remains. PVE is indicated when the future liver remnant (FLR) is either estimated not to support post-resection hepatic function or marginal in size (Makuuchi et al., 1990). PVE is usually indicated when the future liver remnant accounts for less than 25–40% of the total liver volume (Loffroy et al., 2015). The reason for unresectability is often insufficient remnant liver, which can cause postoperative death (Loffroy et al., 2015). The selection of patients is traditionally based on CT volumetry (Van Lienden et al., 2013). PVE induces liver hypertrophy by increasing the production of hepatic growth factor (HGF) and transforming growth factor (TGF) (Hayashi et al., 2010). Contraindications to PVE include unresectable disease, uncorrectable coagulopathy, tumor invasion to portal vein, portal hypertension and severe renal dysfunction (Loffroy et al., 2015). The mainly

used embolization materials include polyvinyl alcohol particles, gelatin sponge and fibrin glue (Loffroy et al., 2015). Growth speed of the non-embolized segments is maximal during the first 2 weeks and persists, although to a lesser extent during approximately until 6 weeks (Loffroy et al., 2015). Liver resection is performed 2 to 6 weeks after PVE. The increase of the liver remnant varies from 8% to 25% in non-cirrhotic patients, in cirrhotic patients, the increase of the liver is slightly lower, between 6% and 20% (Loffroy et al., 2015). Portal vein ligation (PVL) (Rous & Larimore, 1920) is a more invasive technique compared to PVE, because it is performed under general anesthesia and requires laparotomy/laparoscopy (Isfordink et al., 2017). During the operation, ligation of the left or right portal vein is performed (Isfordink et al., 2017). A meta-analysis involving 2,076 patients demonstrated no significant differences between PVE and PVL regarding the rate of FLR hypertrophy (PVE 43.2%, PVL 38.5%, $p = 0.39$) (Isfordink et al., 2017). The reported 30-day mortality after hepatic resection following PVE and PVL was 3.8% and 2.8%, ($p = 0.795$). The most common cause of mortality was hepatic failure (Isfordink et al., 2017).

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS)

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a strategy to increase the size of the future liver remnant (Schnitzbauer et al., 2012). The indications of ALPPS include patients with future liver remnant of < 30% in normal livers or < 40% in diseased livers (Zhang et al., 2014). The indications also include marginally resectable or locally advanced unresectable liver tumors of any origin with insufficient future liver remnant and is mainly used for extended right hepatectomy (Zhang et al., 2014). Unresectable liver metastases and extra hepatic metastases are contraindications for ALPPS (Alvarez et al., 2013). ALPPS involves two stages: first, the liver parenchyma is transected along the intended line of resection and the future liver remnant is cleaned from tumor tissue (Zhang et al., 2014). The branch of the portal vein that feeds the removable liver lobe is ligated. At the second stage and after performing 3D reconstruction and volumetric analysis, liver resection of the deportalized liver part is performed. (Zhang et al., 2014) The reported mean time between the two operations ranges from 6 to 9 days (Zhang et al., 2014). Studies have reported 74% to 80% increase of future liver remnant (Alvarez et al., 2013; Li et al., 2013; Sala et al., 2012; Schnitzbauer et al., 2012; Torres et al., 2013). High operative morbidity and

mortality rates after ALPPS have been reported. The rates range from 16% to 64% and from 12% to 23% of patients (Alvarez et al., 2013; Li et al., 2013; Sala et al., 2012; Schnitzbauer et al., 2012; Torres et al., 2013). Hepatic insufficiency is the main cause of mortality (Zhang et al., 2014). One RCT comparing traditional two-stage hepatectomy (TSH) with the ALPPS for the patients with colorectal liver metastasis (CRLM) and insufficient FLR has been carried out (Sandström et al., 2018). The resection rate was significantly higher with ALPPS than with TSH, with similar complication and mortality rates and negative surgical margins in the liver (Sandström et al., 2018). In a follow-up study, the oncological outcome of patients with CRLM was evaluated (Hasselgren et al., 2021). The resection rate was higher in ALPPS group, compared with TSH group (92% vs 80%, p=0.091) and ALPPS was associated with higher median survival, compared with TSH (46 months vs 26 months, p=0.028). There was no difference regarding postoperative complications, including 90-day mortality for patients randomized to ALPPS and TSH. Four patients (8.3%) randomized to ALPPS died within 90 days from the second intervention compared with 3 (6.1%) patients randomized to TSH. (p=0.68). (Hasselgren et al., 2021).

2.1.8 Histological classification of hepatocellular carcinoma

Macroscopic appearance of HCC

The macroscopic appearance of HCC can vary from green to yellow to light tan, depending in part on their fat and bile content (Lokuhetty et al., 2019). Especially in cirrhotic livers, tumors have a pseudocapsule composed of inflamed and fibrotic tissue (Lokuhetty et al., 2019). Tumor nodules usually exhibit four different macroscopic patterns of HCC that are important for clinical staging purposes: a single distinct nodule, a large dominant nodule with multiple smaller satellites that usually present within 2 cm of the primary tumor, many small nodules (up to hundreds) that are approximately the same size and shape as the cirrhotic nodules, and multiple distinct nodules of HCC that represent independent primaries (Lokuhetty et al., 2019)

Microscopic appearance of HCC

In the histopathological aspect, tumor cells of HCC show hepatocytic differentiation by morphology and/or IHC (Gonzalez et al., 2015). HCC phenotype appears to be closely related to particular gene mutations, tumour subgroups and/or oncogenic pathways (Calderaro et al., 2019). The tumor has lost its normal hepatic architecture, such as portal tracts and reduction of the normal reticulin framework (Shafizadeh & Kakar, 2011). Cytological atypia varies from minimal to marked and tumor cells show increased proliferation (Evason et al., 2013). Typically, increased arterialization is shown in HCC, with aberrant arterioles in the parenchyma (Schlageter et al., 2014). Non-proliferative tumors display often a well-differentiated phenotype, among this subgroup *CTNNB1*-mutated HCCs constitute homogenous subtype, exhibiting cholestasis and microtrabecular and pseudoglandular patterns (Calderaro et al., 2019). In contrast, proliferative HCCs are most often poorly differentiated and notably include tumors with progenitor features (Calderaro et al., 2019). HCCs have four principal growth patterns: trabecular, solid, pseudoglandular, and macrotrabecular, which is associated with a worse prognosis (Lokuhetty et al., 2019; Schlageter et al., 2014). Approximately 50% of resected HCCs have mixed patterns, usually trabecular plus one or two others (Lokuhetty et al., 2019). Subsets of HCC show characteristic cellular changes which include bile production, lipofuscin deposits and glycogen accumulation (Lokuhetty et al., 2019). The tumor cells can develop hyaline bodies, Mallory-Denk bodies or pale bodies (Lokuhetty et al., 2019). Some HCCs exhibit two or more distinct morphologies, which can include differences in architectural pattern, morphological subtype, and/or tumor grade (Lokuhetty et al., 2019). Subtypes of HCC include fibrolamellar, scirrhous, clear cell type, steatohepatitic, macrotrabecular massive, chromophobe, neutrophil-rich and lymphocyte-rich types; as many as 35% of HCCs can be further subclassified into distinct subtypes (Kim et al., 2020; Lokuhetty et al., 2019; Schlageter et al., 2014). All subtypes except the fibrolamellar subtype have been described in cirrhotic and non-cirrhotic livers; the fibrolamellar subtype only occurs in non-cirrhotic livers (El-Serag & Davila, 2004; Schlageter et al., 2014; Soreide et al., 1986). A novel macrotrabecular-massive subtype exhibits a very aggressive phenotype, with frequent satellites and macrovascular and/or microvascular invasion (Calderaro et al., 2019). Macrotrabecular phenotype represents 10-20% of all cases of HCC (Calderaro et al., 2019).

Histological grading of HCC

Histological grading in HCC identifies the degree of differentiation based on HE staining as compared with the morphology of a mature benign hepatocyte (Lokuhetty et al., 2019). A three-tiered grading system has been favored: well, moderately and poorly differentiated (Lokuhetty et al., 2019). Some HCCs can have more than one grade, in which case the worst grade and the predominant grade are reported (Lokuhetty et al., 2019). Tumor grade predicts the patients' survival and disease-free survival after resection with curative intent in cirrhotic and non-cirrhotic livers as well as after transplantation (Lokuhetty et al., 2019). In well-differentiated grade, diagnosis is often made only by loss of reticulin or by aberrant expression of immunostains (Lokuhetty et al., 2019). Cytology and morphology require distinction from hepatic adenoma and dysplastic nodule (Schlageter et al., 2014). In poorly differentiated grade, immunostaining is often needed to confirm hepatocytic differentiation (Zimmermann & Zimmerman 2017). Typically used histopathological markers are discussed in Chapter 2.1.5. In addition, besides these markers, several other tissue biomarkers are used to clarify hepatocytic differentiation. These biomarkers include Arginase-1, Hep-Par-1, Polyclonal CEA, CD10 and AFP (Lokuhetty et al., 2019).

Premalignant lesions

Premalignant lesions for HCCs are typically found in cirrhotic livers and include dysplastic foci and dysplastic nodules (Schlageter et al., 2014). Distinguishing between high-grade early HCC and dysplastic nodules is challenging. Dysplastic foci are microscopic lesions <1 mm in diameter and are incidental lesions, whereas dysplastic nodules are most identified by imaging (Schlageter et al., 2014; Wanless, 1995). There is no consensus on the need for reporting incidentally identified dysplastic foci (Lokuhetty et al., 2019). Dysplastic nodules are usually 5–15 mm in diameter and are detected macroscopically or by imaging, usually in cirrhotic livers, and can be classified into high-grade and low-grade depending on the degree of cytological and architectural atypia (Lokuhetty et al., 2019). Dysplastic nodules have a relative risk of developing into HCC that ranges from 9% to 31%, with the greatest risk found in high-grade dysplastic nodules (Lokuhetty et al., 2019). Imaging-based surveillance and pathological evaluation of liver biopsy are critical for patient management (Lokuhetty et al., 2019)

Early and small progressed HCC

Small HCCs are defined as being ≤2 cm in diameter and are divided into early HCC and small progressed HCC (Lokuhetty et al., 2019). Early HCCs are microscopically well differentiated and commonly show stromal invasion, but no vascular invasion (Lokuhetty et al., 2019). Early HCCs may show a few portal tracts with portal veins and fewer unpaired arteries, whereas small progressed HCC show no portal tracts and no more impaired arteries (Lokuhetty et al., 2019). Small progressed HCC show distinct margins on gross examination, usually have a tumor capsule, and show infiltrative growth patterns. Small progressive HCCs are more likely to show similar histological features as larger HCCs (Lokuhetty et al., 2019).

2.1.9 Prognostic factors

Generally, the prognosis of patients with HCC is poor, most studies reporting < 5% 5-year survival rate, particularly in advanced stages of HCC (Lokuhetty et al., 2019). Long-term survival is likely only in small HCCs that can be radically treated with liver resection, thermal ablation or liver transplantation (Lokuhetty et al., 2019).

Clinical/imaging prognostic factors

Clinical prognostic features include serum alpha-fetoprotein (AFP), which is the most widely used serological marker to establish the diagnosis of HCC and is associated with pathological grade, progression and survival (Bai et al., 2017) and des-γ-carboxyprothrombin (DCP), which is an abnormal prothrombin molecule that is generated as a result of a defect in posttranslational carboxylation of the prothrombin precursor in malignant cells; however, neither AFP nor DCP alone is optimal for detecting HCC (Lok et al., 2010). However, DCP is widely used in Japan for HCC diagnosis and surveillance (Makuuchi et al., 2008). As well, tumor size and number are independent prognostic factors for HCC (Wang & Li, 2019; Wu et al., 2018). Invasion of major vessels on imaging, extrahepatic spread, comorbidities and liver function (portal hypertension, ascites, Child-Pugh score) are also known prognostic factors of HCC (Llovet et al., 1999; Lokuhetty et al., 2019; Vogel et al., 2018).

Morphological/molecular prognostic factors

Morphological prognostic features include tumor grade (Lokuhetty et al., 2019; Martins-Filho et al., 2017) microvascular invasion, which is a marker of aggressive biological tumor behavior that critically weakens the disease prognosis (Rodríguez-Perálvarez et al., 2013) intrahepatic metastasis that originates from the primary cancer (Yang et al., 2017), tumor stage (see also chapter 2.1.6) (Vogel et al., 2018), tumor subtype (El Jabbour, 2019) and presence or absence of cirrhosis (Manghisi et al., 1998). As well, immunohistochemical expression of CK19, which is a cytoskeletal intermediate filament presenting in both normal and malignant cells, is a factor for poor prognosis in HCC (Uenishi et al., 2003), while vascular endothelial growth factor (VEGF) is associated with poor outcomes in HCC (Stroescu et al., 2008) and angiopoietin 2 has shown to have independent prognostic value in HCC (Llovet et al., 2012). Molecular features include fibroblast growth factor 19 (FGF19) amplification (Gao et al., 2019). FGF19 is a highly conserved gene with a wide spectrum of effects: it regulates glucose homeostasis and bile acid synthesis, but is also identified as a tumor-promoting gene (Gao et al., 2019). High amplification of FGF19 is associated with poor prognosis in HCC patients (Raja et al., 2019). Gene expression profiling has also revealed that the prognosis of HCC can be predicted from gene expression profiles in the primary tumors (Lee et al., 2004; Lokuhetty et al., 2019).

2.2 Tumor microenvironment

The tumor microenvironment is a complex network of tumoral cells and stromal cells including angiogenic cells, immune cells and cancer-related fibroblastic cells (CAFs) that is essential for tumor progression (Hernandez-Gea et al., 2013). During chronic liver injury, fibrosis results from deposition of the extracellular matrix that weakens oxygen exchange (Ghouri et al., 2017). Increasing evidence highlights the crosstalk between tumoral cells and the surrounding stroma as the key modulator of hepatocarcinogenesis, epithelial mesenchymal transition (EMT), tumor invasion and metastasis (Hernandez-Gea et al., 2013; Wu et al., 2012). The tumor microenvironment can be roughly divided into cellular and non-cellular components (Yang et al., 2011). The key components of the cellular division include hepatic stellate cells (HSCs), fibroblasts, immune cells and endothelial cells (Yang et al., 2011). These cells produce the non-cellular components of the tumor stroma, such as proteins of the extracellular matrix (ECM), proteolytic enzymes,

growth factors and inflammatory cytokines. The non-cellular tumor stroma modulates the biology of HCC by cancer signaling pathways in tumor cells, tumor invasion and metastasis (Yang et al., 2011). Genetic research has revealed that the tumor microenvironment is an important factor in the biologic and prognostic classification of HCC (Yang et al., 2011). Chronic inflammation of the liver, infections by the hepatitis viruses, as well as the produce of cytokines and growth factors, lead to a complicated microenvironment that initiates various cascades (see Chapter 2.1.3) to regenerate the liver and can also induce hepatocarcinogenesis (Leonardi et al., 2012).

Cellular components of tumor microenvironment

Hepatic stellate cells (HSC) activate due to repeated liver injury, leading to development of hepatic fibrosis (Sokolović et al., 2010; Wynn, 2008). In the initial phase, various cytokines, chemokines and growth factors are released (Bachem et al., 1992; Bataller & Brenner, 2005). Cancer-associated fibroblasts (CAFs) are a known cell type within tumor stroma and play a key role in wound repair, deposition of extracellular matrix, tissue maturation and inflammatory response, but also in tumor-stroma interactions (Birgani & Carloni, 2017; Fransvea et al., 2009; Yang et al., 2011). The immune response of the tumor and its microenvironment activate cells of the innate and adaptive immunity (Birgani & Carloni, 2017). Macrophages are leukocytes with antigen presentation capacity that are involved in tissue remodeling, phagocytosis and scavenging (Birgani & Carloni, 2017). When the macrophages come around the tumoral region, they are called tumor-associated macrophages (TAMs) (Birgani & Carloni, 2017). Although macrophages have a role in antitumor immunity, there is growing evidence that TAMs enhance tumor progression via promoting neoangiogenesis, metastasis and suppression of adaptive immunity of inflammation-related HCC (Capece et al., 2013).

Non-cellular components of tumor microenvironment

The non-cellular components of the tumor microenvironment include various growth factors and cytokines, such as TGF β , which is released in the ECM and activated by matrix metalloproteinases (MMP) (Birgani & Carloni, 2017). TGFβ is highly expressed in cirrhotic livers and plays a key role in liver fibrosis and hepatocarcinogenesis (Leask & Abraham, 2004; Paik et al., 2003). TGFβ is a potent

inducer of HSCs and collagen production (Yang et al., 2011). Normally, TGFβ acts as a tumor suppressor, stopping the cell cycle to stop proliferation, induces differentiation, and promotes apoptosis. When the normal TGFβ signaling pathways become disturbed, oncogenic manners of TGFβ arise (Yang et al., 2011). Other growth factors, such as platelet-derived growth factor (PDGF), VEGF, fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) have a role in hepatocarcinogenesis via promoting fibrogenesis, neoangiogenesis, cell proliferation and invasion of HCC cells (Campbell et al., 2005; Neaud, 1997; Ogasawara et al., 1996; Yang et al., 2009). Matrix metalloproteinases are zinc-dependent endopeptidases that play roles in physiologic tissue remodeling, development and regulation during inflammation (Gross & Lapiere, 1962; Parks et al., 2004). They are also shown to play an important role in the development of liver cirrhosis, tumor cell growth, migration, invasion, metastasis and modulation of the tumor environment (Kessenbrock et al., 2010). Various inflammatory cytokines, such as interleukin-6 (IL-6) (Benedicto et al., 2008; Tilg et al., 1992; Wong et al., 2009), tumor-necrosis factor-α (TNF-α) (Ataseven et al., 2006) and interleukin-1 (IL-1) (Ataseven et al., 2006) are also known mediators of HCC progression from chronic liver injury.

2.2.1 Extracellular matrix

The extracellular matrix (ECM) is a non-cellular component network surrounding all tissues and organs that is dynamic and functional, regulating cell number, morphology, movement and adhesion (Frantz et al., 2010). It is also involved in tissue survival, homeostasis, growth and differentiation (Frantz et al., 2010). ECM contains various proteoglycans and collagen types that are crucial for the normal and carcinogenetic functions of ECM (Frantz et al., 2010). Dysfunction and genetic abnormalities of ECM proteins can be demonstrated in various syndromes and diseases, including various cancers (Frantz et al., 2010). Chronic liver damage induces liver injury through reactive oxygen species (ROS) as well as in various other ways; the hepatic response to this involves the activation of HSCs and macrophages that produce components of the ECM (Hernandez-Gea et al., 2013). Various growth factors are also induced, promotion of endothelial cell migration occurs, as well as neoangiogenesis and fibrosis (Hernandez-Gea et al., 2013). On the one hand, ECM controls the proliferation, differentiation and metastasis of HCC. On the other hand, HCC cells remodel ECM to work as a permissive soil for cancer cells to grow, proliferate, block apoptosis and invade the surrounding tissue (Wu et

al., 2006). In HCC, heparan sulphate proteoglycans (HSPGs) work as co-receptors or as storage for various tumor-progressing growth factors, such as FGF, HGF, PDGF and VEGF (Campbell et al., 2005; Neaud, 1997; Ogasawara et al., 1996). ECM is essential in supporting the architecture of the liver, interacting with the environment (Hernandez-Gea et al., 2013). In disease, such as HCC, the biomechanical and physical composition of the ECM has changed in tumoral stroma and the activity of the ECM enzymes is dysregulated, leading to a fibrotic microenvironment (Birgani & Carloni, 2017). Increased stiffness has been observed in HCC tumors, which may be due to the overexpression of lysyl oxidase (LOX), producing collagen, which interacts with other components of ECM (Birgani & Carloni, 2017; Levental et al., 2009). This leads to deposition of fibrillar collagen types I and II and fibronectin in the liver (Hernandez-Gea et al., 2013). Tumor growth, survival and proliferation through regulation of integrin family occur (Yang et al., 2003). It is also important to note that the collagen fibrils are more oriented in cancers compared to normal tissue and it seems that such topography facilitates tumor angiogenesis and invasion (Levental et al., 2009; Provenzano et al., 2006). Furthermore, MMPs have been observed to be overexpressed in cancerous ECMs and might be involved in the invasive ability of HCC (Birgani & Carloni, 2017).

2.2.2 Tumor budding

Tumor budding is defined as a single tumor cell or cluster of up to four tumor cells at the invasive front of the carcinoma (Imai, 1960). It is a well-established, independent prognostic factor in colorectal cancer that is considered along with other clinicopathological features (Lugli et al., 2017). Biologically, tumor buds are part of the tumor microenvironment and are associated with EMT (Grigore et al., 2016). In colorectal cancer, tumor buds have shown disruption of E-cadherin expression, which is a hallmark of EMT, and loss of β-catenin expression at the cell membrane (Grigore et al., 2016). Loss of β-catenin is sometimes accompanied by nuclear translocation of β-catenin, suggesting that Wnt pathway signaling might be activated in these cells, thus leading to loss of cell-cell adhesion (Grigore et al., 2016). As well, tumor buds overexpress markers that are linked to cell invasion, migration, survival and EMT (De Smedt et al., 2017). Activation of TGFβ has also been identified as a key regulator of EMT-associated gene expression in tumor buds (Jensen et al., 2015). Tumor buds seem to have the capacity to degrade ECM and to invade and migrate through the stroma (Lugli et al., 2020). The poor prognostic

impact of tumor budding has been observed in various cancer types, including gastric adenocarcinoma (Kemi et al., 2019), pancreatic cancer (Karamitopoulou et al., 2013), esophageal cancer (Koike et al., 2008), head and neck cancer (Almangush et al., 2014), squamous cell carcinoma of the lung (Neppl et al., 2020), breast cancer (Liang et al., 2013), bladder cancer (Soriano et al., 2019), endometrial cancer (Koyuncuoglu et al., 2012) and cervical cancer (Huang et al., 2016). Recent studies have also implicated tumor budding with poor prognosis in HCC patients (Wei et al., 2019). In addition, tumor budding has been linked to a variety of different clinicopathological outcomes, such as lymph node metastasis, stage, lymphovascular invasion and histological grade (Lugli et al., 2020).

2.2.3 Tumor-Stroma Ratio

Tumor-Stroma ratio (TSR) is defined as the percentage of tumor cell component relative to the surrounding, tumor-associated stroma, where low TSR stands for high proportion of stroma and high TSR for low proportion of stroma (Wu et al., 2016). As the main component of the tumor surrounding microenvironment, the stroma is a crucial factor for interaction with the tumor component (Wu et al., 2016). Tumor growth, progression, invasion and angiogenesis are facilitated by the surrounding stroma and its cancer-related stromal components (Bissell & Radisky, 2001). Tumor aggression is significantly related to the surrounding stroma and interactions with the microenvironment (Wu et al., 2016). The crosstalk between tumor and stroma is discussed in chapter 2.2. TSR is a prognostic factor in various cancers, such as gastric adenocarcinoma (Kemi et al., 2018), cervical cancer (Liu et al., 2014), breast cancer (de Kruijf et al., 2011), non-small cell lung cancer (Zhang et al., 2015) and esophageal cancer (Wang et al., 2012). In HCC, the prognostic value of HCC is still unclear (Lv et al., 2015). The mechanism leading to poorer prognosis of patients with high TSR is still incompletely understood (Wu et al., 2016).

2.3 Innate Immunity

The immune system can be divided into three levels: anatomical and physiological barriers, innate immunity, and adaptive immunity (Turvey & Broide, 2010). Innate immunity serves as the initial defense against harmful material. Innate immunity can be divided into four types of defensive barriers: anatomic, physiologic, endocytic and inflammatory (Marshall et al. 2018). The task of protection is

performed by both hematopoietic cells and non-hematopoietic cells. Hematopoietic cells include macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer (NK) cells and NK T-cells, whereas non-hematopoietic cells include epithelial cells (Turvey & Broide, 2010). In addition, innate immunity includes a humoral component which includes well-characterized components, complement proteins, LPS binding protein, C-reactive protein, defensins, collectins and different receptors, such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRS) (Turvey & Broide, 2010). Innate immunity is the first line of defense and the protection against pathogens relies on pattern recognition receptors (PRRs) which allow a limited range of immune cells to detect and respond rapidly to a wide range of pathogens that share common structures called pathogen-associated molecular patterns (PAMPs) (Marshall et al., 2018). Examples of PAMPs are lipopolysaccharides, which are bacterial cell wall components, and double-stranded ribonucleic acid (RNA), which is produced during viral infection. Innate immunity is an antigen-independent defense mechanism without immunologic memory (Marshall et al., 2018). Cytokine production during innate immunity mobilizes various defense mechanisms rapidly throughout the body while also activating local cellular responses. The most important cytokines include tumor necrosis factor (TNF), interleukin 1 (IL-1) and interleukin 6 (IL-6), which initiate cell recruitment and local inflammation (Marshall et al., 2018). Phagocytes' function is to phagocytose microbes and kill them, but they are also involved in antigen presentation to T cells (Marshall et al., 2018).

2.4 Pattern recognition receptors

Pattern recognition receptors (PRRs) are germline-encoded receptors that are responsible for sensing the presence of pathogens. PRRs recognize structures of microbial species that are called pathogen-associated molecular patterns (PAMPs). PRRs also recognize endogenous molecules of damaged cells termed damage-associated molecular patterns (DAMPs) (Takeuchi & Akira, 2010). PRRs can be found associated to subcellular compartments, cellular and endosomal membranes, and cytosol, but also, extracellularly in the bloodstream and interstitial fluids (Amarante-Mendes et al., 2018). Currently, four different classes of PRR families have been identified: transmembrane proteins, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), cytoplasmic proteins, such as Retinoic acid-inducible gene-I like receptors (RLRs) and Nucleotide-binding oligomerization

domain-like receptors (NLRs) (Amarante-Mendes et al., 2018). These PRRs are expressed beyond the macrophages and dendritic cells, such as in various non-professional immune cells, for example in epithelial cells, endothelial cells and fibroblasts (Takeuchi & Akira, 2010). The PRRs recognition of PAMPs or DAMPs initiates upregulation of transcription genes involved in inflammatory responses (Takeuchi & Akira, 2010). The transcription genes encode proinflammatory cytokines, type I interferons (IFNs), chemokines and antimicrobial proteins (Takeuchi & Akira, 2010)

2.5 Toll-like receptors

TLRs (Anderson et al., 1985) are a class of PRRs that serve as an important part of the innate immunity system by recognizing pattern-associated molecular patterns of various pathogens and endogenous threats, as well as recognizing self and non-self antigens, bridging innate and adaptive immunity and regulation of cytokine production, proliferation and survival (Kawasaki & Kawai, 2014; Nie et al., 2018). TLRs are expressed in dendritic cells and macrophages, but also in non-immune cells such as fibroblasts and epithelial cells (Kawasaki & Kawai, 2014). TLRs contain three structural domains known as leucine-rich repeats (LRRs) motif (pathogen recognition), transmembrane domain and a cytoplasmic Toll-/IL-1 receptor (TIR) domain (signal transduction and signal initiation) (Nie et al., 2018). To date, ten TLRs have been identified in man (Nie et al., 2018). Different TLRs recognize the different molecular patterns of micro-organisms and self-components (Takeuchi & Akira, 2010). TLRs can be divided into two subfamilies based on their localization. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, whereas intracellular TLRs include TLR3, TLR7-9 in man (Kawasaki & Kawai, 2014). Cell surface TLRs mainly recognize microbial membranes components such as lipids, lipoproteins and proteins (Kawasaki & Kawai, 2014). Intracellular TLRs recognize nucleic acid derived from bacteria and viruses, but also self-nucleic acids in disease such as autoimmunity (Kawasaki & Kawai, 2014).

2.5.1 Toll-like receptor signaling

TLRs signaling pathways are classified into two types, myeloid differentiation primary response protein 88 (MyD88)-path and the TIR domain-containing-adaptor inducing interferon-beta (IFNβ) (TRIF) dependent pathways (Nie et al., 2018). MyD88 is utilized by all TLRs and it activates NF-κB and mitogen-activated

protein kinase (MAPKs) (Kawasaki & Kawai, 2014). Individual TLRs differentially recruit members of TIR-domain-containing adaptors such as MyD88, TRIF, TIR domain containing adaptor protein/MyD88 adaptor-like (TIRAP/MAL) or TRIF-related adaptor molecule (TRAM) (Kawasaki & Kawai, 2014). TIRAP is a sorting adaptor that recruits MyD88 to the cell surface, associating with both cell surfaces and endosomal TLRs (Kawasaki & Kawai, 2014). TRIF is required for TLR3 and TLR4 signaling and promotes an alternative pathway that leads to activation of interferon regulatory factor 3 (IRF3), NF-κB and MAPKs for induction of inflammatory cytokines, whereas TRAM is selectively required for TLR4, but not TLR3, to link between TRIF and TLR4 (Kawasaki & Kawai, 2014). For example, TLR4 activates both the MyD88-dependent and TRIF-dependent pathways, which are controlled by several molecules to induce appropriate responses (Kawasaki & Kawai, 2014).

MyD88-dependent pathway

The MyD88-dependent response is utilized by almost all the TLRs except TLR3 (Nie et al., 2018). Homo- or heterodimer formation initiates signaling to Myd88, and TIRAP conducts the signaling from TLR to MyD88 (Kawasaki & Kawai, 2014). MyD88 binds to the TIR domain of the corresponding TLR (Nie et al., 2018). Subsequently, IL-1 receptor-associated kinase 4 (IRAK4) is recruited through the death domain of Myd88, which leads to formation of the Myddosome complex (MyD88, IRAK1 and IRAK4) (Gorjestani et al., 2012) and autophosphorylation of IL-1 receptor-associated kinase 1 (IRAK1) (Nie et al., 2018). Later, tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) is activated by IRAK1, which activates the TAK/TGF-β-activated kinase (TAB) complex through K63-linked ubiquitination of transforming growth factor-β-activated kinase (TAK1) and TRAF6 (Gorjestani et al., 2012). Afterwards, TAK1 activation leads to the activation of IκB kinase-complex-NF-κB (IKK) and MAPK (Nie et al., 2018). MAPK activation leads to activating protein (AP-1) transcription factor activation, which is responsible for regulating inflammatory responses (Kawasaki & Kawai, 2014). IKK-complex (IKKα and IKKβ and the regulatory subunit NEMO) phosphorylates the NF-κB inhibitory protein I kappa B alpha (IκBα), which leads to nuclear translocation of the transcription factor NF-κB; this induces the transcription genes of encoding inflammatory cytokines (Figure 2.) (Kawasaki & Kawai, 2014).

TRIF-dependent pathway

The TRIF-dependent pathway is considered to be specific in only a few TLRs, such as TLR3 and TLR4 in mammals (Nie et al., 2018). Various transcription factors, such as NF-κB, AP-1, and interferon regulatory factor (IRF) family members can be activated by the TRIF-dependent pathway (Nie et al., 2018). TRIF interacts with TRAF6 and TRAF3 (Kawasaki & Kawai, 2014). TRAF6 recruits the receptor interacting serine/threonine 1 (RIP-1) kinase, which activates the TAK1 complex, subsequently leading to activation of NF-κB and MAPKs and induction of inflammatory cytokines, in the same manner as the MyD88-dependent pathway (Kawasaki & Kawai, 2014). In contrast, TRAF3 recruits the IKK-related kinases TANK-binding kinase-1 (TBK1) and kinase I kappa B kinase i (IKKi) along with NEMO for IRF3 phosphorylation (Kawasaki & Kawai, 2014). Afterwards, IRF3 forms a dimer and translocates into the nucleus from the cytoplasm, where it induces the expression of type I IFN inflammatory cytokines (Kawasaki & Kawai, 2014). Also, Pellino family E3 ubiquitin ligases are linked in TLR signaling (Jiang & Chen, 2012). Pellino-1 is phosphorylated by TBK1/IKKi and thereby facilitates ubiquitination of RIP-1 (Jiang & Chen, 2012). Pellino-1 also regulates IRF3 activation by binding to deformed epidermal autoregulatory factor-1 (DEAF-1), which facilitates binding of IRF3 to the IFNβ promoter (Figure 2.) (Jiang & Chen, 2012).

2.5.2 Toll-like receptor 2

TLR2 recognizes ligands of various pathogens such as bacteria, mycoplasma, fungi and viruses by forming a heterodimer with either TLR1 or TLR6 (Oliveira-Nascimento et al., 2012). The wide range of ligands includes diacyl and triacylglycerol moieties, proteins and polysaccharides (Oliveira-Nascimento et al., 2012). When forming heterodimers with TLR1, initiation of the cytokines occurs via MyD88-dependent pathway with the recruitment of TIRAP (Figure 2.) (Takeuchi & Akira, 2010). Endogenous ligands known as “alarmins” that indicate tissue damage and potential tumor cells have also been described as TLR2 ligands (Oliveira-Nascimento et al., 2012).

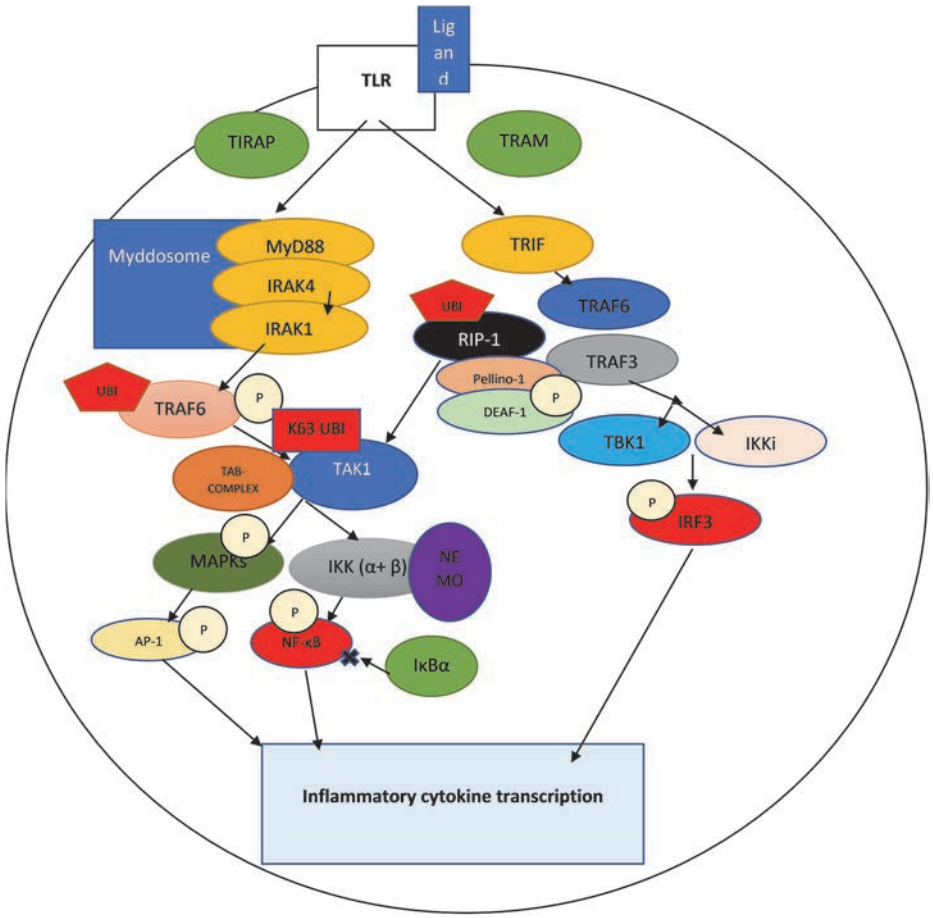

Fig. 2. Toll-like receptor signaling MyD88-dependent and Myd88 independent pathways presented.

2.5.3 Toll-like receptor 4

TLR4 recognizes lipopolysaccharide (LPS) together with myeloid differentiation factor (MD2) on the cell surface (Nie et al., 2018). Also, glycosylphosphatidylinositol-anchored membrane protein (CD14) associates with TLR4 to form a functional LPS-receptor (Park et al., 2009). Thus, the active LPS receptor contains TLR4, CD14 and MD2 (Park et al., 2009). LPS is a component of the outer membrane of Gram-negative bacteria (Flo et al., 2002). TLR4 is also involved in the recognition of viruses by binding to viral envelope proteins and

recognizing DAMPs (Takeuchi & Akira, 2010). TLR4 signaling is mediated by two distinct pathways, MyD88-dependent and MyD88-independent pathway, which are described in Chapter 2.5.1. To mediate a comprehensive immune response, TLR4 requires four different adapters: MyD88, TIRAP, TRIF and TRAM (Vaure & Liu, 2014). TLR4 is activated via TIRAP-Myd88-pathway NF-κB and related inflammatory cytokine production, such as IL-12 (Vaure & Liu, 2014). The TRIF-TRAM-pathway effectuates the subsequent up-regulation of genes encoding type I interferons (IFNs) and co-stimulatory molecules (Vaure & Liu, 2014). As well, it activates TNF-α production and secretion, which leads to NF-κB activation (Vaure & Liu, 2014). The Myd88-independent pathway is responsible for the majority of the LPS response (Vaure & Liu, 2014).

2.5.4 Toll-like receptor 5

TLR5 is expressed in epithelial cells and immune cells, such as monocytes and immature dendritic cells (Yang & Yan, 2017). TLR5 is highly expressed in dendritic cells of the lamina propria (LPDCs) in the small intestine, where it recognizes bacterial flagellin, a structural protein expressed by several Gram negative and positive bacteria (Cario & Podolsky, 2000; Muzio et al., 2000; Takeuchi & Akira, 2010). LPDSs can detect pathogenic bacteria and produce proinflammatory cytokines in a TLR5-dependent manner. In the liver, TLR5 has been found to detect flagellin, promote bacterial clearance, and protect the liver against chronic inflammatory diseases (Yang & Yan, 2017). TLR5 utilizes the MyD88-dependent pathway to induce inflammatory responses, but also TRIF is involved in mediating TLR5-induced NFκB and MAPKs activation and inflammatory responses (Choi et al., 2010).

2.5.5 Toll-like receptor 8

TLR8 is an endosomal sensor in human monocytes, macrophages and dendritic cells that recognizes viral or bacterial single-stranded RNAs, endogenous RNAs, and self-RNAs from dead or dying cells, and is activated by uridine- and guanosine-rich ssRNA as well as by certain synthetic chemicals (Tanji et al., 2015). TLR8 utilizes adaptor protein MyD88 to signal through NF-κB and interferon regulatory factor 7 (IRF7) to induce inflammatory cytokine activation (Cervan
tes et al., 2012).

2.5.6 Toll-like receptors in cancer

TLR2

TLR2 signaling is one of the mechanisms of chronic inflammation, but it can also mediate tumor cell immune escape and tumor progression (Khan et al., 2016). On the one hand, TLR2 has been linked to tumor progression (Huang et al., 2012; Mohamed et al., 2020; Shi et al., 2014) and on the other hand, TLR2 has shown antitumoral effects (D'Agostini et al., 2005; Fuge et al., 2015; Luo et al., 2011). In HCC, the tumor-promoting role of TLR2 has been observed (Huang et al., 2012; Shi et al., 2014; Zhe et al., 2016). These studies observed tumor proliferation, invasion and migration, induced NF-κB/P65 expression, and inhibited cells apoptosis *in vivo* (Shi et al., 2014). Also, HCC proliferation was promoted by heat shock protein 70 (HSP70) through activation of TLR2 and initiated subsequent activation of the intracellular JNK1/2/MAPK signaling pathway (Zhe et al., 2016). As well, the tumor-inhibiting role of TLR2 via reducing the IL-18 mediated pathway has been observed *in vitro* (Li et al., 2015).
In other cancers such as laryngeal squamous cell carcinoma (Wang et al., 2013), ovarian cancer (Chefetz et al., 2013) and gastric cancer (Tye et al., 2012; Yang et al., 2014). TLR2 expression has been observed to be related to cancer progression. These studies suggested that tumor repair and recurrence were induced via TLR2-MyD88-NFκB pathway (Chefetz et al., 2013) and upregulation of TLR2 via oncogenic transcription factor STAT3, leading to increased tumorigenesis and cancer proliferation (Tye et al., 2012) and increased expression of metastatic genes of VEGF-C and MMP-9, resulting in increased metastatic behavior and invasion via expression of TLR2 (Yang et al., 2014). Adversely, in some studies, for example in multiple myeloma (Abdi et al., 2013) and breast cancer (Wang et al., 2020). TLR2 seems to have an antitumoral role, but the mechanism is still unclear. The significance of TLR2 in cancer progression is still unclear (Khan et al., 2016).

TLR4

The role of TLR4 in hepatocarcinogenesis has been observed in several studies (Dapito et al., 2012; Seki et al., 2007; Sepehri et al., 2017). Arguably, TLR4 interacts with the surrounding microenvironment, thus promoting tumorigenesis (Zheng et al., 2015). As well, the signaling pathways of TLR4 resulting in the activation of MAPKs, NF-κB and various cytokines have tumor progressing

abilities (Zheng et al., 2015). TLR4 activation by LPS from intestinal microbiota contributes to injury- and inflammation-driven tumor promotion (Dapito et al., 2012). Eiró and colleagues observed high expression of TLR4, along with TLR3 and TLR9, to be associated with tumor size, poor prognosis and tumor aggressiveness. Expression of TLR4, inducing NF-κB, was suggested as one of the reasons behind these findings (Eiró et al., 2014). As well, Kang and colleagues observed that the TLR4/MyD88 signaling pathway may be involved in hepatocellular carcinogenesis via upregulating the IL-23/IL-17A axis (Kang et al., 2018). Also, LPS-induced TLR4 expression was observed to increase metastasis and invasion of HCC cells via the MKK4/JNK pathway (Dong et al., 2015). Liu and colleagues observed positive TLR4 expression to be related to enhanced migration and invasion of HCC cells; they also observed positively expressed markers (Snail and E-cadherin) that are typical characteristics of EMT (Liu et al., 2015). As well, EMT-related tumor progression via LPS-TLR4 results and conclusions were offered by Jing and colleagues (Jing et al., 2012). Lin and colleagues demonstrated that intestinal flora contributes to inflammation-driven HCC promotion and TLR4 was functionally expressed in HCC cells. It was also demonstrated that TLR4 signaling could promote proliferation of HCC, where COX-2/PGE_2/STAT3 positive feedback loop played an important role (Lin et al., 2016). The role of TLR4 expression in cancer progression has also been observed in breast cancer (González-Reyes et al., 2010), colon cancer (Wang et al., 2010), esophageal squamous cell carcinoma (Kauppila & Selander, 2014), ovarian cancer (Li et al., 2016), gastric cancer (Yuan et al., 2013) and lung cancer (He et al., 2007).

TLR5

Flagellin is a potent activator of various cell types that are involved in innate and adaptive immunity (Hayashi et al., 2001). To date, the role of flagellin-induced TLR5 activation in tumor prognosis is unclear (Etienne-Mesmin et al., 2016; Helminen et al., 2016;. Kauppila et al., 2013; Pimentel-Nunes et al., 2011; Song et al., 2011). Flagellin-induced acute liver function abnormality and damage was observed by Xiao and colleagues. The study revealed that high-dose flagellin-induced TLR5 expression caused the production of pro-inflammatory cytokines and liver damage (Xiao et al., 2015). In a study by Yang and colleagues, TLR5 expression was examined *in vitro*. It was found that xenograft hepatocarcinoma tumor tissue exhibited higher levels of TLR5 compared to normal liver tissue (Yang et al., 2014). TLR5 has previously been associated with gastric cancer (Schmaußer

et al., 2005), cervical neoplasia progression (Kim et al., 2008), and squamous cell carcinoma of the tongue (Kauppila et al., 2013). On the contrary, antitumoral activity of TLR5 has been observed in colon carcinoma *in vitro*, suggesting that TLR5/MyD88-dependent signaling mediates anti-tumor activity in a mouse xenograft model of human colon cancer (Rhee et al., 2008). In addition, antitumoral effects of TLR5 have been observed in breast cancer (Cai et al., 2011).

TLR8

To date, the relationship of TLR8 and HCC remains unclear. Antitumoral effects of TLR8 have been observed *in vitro*, suggesting that TLR8 agonists are effective immunomodulators by inducing apoptosis, but also by promoting the activation of natural killer cells (Zhou et al., 2015). In colorectal cancer, expression of TLR8 has been associated with tumor progression and reduced tumor survival; it has been suggested that inflammation through the TLR signaling followed by activation of NF-κB may potentially enable tumor-initiating cells to maintain themselves as well as re-establish tumor heterogeneity by enhancing their resistance to apoptosis (Grimm et al., 2010). In an *in vivo* study by Zhang and colleagues, the expression of TLR8 in patients with cervical cancer was upregulated, suggesting possible correlation with tumor prognosis, and TLR8 expression was associated with Bcl-2 and VEGF, which play an important role in tumor angiogenesis (Zhang et al., 2014). In human lung cancer, high expression of TLR8 was associated with inflammation, tumor growth and resistance to apoptosis induced by chemotherapy; activation mainly through NF-κB pathway was observed (Cherfils-Vicini et al., 2010). In primary ductal pancreatic cancer, increased TLR8 expression in a human pancreatic cell line resulted in elevated NF-κB and COX-2 expression, increased cancer cell proliferation, and reduced chemosensitivity (Grimmig et al., 2015).

2.5.7 TP53 and Ki67

TP53

Tumor protein 53 (TP53) is the most frequently mutated gene in human cancer (Kastenhuber & Lowe, 2017). The TP53 gene encodes protein 53 (p53) (Kastenhuber & Lowe, 2017). P53 is a sequence-specific DNA binding protein that regulates transcription and suppresses growth and oncogenic transformation in cell

culture. In normal functions, p53 is activated by stress stimuli and when activated, it governs a complex anti-proliferative transcriptional program of biological responses (Kastenhuber & Lowe, 2017). The best-known functions of p53 are the ability to promote cell cycle arrest and apoptosis. DNA damage response (DDR) kinases phosphorylate p53, driving cell cycle arrest, senescence or apoptosis (Kastenhuber & Lowe, 2017). P53 also stimulates DNA repair by activating target genes that encode components of DNA repair. Inactivating TP53 mutations are common in human tumors (Kastenhuber & Lowe, 2017).

In hepatocellular carcinoma, the frequency of TP53 mutations and the mutation spectra seem to vary in different geographical areas and presumably reflect the differences in etiology in HCC (Hussain et al., 2007). The p53 cell cycle pathway is altered in at least half of HCC patients (Kunst et al., 2016). Disruption of the p53 pathway in HCC can occur by the mutations of the p53 gene itself (Hussain et al., 2007; Schilling et al., 2010; Zalcenstein et al., 2003) or by alterations such as p14 alternate reading frame protein (ARF) inactivation (Anzola et al., 2004), or as a result from the amplification or overexpression of its specific inhibitors murine double minute 2 (MDM2) and murine double minute 4 (MDM4) (Biderman et al., 2012; Wade et al., 2013). Mutation of somatic TP53 occurs in almost every type of cancer (Olivier et al., 2010).

Ki67

Ki67 is a nuclear protein attaching to nuclear genes that codes two protein isoforms and is present during all active phases of the cell cycle but absent in resting cells (Li et al., 2015). Ki67 also has a role in mitotic cells and its cellular distribution changes dramatically during cell cycle progression (Sun & Kaufman, 2018). The expression of Ki67 is associated with proliferative activity of intrinsic cell populations, allowing it to be used as a marker of tumor aggressiveness (Li et al., 2015). The expression of Ki67 reflects the tumor proliferation rate and correlates with initiation, progression, metastasis and prognosis in various cancer types (Li et al., 2015; Machowska et al., 2014; Nielsen et al., 2013; Nielsen et al., 2012; Palmqvist et al., 1999).

In HCC, Ki67 has been observed to be associated with poor prognosis and tumor grade. Shi and colleagues speculated that overexpression of Ki67 might result in poor prognosis by regulating the level metastasis-associated tumor gene 2 (MTA2) (Shi et al., 2015). A correlation between Ki67 expression and poor prognosis in HCC has also been identified in various studies (Ba et al. 2017; King

et al., 1998). A meta-analysis by Luo and colleagues revealed that high expression of Ki67 correlated strongly with histological grade, tumor size, number of tumor nodes, metastasis, vein invasion and cirrhosis, as well as poor prognosis (Luo et al., 2015)

3 Aims of this study

The aim of this study was to examine the treatment trends and outcomes of hepatocellular carcinoma treatment in Oulu University hospital and the prognostic value of tumor budding, Tumor-Stroma ratio, TLR2, TLR4, TLR5 and TLR8 expression. Particularly, the objectives of this study were:

1. To examine the treatment trends and outcomes of hepatocellular carcinoma in Northern Finland and find areas for improvement.
2. To examine the prognostic value of tumor budding and TSR in hepatocellular carcinoma.
3. To examine the prognostic value of TLR2 and TLR4 tumor expression in hepatocellular carcinoma.
4. To examine the prognostic value of TLR5 and TLR8 tumor expression in contrast with the previously known risk markers Ki67 and p53.

4 Materials and methods

4.1 Study setting and patients

This study, containing studies I-IV, was a retrospective cohort study in a single institution tertiary care hospital in Northern Finland. The patients were identified from archives using ICD-10 code C22.0& (hepatocellular carcinoma). Patient survival data was acquired from Statistics Finland. The Oulu University Hospital Ethics Committee approved the study and the need to obtain informed consent from the study patients was waived by the Finnish National Authority for Medicolegal Affairs (VALVIRA). All diagnoses were confirmed with histological examination. The 8th edition of the TNM classification was used in staging. A gastrointestinal pathologist (Vesa-Matti Pohjanen (V-M.P.)) re-evaluated and confirmed the diagnoses of all patients included in studies I-IV. All cases were also re-graded for histological grade of differentiation by a gastrointestinal pathologist (V-M. P.).

Hematoxylin and eosin stained (HE) slides were used. In studies II-IV, at first, multiple sections from each patient were viewed with a light microscope before further examination. A representative slide with visible tumor component was selected and digitized using Aperio AT2 (Leica Biosystems, Wetzlar, Germany).

Study I

The clinical data was collected from Oulu University Hospital patient records. The whole cohort consisted of 298 HCC patients diagnosed in Oulu University Hospital between January 1, 1983 and March 12, 2018, of whom 49 underwent surgical resection, 25 RF, laser ablation or PEI, 48 were treated with TACE, TAE or SIRT, and 151 were treated with palliative treatment or best supportive care. Some patients received more than one treatment. Twenty-five patients were originally excluded from Study I due to lack of information from the patient files, equaling 273 consecutive patients in Study I. The complications in Study I were classified primarily with the Accordion Severity Grading System (Strasberg et al., 2009) and secondarily with the Clavien-Dindo classification system (Dindo et al., 2004).

Studies II-IV

In Study II, representative samples from 259 patients were available for analysis and were included in the present study. In Study II, four patients treated surgically had only biopsy samples available. Eight non-surgically treated patients had surgical resection samples available. Patients were divided into two groups based on treatment (surgically and non-surgically treated). In Studies II-IV, the histological material consisted of surgical resection samples and core needle biopsies. If the patient had a surgical resection sample and a biopsy sample available, the surgical resection sample was used for the analysis. Due to introduction of a new TNM 8th staging edition during Study II, all surgical patient samples were reviewed by a gastrointestinal pathologist (V-M.P.) to properly evaluate vascular invasion (and T-class). In non-surgical patients, vascular invasion was impossible to determine from core needle biopsies and therefore these were based on radiology. A total of 11 patients were upstaged. In Study III, a total of 203 and in Study IV, 182 patients with representative tissue samples were included.

4.2 Tissue micro array (studies III and IV)

Tissue microarrays (TMAs) were constructed using a method described earlier (Nocito et al., 2001). The most representative tumor areas were chosen from the HE-stained slides, confirmed by a gastrointestinal pathologist (V-M.P.). TMAs were then constructed with the Galileo CK4500 tissue microarray platform. Tissue cores with a diameter of 1.0 mm were taken from the tumor, using the chosen scanned slides as a guideline. One core was taken per sample block.

4.3 Assessment of tumor budding (Study II)

When investigating tumor budding in Study II, a bud was defined as a single tumor cell or a cell cluster of up to four tumor cells that seemed to be detached from the main tumor. Tumor budding was analyzed from scanned sections (see Chapter 4.1) by using Aperio ImageScope by two independent investigators (Valtteri Kairaluoma (V.K.) and Niko Kemi (N.K.)) blinded to the clinical and outcome data. If the sample estimates were on the different sides of the cut-offs, the sample was re-assessed and consensus was reached. A few dubious cases were reassessed by a third investigator (V-M.P.) The hotspot method was used, which is highly recommended when investigating tumor budding in colorectal cancer (Lugli et al.,

2017). In sample investigation, the tumor area was first screened with low magnification to find the area with the most tumor budding. Then, the number of buds was counted from a single field of view at 200x total magnification. After assessment, the patients were divided into low and high budding groups. The cases were classified as bud-negative if no tumor buds were found and as bud-positive if at least one tumor bud was present because the median number of tumor buds was found to be zero. Examples of tumor budding are presented in Figure 3.

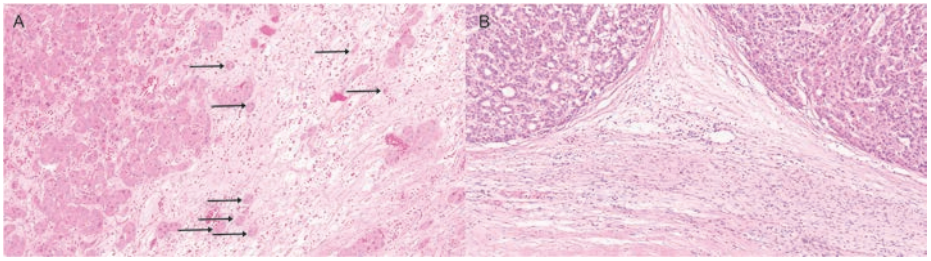

Fig. 3. Examples of tumor budding; A) Bud positive (arrows pointing at tumor buds), B) Bud negative.

4.4 Assessment of Tumor-Stroma ratio (Study II)

Tumor-Stroma ratio was examined from the same samples as those used for the assessment of tumor budding (see Chapter 4.3). TSR was analyzed from scanned sections (see Chapter 4.1) by using Aperio ImageScope by two independent investigators (V.K. and N.K.) blinded to the clinical and outcome data. If the sample estimates were on different sides of the cut-offs, the sample was re-assessed and consensus was reached. A few dubious cases were reassessed by a third investigator (V-M.P.) Different methods were used for assessing TSR from surgical resection and biopsy samples. The surgical resection samples were viewed at low magnification and the area with the most stroma compared to tumor cells was identified. The area of stroma compared to the area of tumor cells was estimated from a single field of view using 100x total magnification. The presence of tumor cells was confirmed on all four sides of the field of view before the assessment was performed. The percentage of stroma on a selected area compared to the tumoral cell component was estimated and scored at 10% intervals (10, 20, 30% etc.). Necrosis and normal hepatocytes were excluded. In biopsy samples, the area between the most remote tumor cells in the section was analyzed. If the biopsy

sample had been shattered into more than two pieces, all of which contained stroma and tumor cells at both ends of the sample, the whole biopsy sample was used for estimation. The amount of stroma in the selected area was scored in the same way as in the surgical resection samples. Cut-off value was set at 50% and patients were divided into high TSR (<50% stroma) and low TSR (⩾50% stroma) groups. Examples of Tumor-Stroma ratio are presented in Figure 4.

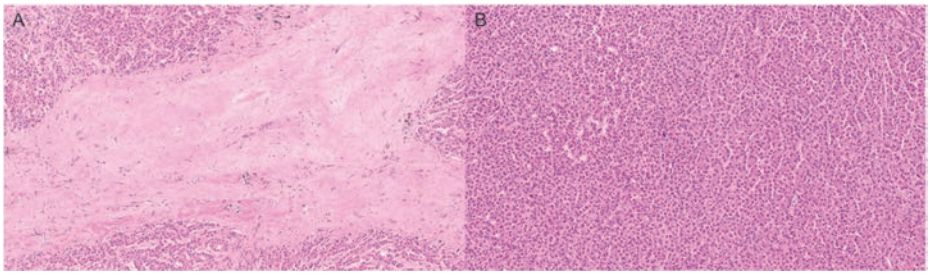

Fig. 4. Examples of Tumor-Stroma ratio; A) low TSR, B) high TSR.

4.5 Assessment of TLR2 and TLR4 (Study III)

Immunohistochemical staining of TLR2 and TLR4 was evaluated by two independent investigators (V.K. and N.K.) blinded from the clinical data. Cytoplasm intensity, cytoplasm percentage (e.g. percentage of cells with detectable cytoplasmic expression), nuclei percentage and membrane percentage were evaluated from TMA sections independently by two investigators (V.K. and N.K.). Assessment of the staining intensity was performed using a 4-point scale from 0 (negative) to 1 (weak), 2 (moderate) and 3 (strong) according to the most prevalent positive expression score. The extent of staining was estimated from 0 to 100% to express the percentage of positive cytoplasm and nuclei. Thus, all values are means of intensities and percentages from two investigators. For statistical evaluation, each staining (TLR4 and TLR2) was dichotomized based on median value into two groups. The following groups were formed: cytoplasm intensity (weak and strong), percentage of stained cytoplasmic cells (low and high) and nucleic cells (low and high). Cutoffs were determined according to median values: TLR4 cytoplasm intensity (<=2), TLR4 cytoplasm percentage (<100) and TLR4 nucleic percentage (<=50.0). TLR2 cytoplasm intensity (<=2), TLR2 cytoplasm staining percentage (<100) and TLR2 nucleic percentage (<=0.0). To exclude possible bias related to technical staining differences TLR4 and TLR2 cytoplasm intensities were

compared between surgical resection samples and core needle biopsies with Mann-Whitney U test. No significant differences were observed. There were no differences between old (1983–2005) and new (2006–2018) samples, either.

TLR2 expression was only found on cell membranes in samples from 3 (1.6%) patients. Because cytoplasmic percentage was under 100% in only a few cases, it was not used in statistical testing. In addition, since TLR2 membrane expression was present in only three patients, it was not used in statistical testing. Cytoplasmic TLR2 staining was technically unreliable in three patients and they were excluded. In TLR4, cytoplasmic percentage was under 100% in only a few cases, which is why it was not used in statistical testing. Cytoplasmic TLR4 staining was technically unreliable in three patients and they were excluded. TLR4 expression was not found on cell membranes. Examples of TLR2 and TLR4 are presented in Figures 5-8.

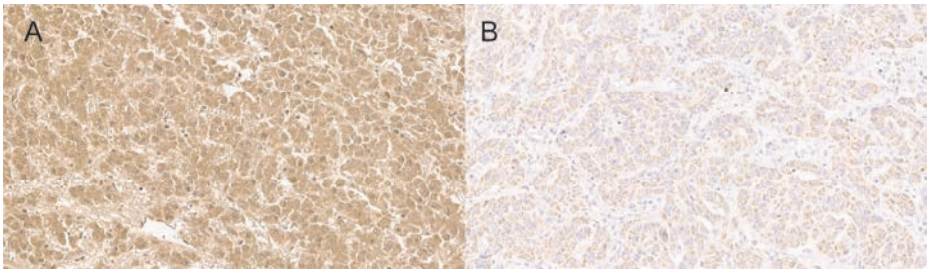

Fig. 5. TLR2 cytoplasm intensity staining. A) represents strong cytoplasmic intensity, B) represents weak cytoplasmic intensity.

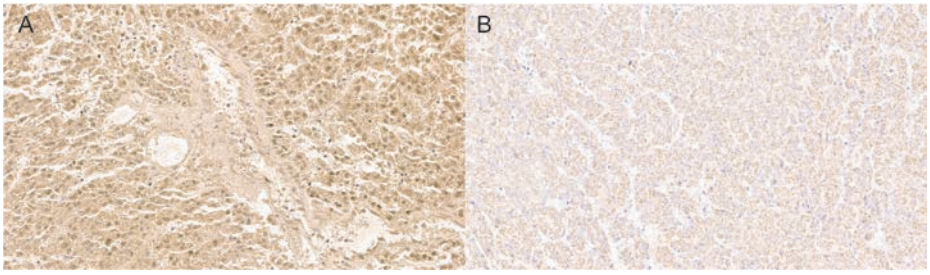

Fig. 6. TLR2 nuclei percentage staining. A) represents high nuclei staining percentage, B) represents low nuclei staining percentage.

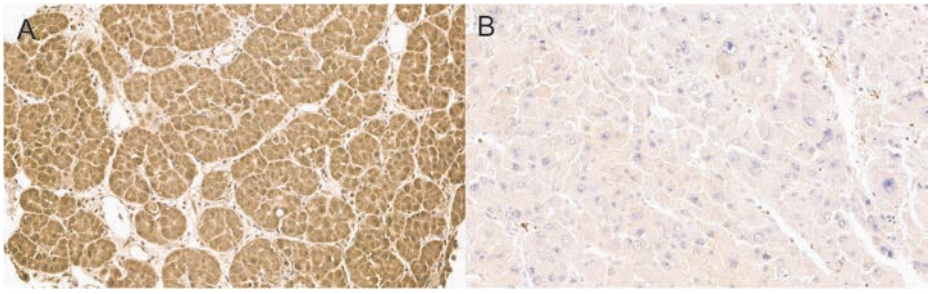

Fig. 7. TLR4 cytoplasm intensity staining. A) represents strong cytoplasmic intensity, B) represents weak cytoplasmic intensity.

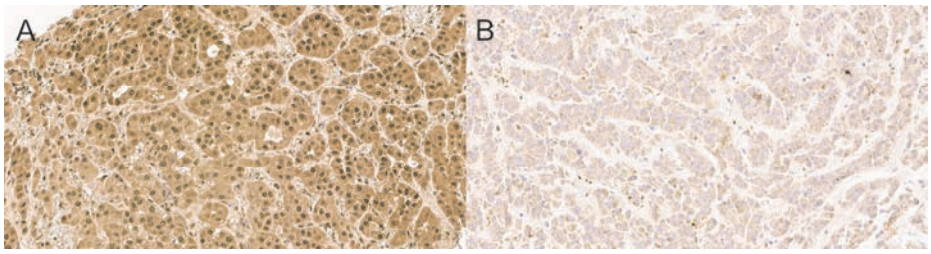

Fig. 8. TLR4 nuclei percentage staining. A) represents high nuclei staining percentage, B) represents low nuclei staining percentage.

4.6 Assessment of TLR5, TLR8, Ki67 and p53 (Study IV)

All histological analyses were performed independently by two investigators (V.K. and N.K.) blinded to the clinical data. The assessment of cytoplasm intensity was evaluated using a 4-point scale from 0 (negative) to 1 (weak), 2 (moderate) and 3 (strong) according to the most prevalent positive expression score. The extent of staining was estimated from 0 to 100% to express the percentage of positive cytoplasm and nuclei. All values are means of intensities and percentages from two investigators. For statistical evaluation, each stain (TLR5, TLR8, Ki67 and p53) was dichotomized by median value into two groups. Ki67 was evaluated by using QuPath 0.2.1 software (Bankhead et al., 2017) to detect positively stained Ki67 cells, which has shown great reproducibility in breast cancer (Acs et al., 2019). The cut-offs were as follows: TLR5 cytoplasm intensity <=1.0, TLR5 nuclei percentage <=95.0, TLR5 cytoplasm percentage <100.0, TLR8 cytoplasm intensity <=2.0, TLR8 nuclei percentage <=27.5, TLR8 cytoplasm percentage <100.0, Ki67 nuclei percentage <=8.0, and p53 nuclei percentage <=10.0. To exclude possible bias related to sample staining intensity, TLR5 and TLR8 cytoplasm intensities were

compared between surgical resection samples and core needle biopsies with Mann-Whitney U test. A significant difference between groups was observed ($p<0.001$). Since technical reasons related to the smaller staining area could not be excluded, given treatment was adjusted to exclude possible bias.

Cytoplasmic TLR5 staining was unreliable in 6 patients and they were excluded. TLR5 expression was not found on cell membranes. Cytoplasmic TLR8 staining was unreliable with 7 patients and they were excluded. Because cytoplasmic percentage was under 100% in only a few cases, it was not used in statistical testing. TLR8 expression was not found on cell membranes. Examples of TLR5, TLR8, Ki67 and p53 are presented in Figures 9–14.

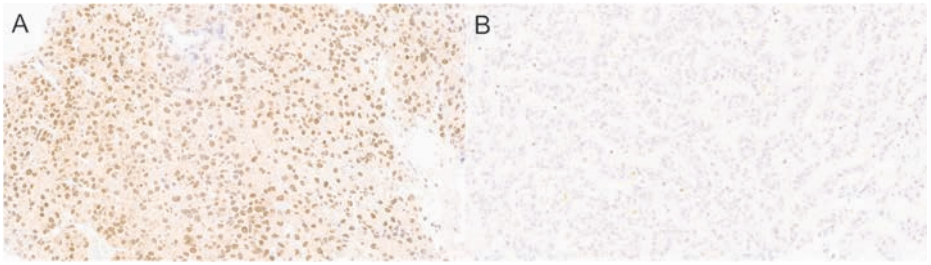

Fig. 9. TLR5 cytoplasm intensity staining. A) represents strong cytoplasmic intensity, B) represents weak cytoplasmic intensity.

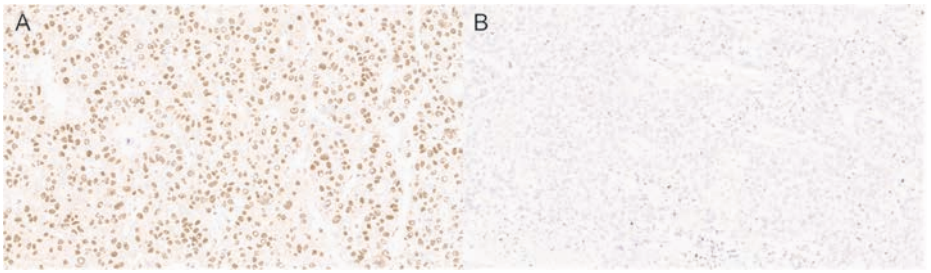

Fig. 10. TLR5 nuclei percentage staining. A) represents high nuclei staining percentage, B) represents low nuclei staining percentage.

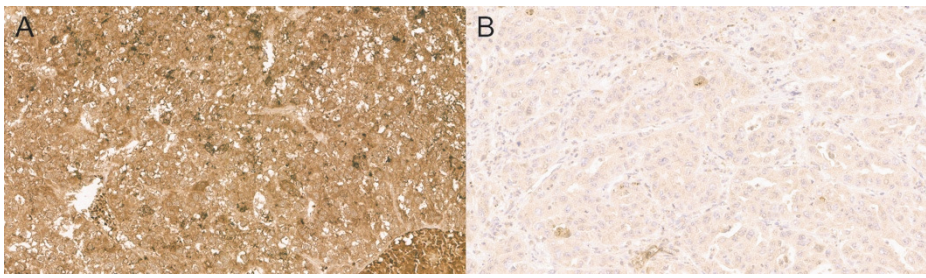

Fig. 11. TLR8 cytoplasm intensity staining. A) represents strong cytoplasmic intensity, B) represents weak cytoplasmic intensity.

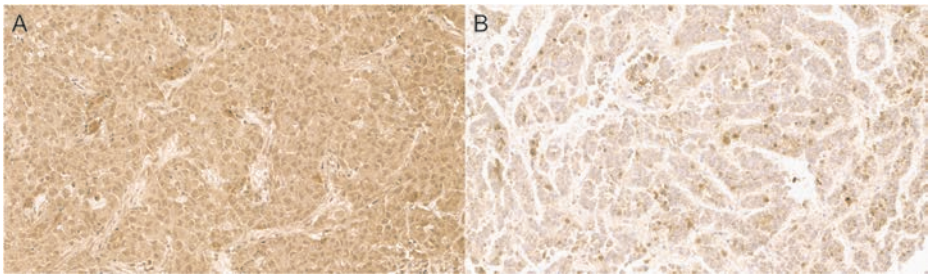

Fig. 12. TLR8 nuclei percentage staining. A) represents high nuclei staining percentage, B) represents low nuclei staining percentage.

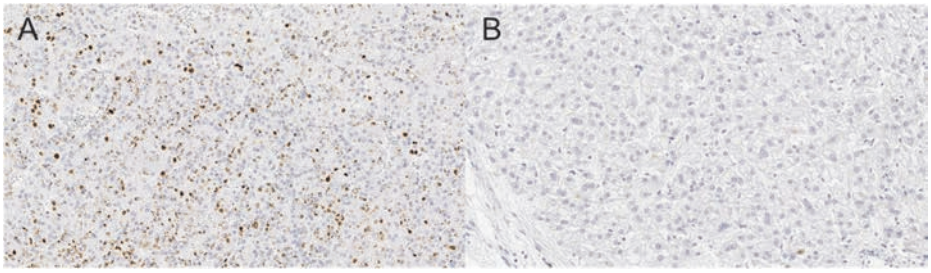

Fig. 13. Ki67 nuclei percentage staining. A) represents high nuclei staining percentage, B) represents low nuclei staining percentage.

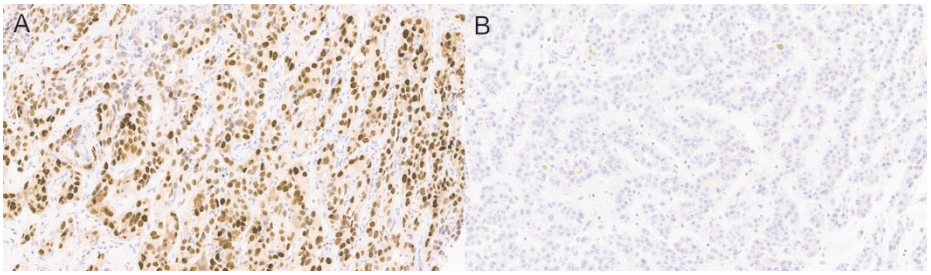

Fig. 14. p53 nuclei percentage staining. A) represents high nuclei staining percentage, B) represents low nuclei staining percentage.

4.7 Immunohistochemistry (III-IV)

Immunohistochemistry was performed on tissue cores, which were selected on the basis of HE-staining as representative for tumor tissue. Antigen retrieval was performed by exposure to high temperature (microwaving 2 minutes with 800W + 15 minutes with 300W) and in Tris-EDTA buffer for 15 min (pH 9.0). The kit used was Dako REAL EnVision Peroxidase/DAB+, Rabbit/Mouse, REF K5007 (Dako, Copenhagen, Denmark). The same kit was used for detection of the first antibody binding. The reaction was visualized by Dako REAL™ DAB+ Chromogen. As negative control, we used omission of the primary antibody and replacement of the primary antibody with non-specific mouse primary antibody isotype.

Study III

Immunostaining was performed manually with rabbit antibodies against TLR2 (ROCKLAND 600-401-956, Rockland Immunochemicals, Inc. Limerick, PA. USA) at a dilution of 1:500, 60 minutes in room temperature, TLR4 (H00007099-M02, IgG2a, clone 3B6, Abnova, Taipei, Taiwan) at a dilution of 1:1.000, 60 minutes in room temperature (Study III).

Study IV

Immunostaining was performed manually with mouse antibodies against TLR5 (NBP2-24787) at a dilution of 1:75, overnight in refrigerator, TLR8 (NBP-2-24917) at a dilution of 1:850, 60 minutes in room temperature, Ki-67 (Bond, Leiga REF PAO230) without dilution, 60 minutes in room temperature, p53 (DAKO

monoclonal mouse clone DO-7), at a dilution of 1:400, 30 minutes in room temperature.

4.8 Statistical analysis

Mann-Whitney U test was used to compare differences between two independent groups with continuous variable. For categorical data-analysis $\chi 2$-test, or Fisher-test, in case of frequency <5, were used. The threshold for significance was set at $P < 0.05$. Cohen's kappa was calculated to analyze interobserver agreement (Studies II-IV) where values between 0.01–0.20 indicate none to slight, 0.21–0.40 fair, 0.41– 0.60 moderate, 0.61–0.80 substantial, and 0.81–1.00 almost perfect agreement (McHugh, 2012). In all continuous variables, median and interquartile range was presented. For survival data, Kaplan-Meier with log-rank test was used. Cox-regression analysis was used to analyze survival in groups adjusting with the following covariates: sex (female/male), age (continuous), Charlson comorbidity index (0-1, 2 or higher), cirrhosis (no/yes), Child-Pugh index A, B, C (Study I), Child-Pugh index A-B or C (studies II-IV), ASA status 1, 2, 3, 4 or more (Study I), year of operation (1983–2005, 2006–2018), Stage 1, 2, 3, 4 (Study I), Stage 1– 2 or higher (studies II-IV), tumor grade 1-2 or 3 (Study II–IV) and given treatment (surgery, local ablation, transarterial chemoembolization, palliative treatment) (studies III–IV). Hazard ratios (HR) with 95% confidence intervals (CI) were provided. For comparison of survival trends over time, patients were divided into equal sized groups based on year of operation 1983-2000 and 2001-2018 for surgery, 1983-2012 and 2013-2018 for local ablation, 1983-2011 and 2012-2018 for angiological treatment and 1983-2011 and 2012-2018 for palliative treatment. (Study I). Statistical analysis was performed with IBM SPSS statistics 24.0 (IBM Corp., Armonk, NY).

5 Results

5.1 Outcomes of the hepatocellular carcinoma patients treated in Oulu University Hospital (Study I)

Based on Study I, resection rate of HCC in Oulu University Hospital was 17.9% during 1983–2018. The patient characteristics are presented in Table 4. In overall and disease-specific survival, surgical resection was associated with higher survival rates compared to angiological and palliative groups (Table 5 and Table 6). There was no statistically significant difference between surgical resection and local ablation groups. In Cox regression analysis adjusted for confounding factors, local ablation (HR 2.56, 95% CI 1.10–5.97) and angiological treatment (HR 3.42, 95% CI 1.61–7.27) were associated with increased risk for long-term overall mortality compared to resection group (Study I). Overall complications occurred more frequently in surgical resection group than in local ablation group (71.5% vs. 32.0%, $p<0.001$). The difference between groups (surgery vs. local ablation) could not be calculated in tumor recurrence due to the low number of patients. Local recurrence occurred more often in local ablation group compared to surgery group (32.0% vs. 4.1%, $p<0.001$).

When the cohort was divided into two equal sized cohorts based on year of operation (see Chapter 4.8), a significant difference in disease-specific survival between the groups was observed at 5 years. Disease-specific survival in the combined old cohort at 1, 3 and 5 years was 50.8%, 27.6% and 16.5%, respectively. Disease-specific survival in the combined old cohort at 1, 3 and 5 years was 50.8%, 27.6% and 16.5%, respectively. The survival rates in the new cohort were 58.0%, 40.6% and 37.2% ($p=0.035$ between groups at 5-years). Since the introduction of ethanol injections (year 1997), TACE (year 2000) and RF (year 2006) the rate of surgery has declined with a corresponding rise in local ablation and other invasive treatments. Still, absolute surgery volume has been stable with annual means of 1.3, 1.4, 1.4, 1.0 and 2.0 surgeries/year during presented time intervals (1983-1993, 1994-2003, 2004-2008, 2009-2013, 2014-2017). (Figure 15).

Table 4. Clinical characteristics of HCC patients treated in Oulu University hospital between 1983-2018.

Patient clinical data	n (%)
Median age at the time of diagnosis (IQR)	71.5 (65.0-78.4)
Sex (%)	Female 81 (27.7), Male 192 (65.8)
Surgical treatment (%)	49 (17.9)
Year of treatment	≤ 2005, 75 (27.5), >2005 198 (72.5)
CCI[1]	≤1 116 (42.5), ≥2 156 (57.1)
Cirrhosis	99 (36.3)
Child-Pugh index[2]	A 205 (75.1), B or C 68 (24.9)
Stage[3]	1 114 (41.8), ≥2 156 (57.1)

[1] Charlson comorbidity index (0-1, 2 or higher)

[2] Child-Pugh score (A,B or C)

[3] Stage 1, 2 or higher

Table 5. Overall survival rates in the surgical resection group, local ablation group, angiological group and palliative group.

Patient group	30 days	90 days	1 year	3 years	5 years	Median survival. years, (IQR)
Surgical resection group	95.9%	95.9%	85.1%	59.0%	51.2%	5.9 (IQR 1.6-9.7)
Local ablation group	100.0%	100.0%	86.1%	43.1%	18.8%	2.6 (IQR 2.0-4.0)
Angiological group	95.8%	93.6%	56.1%	26.3%	6.6%	1.5 (IQR 0.7-3.3)
Palliative group	81.4%	60.7%	26.0%	4.9%	0.0%	0.4 (IQR 0.1-1.1)

Table 6. Disease-specific survival rates in the surgical resection group, local ablation group, angiological group and palliative group.

Patient group	30 days	90 days	1 year	3 years	5 years	Median survival. years, (IQR)
Surgical resection group	95.9%	95.9%	85.1%	63.7%	58.2%	7.7 (IQR 1.6-non est)
Local ablation group	100.0%	100.0%	90.2%	67.4%	36.8%	3.4 (IQR 2.7-non est)
Angiological group	95.8%	93.6%	61.8%	47.2%	15.7%	2.7 (IQR 0.7-4.7)
Palliative group	85.9%	65.9%	33.9%	7.8%	0.0%	0.5 (IQR 0.2-4.1)

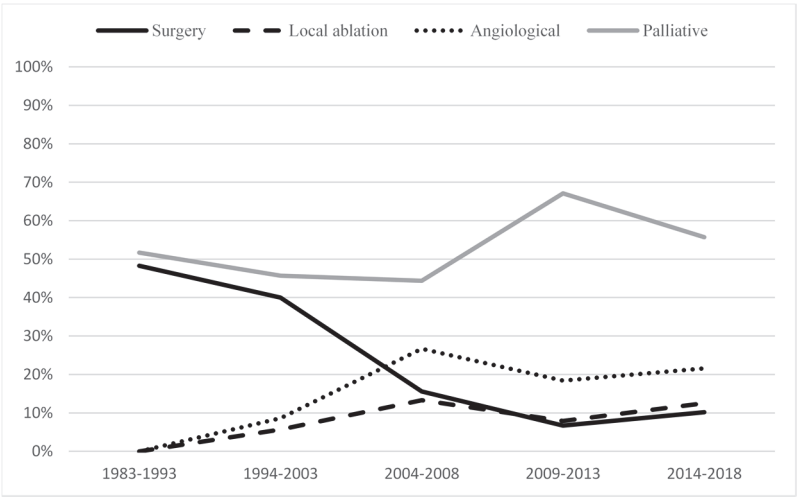

Fig. 15. Percentual proportions (not absolute numbers) of HCC treatments in Oulu University Hospital 1983–2018.

5.2 Tumor budding and Tumor-Stroma ratio in hepatocellular carcinoma (Study II)

In Study II, patients were divided into bud-negative (n=154, (59.5%)) and bud-positive (n=105, (40.5%)) groups (range 0-23 buds). Overall 5-year survival was 72.1% in bud-negative patients and 29.2% in bud-positive patients, p=0.009. Disease-specific 5-year survival was 86.5% and 35.1% (p=0.002), respectively.

In univariable analysis, positive tumor budding was associated with increased risk for 5-year overall (HR 3.30, 95% CI 1.27–8.57) and disease-specific (HR 5.64, 95% CI 1.62–19.73) long-term mortality compared to negative tumor budding. In multivariable analysis adjusted for confounding factors, positive tumor budding was associated with increased risk for 5-year overall (HR 3.87, 95% CI 1.10–13.61) and disease-specific (HR 6.17, 95% CI 1.19–31.90) mortality compared to negative tumor budding.

In TSR, patients were divided into two groups, high TSR (<50%) (N=206, 79.5%) and low TSR (⩾50%) (N=53, 20.5%). Overall 5-year survival was 53.1% in patients with high TSR and 41.9% in patients with low TSR (p=0.711). Disease-specific 5-year survival was 65.6% and 41.9% (p=0.384). In univariable or

multivariable analysis, low TSR was not associated with increased 5-year mortality compared to high TSR.

Cohen's Kappa value for surgical resection samples was 0.801 in tumor budding and 0.876 in TSR. Cohen's Kappa value for biopsy samples was 0.729 in tumor budding and 0.814 in TSR.

5.3 TLR2 and TLR4 in hepatocellular carcinoma (Study III)

No significance was observed in TLR2 survivals. The overall and disease-specific survivals as well as patient numbers of TLR2 are presented in Table 7. In TLR4, significant differences were observed between the TLR4 cytoplasm intensity groups (strong vs. weak) and between the TLR4 nuclei percentage groups (high vs. low) in overall and disease-specific survival. TLR4 patient survivals and patient numbers are presented in Table 8.

Multivariable analysis was not performed in TLR2 due to non-significant differences in crude survival between groups. In univariable analysis, strong TLR4 cytoplasm intensity was associated with increased risk for 5-year disease-specific mortality (HR 1.74, 95% CI 1.19–2.54). In multivariable analysis adjusted for confounding factors, strong TLR4 cytoplasm intensity was not associated with increased risk for 5-year overall mortality in adjusted model 2, (HR 1.24, 95% CI 0.87–1.77) but was associated with increased 5-year disease-specific mortality (HR 1.53, 95% CI 1.02–2.28). In adjusted model 1, when given treatment was not taken into account, increased risk for 5-year overall mortality (HR 1.56, 95% CI 1.01–2.23) and disease-specific mortality (HR 1.95%, 95% CI 1.30–2.92) was observed.

In univariable analysis, high TLR4 nuclei expression was associated with increased risk for 5-year overall (HR 1.49, 95% CI 1.07–2.08) and disease-specific (HR 1.65, 95% CI 1.13–2.41) mortality. In multivariable analysis, high TLR4 nuclei percentage was not associated with increased risk for 5-year overall mortality (adjusted model 2, (HR 0.75, 95% CI 0.50–1.12)) or disease-specific mortality (adjusted model 2, (HR 0.82, 95% CI 0.52–1.30). In adjusted model 1, when given treatment was not taken into account, increased risk for 5-year overall mortality (HR 1.52, 95% CI 1.06–2.16) and disease-specific mortality (HR 1.78, 95% CI 1.19–2.68) was observed.

TLR4 nuclei percentage was associated with tumor recurrence ($p=0.009$), local recurrence ($p<0.001$) and cirrhosis ($p=0.034$). In TLR2 cytoplasm intensity, Cohen's Kappa value was 0.984 and for TLR2 nuclei percentage, 0.950. Cohens's

Kappa value for TLR4 cytoplasm intensity was 0.973 and for TLR4 nuclei percentage, 0.763.

Table 7. Overall and disease-specific 5-year survival in TLR2.

Survivals	TLR 2 strong cytoplasm, N=46 (25.0%)	TLR2 weak cytoplasm, N=138 (75.0%)	p-value	TLR2 high nuclei percentage, N=17 (9.1%)	TLR2 low nuclei percentage, N=170 (90.9%)	p-value
Overall 5-year survival	14.4%	15.2%	0.986	27.9%	14.0%	0.238
Disease-specific 5-year survival	23.2%	24.3%	0.873	49.6%	22.5%	0.130

Table 8. Overall and disease-specific 5-year survival in TLR4.

Survivals	TLR 4 strong cytoplasm intensity, N=79 (43.9%)	TLR4 weak cytoplasm intensity, N=101 (56.1%)	p-value	TLR4 high nuclei percentage,N=89 (48.6%)	TLR4 low nuclei percentage, N=94 (51.4%)	p-value
Overall 5-year survival	11.9%	18.3%	*0.050*	7.5%	22.7%	*0.019*
Disease-specific 5-year survival	14.3%	33.7%	*0.004*	12.1%	35.8%	*0.009*

5.4 TLR5 and TLR8 in hepatocellular carcinoma (Study IV)

A significant difference was observed between TLR5 cytoplasm intensity groups (strong vs. weak) and in TLR5 cytoplasm percentage groups (high vs. low) in 5-year overall and disease-specific survival; in the TLR5 nuclei percentage group (high vs. low) a significant difference was observed only in disease-specific survival (Table 9.). In TLR8 there were no significant differences between groups in TLR8 cytoplasm intensity or in nuclei percentage in survivals (Table 10.). In Ki67 and p53 nuclei percentage, significant differences were observed between the

groups in survival (high vs. low) (Table 11.). Patient numbers are presented in Tables 9-11.

In univariable analysis, strong TLR5 cytoplasm intensity was associated with increased risk for 5-year overall mortality (HR 2.36, 95% CI 1.65–3.38) and for 5-year disease-specific mortality (HR 2.48, 95% CI 1.66–3.71). In multivariable analysis adjusted for confounding factors, strong TLR5 cytoplasm intensity remained as a risk for 5-year overall mortality (HR 1.88, 95% CI 1.26–2.81) and 5-year disease-specific mortality (HR 2.00, 95% CI 1.27–3.15). High TLR5 nuclei percentage was associated with increased risk for disease-specific mortality (HR 1.56, 95% CI 1.06–2.28) in univariable analysis, but not in adjusted analysis. High TLR5 cytoplasm percentage was associated with increased risk for 5-year overall (HR 1.53, 95% CI 1.07–2.18) and disease-specific mortality (HR 1.52, 95% CI 1.02–2.27) in univariable analysis, but not in adjusted analysis.

Multivariable analysis was not performed in TLR8 due to non-significant differences in crude survival between groups.

In univariable analysis, but not in adjusted analysis, high Ki67 was associated with increased mortality for 5-year overall and disease-specific survival (HR 1.55, 95% CI 1.11–2.17 and HR 1.85, 95% CI 1.26–2.71) (Study IV). High p53 was associated with increased risk for 5-year overall and disease-specific mortality in univariable (HR 2.29, 95% CI 1.62–3.23 and HR 2.48, 95% CI 1.68–3.66) and adjusted (HR 1.83, 95% CI 1.28–2.62 and HR 1.97, 95% CI 1.31–2.96) analysis (Study IV).

TLR5 cytoplasm intensity and cytoplasm percentage were associated with tumor multifocality (p=0.003 and p=0.048). TLR5 nuclei percentage was associated with local recurrence (p=0.021). TLR8 cytoplasm intensity was associated with AFP (p=0.034). TLR8 nuclei percentage was also associated with tumor stage (p=0.014), tumor recurrence (p=0.040), and tumor multifocality (p=0.008). Ki67 nuclei percentage was associated with histological tumor grade (p=0.001). P53 nuclei percentage was associated with tumor size (p=0.044), tumor stage (p=0.023), and AFP (p=0.008).

Cohen’s Kappa value for TLR5 cytoplasm intensity was 0.984, for TLR5 nuclei percentage 0.840, and for TLR5 cytoplasm percentage, 0.939. In TLR8, the respective values were 0.973, 0.788 and 0.781, and for p53 nuclei percentage, the value was 0.979.

Table 9. Overall and disease-specific 5-year survival in TLR5.

Survivals	TLR 5 strong cytoplasm intensity, N= 80 (45.5%)	TLR5 weak cytoplasm intensity, N=96 (54.5%)	p-value	TLR5 high nuclei percentge, N=78 (43.6%)	TLR5 low nuclei percentage, N=101 (56.4%)	p-value	TLR5 high cytoplasm percentage, N=110 (61.5%)	TLR5 low cytoplasm percentage, N=69 (38.5%)	p-value
Overall 5-year survival	0.0%	23.8%	*<0.001*	11.7%	19.0%	0.121	11.3%	22.4%	*0.018*
Disease-specific 5-year survival	0.0%	34.9%	*<0.001*	16.3%	31.5%	*0.022*	18.8%	32.2%	*0.038*

Table 10. Overall and disease-specific 5-year survival in TLR8.

Survivals	TLR 8 strong cytoplasm intensity, N=86 (49.1%)	TLR8 weak cytoplasm intensity, N=89 (50.9%)	p-value	TLR8 high nuclei percentge, N=88 (50.0%)	TLR8 low nuclei percentage, N=88 (50.0%)	p-value
Overall 5-year survival	10.3%	20.0%	0.354	9.9%	20.9%	0.157
Disease-specific 5-year survival	17.0%	31.2%	0.182	16.8%	32.0%	0.058

Table 11. Overall and disease-specific 5-year survivals in Ki67 and p53.

Survivals	Ki67 high nuclei percentage, N=85 (46.7%)	Ki67 low nuclei percentage, N=97 (53.3%)	p-value	p53 high nuclei percentage, N=83 (46.4%)	p53 low nuclei percentage, N=96 (53.6%)	p-value
Overall 5-year survival	11.2%	17.4%	*0.010*	6.5%	23.3%	*<0.001*
Disease-specific 5-year survival	15.5%	29.9%	*0.001*	10.6%	35.3%	*<0.001*

6 Discussion

In this thesis, the aim was to examine the treatment trends and outcomes of hepatocellular carcinoma treatment in Oulu University Hospital and the prognostic value of tumor budding, Tumor-Stroma ratio, and TLR2, TLR4, TLR5 and TLR8 expression in hepatocellular carcinoma. It was observed that surgical treatment has declined since the introduction of non-surgical treatments, surgical resection of hepatocellular carcinoma could be used more in the Oulu University Hospital, and surgical resection is the most effective treatment considering long-term survival and local recurrences after adjustment of confounding factors. The absolute surgery volume has remained more or less stable. For the first time in a Western population, tumor budding was shown to be an independent prognostic factor in hepatocellular carcinoma. We also showed that TLR4 expression is a useful biomarker for poor prognosis in both surgically resected tissue samples and core biopsy samples with good interobserver agreement. It was also shown for the first time that TLR5 is an independently prognostic biomarker in hepatocellular carcinoma. These findings add new insight into the role of tumor budding and TLRs in pathophysiology of hepatocellular carcinoma.

6.1 Modern treatment of hepatocellular carcinoma (Study I)

Based on the results from Study I in a Northern Finland population, major changes in HCC treatment and improvement in survival have occurred over time. Since the introduction of PEI, TACE and RF, the rate of surgery has declined with a corresponding rise in non-surgical treatments. The resection rate in Oulu University hospital in the whole cohort was 17.9% and for the last 10 years 8.8%. It is significantly lower compared to the overall resection rate (29.6%) in a systematic review (Sotiropoulos et al., 2006.). At the same time, gradual increase of HCC incidence is observable in Northern Finland (1984-1988 7.4/100 000 vs 2014-2018 10.3/100 000) (Finnish Cancer Registry, 2018)

In the surgery group, overall survival rates at 1, 3 and 5 years were 85.1%, 59.0% and 51.2%, respectively. It is notable that the median tumor size in surgically treated patients was 5.0 cm (IQR 3.5–10.0), with a cirrhotic liver in one third of the patients. Better survival rates have been observed in patients with smaller, non-cirrhotic HCC tumors (<5cm) where overall survival rates have varied at 1, 3 and 5 years from 91.3% to 100.0%, from 73.4% to 92.2% and from 61.5% to 75.7%, respectively (Chen et al., 2006; Hasegawa et al., 2008; J. Huang et al., 2010; Lü et

al., 2006; Yamamoto et al., 2001). In cirrhotic liver, overall survival rates of surgically treated patients are lower, ranging from 41.0 to 79.0% at 5 years (Castells et al., 1993; Livraghi et al., 1995; Wu et al., 2005).

Overall survival rates of HCC patients treated with local ablation at 1, 3 and 5 years were 86.1%, 43.1% and 18.8%, respectively. Better results have been observed in previous studies at 1, 3 and 5 years, varying from 90.0% to 100.0%, from 60.0% to 89.0%, and from 40.0% to 72.0%, respectively (Livraghi et al., 2007; Lü et al., 2006; Tateishi et al., 2005; Yang et al., 2016). However, inclusion of PEI may have an effect on the outcomes in this cohort. Local recurrence occurred more often after local ablation compared to surgery, which has been observed before (Zhang et al., 2007).

TACE is the most widely used primary treatment for inoperable HCC; in previous guidelines, it was recommended as first-line therapy for patients with intermediate-stage disease (Galle et al., 2018). In previous studies comparing RF and TACE within Milan criteria, RF led to better long-term results in univariate analysis, but RF was not an independent favorable prognostic factor in adjusted Cox model (Hsu et al., 2011). At 1, 3 and 5 years, survival rates from 29.0% to 95.0%, from 29.0% to 61.7%, and from 12.8% to 38.3%, respectively, have been reported after TACE (Burrel et al., 2012; Lencioni et al., 2016; Llovet et al., 2002; Malagari et al., 2012; Terzi et al., 2012), being slightly superior compared to the results of this study: 56.1%, 26.3% and 6.6% at 1, 3 and 5 years, respectively.

In our study, patients with an untreatable tumor had a dismal prognosis. Previous studies (Giannini et al., 2014; Yim et al., 2016) reported overall survival in untreated patients with intermediate HCC at 1 and 3 years from 41.6% to 73.4% and from 16.8% to 56.4%, respectively.

Liver surgery remains a complex surgical procedure; we reported 14 major complications (28.6%) and 2 (4.1%) postoperative deaths. In previous studies, the reported major complications and mortality rates vary from 27.8% to 55.5% and from 0.0% to 11.0% (Chen et al., 2006; Huang et al., 2010; Poon et al., 2002; Wu et al., 2005). In our study, complications were more in common after surgical resection compared to RF.

We observed a significant rise in other treatment modalities than surgery, which may be due to multiple factors, for example the development of new therapies, histological and radiological examination and patient evaluation. In Finland, alcohol plays a critical role in the etiology of cirrhosis and HCC, which is a known risk factor of surgery (Leon & McCambridge, 2006; Wu et al., 2005). Other reasons might be the long distances in Northern Finland, the patient material, the time delay

in seeking medical treatment, and unwillingness to surgical treatment. Even after adjustment, underlying disease may be a more important determinant of outcome than the treatment the patients received. However, there were no significant differences between the actively treated patient groups in tumor stage and Child-Pugh classification, and despite the cirrhosis and physical status, more patients could possibly be treated with surgery. With standardized reporting of complications and long-term survival, critical evaluation of results can be performed, with a possibility to improve treatment of our patients (Staiger et al., 2018). Laparotomy was the standard surgical approach in our center. In guidelines, hepatic resection is recommended to be performed via laparoscopic/minimally invasive approaches when possible (Galle et al., 2018). The approach can cause confounding when comparing complication profiles to recent reports.

Based on our study on a Northern Finland population, surgical resection of HCC could be used more and it is the most effective treatment considering long-term survival and local tumor recurrence after adjustment for confounding factors. Despite improvements, the overall prognosis of patients diagnosed with HCC remains poor.

6.2 Tumor budding and TSR in hepatocellular carcinoma (Study II)

The results of Study II indicate that tumor budding is reproducible and an independent prognostic factor in hepatocellular carcinoma in patients treated with surgery. Study II is the first to analyze tumor budding in a Western hepatocellular carcinoma cohort.

In a Chinese study of 423 HCC cases mainly related to HBV infection, tumor budding was an adverse prognostic factor (Wei et al., 2020) suggesting that budding is clinically important regardless of HCC etiology. Tumor budding has previously been studied in a variety of carcinomas (Almangush et al., 2014; Kemi et al., 2019; Liang et al., 2013; O'Connor et al., 2015; Okuyama et al., 2003; Roh et al., 2004). A systematic review of colorectal cancer showed that exhibiting tumor budding was associated with lymph node positivity, higher risk for recurrence, and higher risk of cancer-related death at 5 years (Rogers et al., 2016); the studies included in the review varied in cut-off for presence or absence of tumor budding up to >9 buds (Rogers et al., 2016). Cut-offs also seem to vary in the field of tumor budding: Kemi and colleagues set the cut-off at 10 buds in a study of tumor budding in gastric cancer (Kemi et al., 2019), while Liang and colleagues observed that the presence of 8 or more visible buds was associated with dismal prognosis in breast cancer

(Liang et al., 2013). In colorectal cancer, the use of a three-tier system is recommended along with the budding count (Lugli et al., 2017). Tumor budding has been associated with the presence of lymphovascular invasion, larger tumor size, and poorer survival (Almangush et al., 2014; Liang et al., 2013; O'Connor et al., 2015; Roh & Seki, 2013).

One earlier study of TSR in hepatocellular carcinoma has been published (Lv et al., 2015). In the study, TSR was an independent prognostic factor for HCC patients after liver resection or transplantation (Lv et al., 2015). The same 50% cut-off was set as in our study and 0.870 kappa value was achieved, which was similar to that in our study. Low TSR patients' overall survival rates were significantly lower than those of high TSR patients, and this finding was repeated after adjusting for confounding factors (Lv et al., 2015). We could not repeat this finding. The difference in point-estimate magnitude and statistical significance between the studies may be due to our small sample size of surgically treated patients, or potentially due to differences in etiology, namely alcohol in Finland (Gao & Bataller, 2011; Morgan et al., 2004) and viral infections in China (El-Serag & Rudolph, 2007).

Previously, TSR has been studied in a variety of cancers; in esophageal adenocarcinoma, patients with low TSR had poorer disease-free and overall survival (Courrech et al., 2010), and in gastric cancer, TSR has been identified as an independent marker for poor prognosis (Kemi et al., 2018). Similar results have been observed in breast cancer (de Kruijf et al., 2011) and in cervical cancer (Liu et al., 2014). There are also studies reporting a negative association between TSR and survival, for example in esophageal cancer (Leppänen et al., 2017).

Tumor budding is widely believed to provide an important histological basis for invasion and metastasis (Grigore et al., 2016). The findings of down-regulation of epithelial markers and up-regulation of mesenchymal markers have implied that tumor budding is the morphological expression of epithelial-to-mesenchymal transition. However, it has also been observed that most epithelial-to-mesenchymal transition processes in tumor buds are not complete, giving rise to the notion that tumor buds undergo partial epithelial-to-mesenchymal transition (Grigore et al., 2016).

The mechanism leading to poorer prognosis of patients with low TSR is not yet fully understood. Complex interactions between stromal cells and cancer cells have been suggested to be a part of cancer development (Quail & Joyce, 2013). Cancer-associated fibroblasts may play an important role during tumor development from preneoplastic state to metastatic state (Marsh et al., 2013). A

number of studies have implicated reactive stroma, including activated fibroblasts, in accelerating carcinoma development (Marsh et al., 2013). The complex tumor-related stroma components, including extracellular matrix and various cell types, are known to assist the communication between stromal cells and cancer cells (Wu et al., 2016). All in all, tumor budding and TSR are under intense research in order to learn more about tumor development and interaction between tumor cells and stromal cell components.

In non-surgical patients, tumor budding and TSR were not prognostic, possibly due to advanced tumor stage at diagnosis and short survival as shown in Stage IV colorectal cancer (Galon et al., 2006).

The results of Study II have clinical and research-related implications. Our study showed for the first time that tumor budding is an independent prognostic factor in Western patients with surgically treated hepatocellular carcinoma. The analysis of tumor budding can be reliably replicated and routinely analyzed from HE-stained slides without additional immunohistochemistry or costs. According to our study, tumor budding can be used in daily clinical practice, but validation studies are still needed. More studies are also needed to confirm the prognostic value of TSR in hepatocellular carcinoma.

6.3 TLR2 and TLR4 in hepatocellular carcinoma (Study III)

The results of Study III suggest that TLR4 expression is a prognostic factor in HCC. Strong cytoplasmic TLR4 intensity and high TLR4 nuclei percentage were associated with poor 5-year overall and disease-specific survival. In multivariable analysis, strong cytoplasmic TLR4 intensity remained prognostic. TLR2 expression was not associated with patient survival. High TLR4 nuclei percentage was associated with higher tumor recurrence ($p=0.009$) and higher number of local tumor recurrence ($p<0.001$).

In a study by Zhe and colleagues, TLR2 associated with the grade of differentiation, TNM stage, and shorter relapse-free survival (Zhe et al., 2016). Mohamed and colleagues observed TLR2 nuclear expression in HCC cells (Mohamed et al., 2020). In the study by Shi and colleagues, suppression of TLR2 resulted in lower tumor cell proliferation, invasion and migration compared to reference group (Shi, Su, et al., 2014). In most cited studies, TLR2 expression levels were higher in HCC and correlated with poor prognosis, but we were unable to repeat this finding, which may be due to different etiology in Western and Eastern populations (alcohol vs. hepatitis infection).

In previous studies related to TLR4 and HCC, versatile results have been reported. In a study by Eiró and colleagues, the expression level of TLR4 did not associate with overall survival or other clinicopathological variables, but the number of patients studied was small (n=30) (Eiró et al., 2014). Similarly, Zhe and colleagues did not observe a correlation between the expression of TLR4 and relapse-free survival (Zhe et al., 2016).

The relationship between inflammatory response and cancer progression has been known for years (Balkwill & Mantovani, 2001). In several animal models, inflammatory cells and cytokines, such as NF-κB, TNF-α and various interleukins, have shown the ability to promote carcinogenesis with their anti-apoptotic effects, induction of oxidative damage to DNA, and the induction of tissue repair response (Balkwill & Mantovani, 2001; Coussens & Werb, 2002; Karin & Greten, 2005; Rakoff-Nahoum & Medzhitov, 2009). Toll-like receptors are known for their role in host defense, but increasing evidence suggests also a role in cancer progression (Rakoff-Nahoum & Medzhitov, 2009). Infection or injury can induce inflammation, which can promote tumorigenesis through chronic tissue damage and the subsequent induction of tissue repair (Rakoff-Nahoum & Medzhitov, 2009). The unregulated TLR-regulated tissue repair response can drive tumor growth and progression in a positive feedback of unregulated tissue injury and repair, which can trigger TLR-dependent inflammatory responses (Rakoff-Nahoum & Medzhitov, 2009). Multiple mechanisms for TLRs' role in cancer promotion have been suggested. For example, systemic injection of TLR4 ligand, lipopolysaccharide (LPS) of Gram-negative bacteria stimulated breast adenocarcinoma cell migration, invasion and angiogenesis in tumors (Harmey et al., 2002). A study including *in vitro* and *in vivo* experiments demonstrated that TLR4 signaling is required for LPS-induced epithelial-to-mesenchymal transition, tumor cell invasion and metastasis (Jing et al., 2012). Further studies showed that LPS could directly activate NF-κB signaling through TLR4 in HCC cells (Jing et al., 2012). In addition, TLR2-dependent activation of NF-κB caused by Listeria monocytogenes results in increased resistance of tumor cells to chemotherapeutics (Huang et al., 2007). In previous HCC-TLR studies, TLR2 expression has been associated with proliferation and metastasis of tumor cells and lower apoptotic ratio (Zhe et al., 2016). TLR activation by its own ligands seems to induce activation of various cytokines and anti-apoptotic proteins via NF-κB transcription factor and to promote the tumor-stimulating effect; the stimulation of angiogenic factors via TLRs can also boost the tumor angiogenesis and invasive activity of tumor cells (Shchebliakov et al., 2010). The potential role of TLRs in cancer immunotherapy

has also gained a lot of interest (Urban-Wojciuk et al., 2019). The underlying mechanism behind nuclear translocation of TLR4 and the correlation with prognosis is unclear. TLR4 contains several sequences indicating nuclear localization (Brameier et al., 2007). Alternatively, nuclear carrier proteins might be related to nuclear translocation, but no such proteins have been identified. Translocation of membrane-bound TLRs to nucleus might be due to increased amounts of these proteins and related signaling activity (Huhta et al., 2016).
The results of the present study have clinical and research-related implications. Our study showed that strong cytoplasmic TLR4 expression is an independent factor for poor prognosis in HCC. High TLR4 nuclei expression percentage seems to have prognostic impact in HCC, but in this cohort, poor prognosis was seen only in unadjusted analysis, and in multivariable analysis when given treatment was not included as a confounder. High TLR4 nuclei expression percentage was associated with increased tumor recurrence and local recurrence. In the future, replication studies are needed to examine the prognostic role of TLR4 in HCC, especially in different subgroups based on received treatment. Furthermore, optimal cut-offs need to be determined. Based on this study, TLR4 is a useful biomarker for poor prognosis in both surgically resected tissue samples and core biopsies with good interobserver agreement. TLR2 nuclear percentage was detected in 90.6% of the patients, but only 9.1% had high TLR2 nuclei percentage. The absolute survival difference between high and low TLR2 nuclei percentage was nearly 30%, but without statistical significance. The role of TLR2 nuclear percentage in HCC needs further studies.

6.4 TLR5 and TLR8 in hepatocellular carcinoma (Study IV)

In Study IV, we observed for the first time that TLR5 expression is a predictor of poor prognosis in HCC. TLR8 was not associated with patient survival. As shown previously, p53 expression was an independent predictor of poor 5-year overall and disease-specific survival, with similar point estimate as TLR5 cytoplasm intensity.

Toll-like receptor 5 recognizes bacterial flagellin from both Gram-negative and positive bacteria, and the activation of TLR5 mobilizes nuclear factor kappa B (NF-κB) and stimulates tumor necrosis factor- α (TNF-α) production (Hayashi et al., 2001). TLR5 seems to be differently involved in tumor development depending on tissue or cell origin. Previously, an association between TLR5 and cancer progression has been observed in squamous cell carcinoma of the tongue (Kauppila et al., 2013), cervical neoplasia (Kim et al., 2008), gastric dysplasia and carcinoma

(Pimentel-Nunes et al., 2011; Schmaußer et al., 2005), esophageal dysplasia (Helminen et al., 2014) and colon carcinogenesis (Pimentel-Nunes et al., 2012). TLR5 expression has been observed in breast cancer, but to the contrary, TLR5 activation to flagellin resulted in tumor suppressive activation (Cai et al., 2011).

In a mouse model study of human colon cancer, lack of MyD88 or TLR5 expression enhanced tumor growth and inhibited tumor necrosis, indicating anti-tumoral activity of TLR5 (Rhee et al., 2008). In a study by Kasurinen and colleagues (Kasurinen et al., 2019) high TLR5 expression predicted better outcome compared to low TLR5 expression in gastric cancer. In the current study, high TLR5 expression was connected with poor survival compared to low TLR5 expression. One explanation could be a special type of colon and gastric cancer microbiome compared to HCC (Lee et al., 2019; Ponziani et al., 2019; Sokic-Milutinovic et al., 2015). *Fusobacterium nucleatum* and *Helicobacter pylori* are known colon and gastric cancer promoting microbial species and they induce innate immune response partly through TLR5 signaling via NF- κB dependent manner (Kostic et al., 2013; Smith, 2014). Low TLR5 activation might lead to impaired recognition of carcinogenetic species such as F. nucleatum and H. pylori. This would allow harmful species to colonize the colon and gastric tissues and modulate the tumor microenvironment to further promote its growth. In addition, metagenomic analysis has shown enrichment of Bacteroides and Ruminococcus in HCC. In a xenograft model, HCC incidence increased more in wild-type mice compared to TLR5 knock-down mice after modulating the intestinal environment with diet to be more favorable to Bacteroides and Ruminococcus (Ponziani et al., 2019).

TLR8 recognizes viral or bacterial single-stranded RNA promoting innate immune system responses (Tanji et al., 2015). A positive correlation between expression level of TLR8 and Bcl-2 or VEGF was found in cervical cancer samples, correlating with poor prognosis (Zhang et al., 2014). High TLR8 expression has also been observed in various other cancers (Cherfils-Vicini et al., 2010; Grimm et al., 2010; Grimmig et al., 2015).

In our study, TLR8 was not associated with survival, but we noticed that patients with high TLR8 nuclei percentage had more often multifocal tumors. High TLR8 nuclei percentage was also associated with tumor stage and tumor recurrence. These findings have not been reported before.

The expression of Ki67 antigen is associated with proliferative activity of intrinsic cell populations in malignant tumors (Li et al., 2015). In HCC, expression of Ki67 has been linked to poor survival and tumor node metastasis and tumor

recurrence (Cui et al., 2004; King et al., 1998; Li et al., 2018). In our study, high Ki67 nuclei percentage was associated with poor survival, but the prognostic impact did not remain in multivariable analysis.

P53 acts as a tumor suppressor by initiating cell cycle arrests and apoptosis, and abnormalities in the TP53 gene are commonly observed in cancers (Meng et al., 2014). Activation of the p53 family is a central event in tumor progression, DNA-damage response, chemosensitivity and prognosis in HCC (Kunst et al., 2016). A comprehensive systematic review and meta-analysis showed high p53 association for worse overall survival in HCC patients compared with patients with low/undetectable p53 expression (Liu et al., 2012), which is in line with the current study, as high p53 nuclei percentage was an independent prognostic predictor for poor survival.

The results of this study have clinical and research-related implications. This is the first study to show an association with TLR5 and poor prognosis in HCC. The underlying mechanism is not yet fully understood.

Replication studies are needed to examine the prognostic role of TLR5 and TLR8 in HCC. Optimal cut-offs need to be determined in the future in order to use TLR in daily work. Based on this study, TLR5 is a useful biomarker with good interobserver agreement.

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6.5 Strengths and limitations (studies I-IV)

Some strengths and limitations in studies I-IV must be noted. The strengths of studies I-IV include the homogenous study population from a single geographical area where the diagnosis and treatment occurred in the same hospital without selection bias. All patients were treated in a single center with full access to patient records, so we were not restricted to register data, which is often the case in large-scale studies. Good interobserver repeatability was observed in both surgical resection samples and biopsy samples throughout the studies (II-IV), as well in TLR expression. In Study II, the evaluation technique used in the core biopsy samples was used for the first time. Even though the Cohen's Kappa value was good, no significant differences were observed in the study groups that had only biopsy samples available. This could result from underestimation of tumor budding

and TSR from the biopsy samples, which needs to be confirmed in future studies. The difference in samples was also the reason why survival in the surgical and non-surgical cohorts was not compared.

The limitations in this study include its retrospective nature. Also, a single-institution study causes some limitations due to the relatively small number of patients and wide confidence intervals, especially in the surgical cohort, leading to low power of analysis, particularly in short-term survival, and limiting the generalizability of our results. However, in Study I, our aim was to describe the changes and results of HCC treatment and to compare the number and profile of perioperative complications and long-term survival, for which the number of patients is sufficient. In Study I, many patients possibly eligible for surgery were treated with local ablation or angiological therapy, therefore making the comparison between modalities possible. With complete follow-up information on diagnosis, treatment, complications and long-term survival, we were able to provide reliable comparison of treatment modalities.

Also, the long time period of 35 years (1983–2018) may cause confounding due to improvements in HCC treatment over the years. Nevertheless, this limitation was taken into account by adjusting the multivariate analysis for year of surgery and other confounders (studies I-IV). In the most recent HCC cases in the cohort, 5-year follow-up was not reached, which could cause confounding, although follow-up time was taken into account in statistical analyses. The long time period may also have an impact on the histological samples. For example, the TNM staging system has changed a few times over the years. This limitation was taken into account by re-grading to match the present system.

One limitation may be lack of information in patient documents; because there is no standardized patient inquiry, patients' actual alcohol consumption is probably not reliably registered in the patient records. However, Child-Pugh classification and the data on the presence or absence of cirrhosis were available for most of the patients, and heavy alcohol consumption is thus indirectly taken into account.

Furthermore, in Study II, with the present sample size we were restricted to negative/positive cut-off in tumor budding instead of searching for the optimal cut-off value. According to the regression analysis, mortality risk increases with positive tumor budding, and future studies will need to set an optimal cut-off. However, in clinical practice, the presence/absence of tumor budding is more practical compared to three-tier cut-offs.

One possible limitation of the study may be the heterogeneous staining of the tumor that we were unable to observe using only TMA. Since the technical reason

related to the smaller staining sample could not be excluded, given treatment was adjusted to exclude possible bias in studies III and IV.

One strength of studies III and IV is the strong adjustment in multivariable analysis. In Study III, we were able to perform regression analysis with previously recognized confounding factors in adjusted model 1 where both TLR4 cytoplasm intensity and nuclear percentage were independently prognostic. To include the effect of given treatment, adjusted model 2 was performed. However, treatment strongly overlaps with other covariates such as stage, cirrhosis and Child-Pugh index, but to avoid false positive results, adjusted model 2 was used as the primary analysis despite the possibility of over-adjustment. However, despite strong adjustment, TLR4 cytoplasm expression remained prognostic. As well, in Study IV, to include the effect of given treatment, we included surgery, local ablation, TACE or palliative separately in the adjusted model. Despite the strong overlapping of treatment with other covariates, similarly as in Study III, TLR5 cytoplasm expression remained prognostic.

All the TMAs were created and all the samples were stained in the same laboratory. On the one hand, this is a strength, due to similar handling of all samples and long experience of TLR staining in our center. On the other hand, there is no standardized back-up against errors except recognition by researchers during sample examination. As long as histological samples are examined by humans and not by artificial intelligence, human errors are possible. Nevertheless, this limitation was taken into account with blinding the two independent researchers to the clinical data and outcomes and also by having an expert histopathologist examine the dubious cases.

6.6 Clinical relevance and directions for future research

In studies I-IV, we showed significant observations that may have an impact on future research and clinical practice. In Study I, we showed that the resection rate in our center is relatively low and the trends of treatment in hepatocellular carcinoma have moved towards more conservative treatment therapies. As previously known, surgery is a highly complex procedure with a higher number of complications compared to local ablation. Based on data in Study I, there were no significant differences between the actively treated patient groups in tumor stage and Child-Pugh classification, and despite cirrhosis and physical status, more patients could possibly be treated with surgery. As well, based on this cohort, local ablation and TACE are associated with increased risk for long-term mortality

compared to surgery, and local ablation is related to higher local recurrence, which promotes the role of surgery in the treatment of hepatocellular carcinoma. In Study I, the observation was made that all surgery was done via laparotomy even though the modern guidelines support treatment via laparoscopy. After the completion of Study I, laparoscopy has increased significantly in liver surgery in Oulu University hospital; this will reduce the complications of surgical approach. One of the biggest obstacles in the field of hepatocellular carcinoma is the early detection of the tumor. The implementation of screening programs of liver tumors should be improved. For example, it would be necessary to evaluate the need of screening in cirrhotic patients against HCC in Finland. Currently, educating the public about liver diseases could also have a decreasing impact on the incidence of HCC because most risk factors of HCC are acquired. There is also an obvious need to develop more precise and safe mechanisms to evaluate and to grow the future liver remnant. PVE and ALPPS offer interesting inventions in this field.

In Study II, we showed the association between tumor budding and poor survival. The reason behind this is still unclear. Several studies have suggested that tumor budding is the basis of tumor invasion and metastasis, which is a reasonable thought, because buds are located at the invasive front of the tumor and resemble a micro-metastasis. Tumor budding has been related to epithelial-to-mesenchymal transition (EMT); however, it has also been observed that most EMTs in tumor buds are not complete, giving rise to the notion that buds undergo partial EMT (Grigore et al., 2016). The tumor microenvironment, which is related to EMT, surely has a major role in the migration and adhesion of buds. This theory is supported by the findings that tumor buds have shown disruption of E-cadherin expression, which is a hallmark of EMT, and loss of β-catenin expression at the cell membrane (Zlobec & Lugli, 2018).

In Study II, TSR was not associated with poorer prognosis; however, various studies, including one HCC-study (Lv et al., 2015), have shown opposite results. This might be due to the small sample size of surgically treated patients who had surgical resection samples available, or potentially, due to different etiology, namely alcohol in Finland. However, the mechanism leading to poorer diagnosis of patients with low TSR is not known. Likewise, in tumor budding, the complex interactions in the tumor microenvironment are potentially behind the dismal prognosis. Cancer-associated fibroblasts, which are important in wound repair, tissue maturation, as well as tumor-stroma interactions, may play an important role behind this interaction during tumor development from the preneoplastic stage to advanced stages, especially in damaged liver (Marsh et al., 2013). Even though the

knowledge behind TSR is growing, there is much that is still unknown. It can be suggested that with its complex interactions with various cytokines and growth factors, the tumor microenvironment plays an important role behind TSR.

All in all, in Study II, we showed for the first time in a Western population that tumor budding is a prognostic factor that has great reproducibility also in HCC and can be reliably replicated and routinely analyzed from HE-stained slides without additional immunohistochemistry or costs. According to our study, tumor budding can be used in daily clinical practice, but validation studies are still needed. In the future, tumor budding could potentially be used in the treatment evaluation of HCC. However, to make this possible, more studies are needed to validate tumor budding from core needle biopsies. As well, future studies will need to set an optimal cut-off. More studies are also needed to confirm the prognostic value of TSR in hepatocellular carcinoma.

In Study III, we showed that TLR4 expression is a prognostic factor in HCC with good interobserver agreement. Strong cytoplasmic expression and high nuclei percentage associated with dismal prognosis. High TLR4 nuclei percentage was also associated with higher tumor recurrence.

Lipopolysaccharide (LPS), a ligand of TLR4, initiates MyD88-dependent and -independent pathways resulting in cytokine production. The question is, what turns around the protecting role of TLR4 to a tumor progressive receptor? The continuous liver damage and inflammation may cause mutations that may change the regulation of normally protective cytokines, thus making them tumor progressive. In addition, changes in feedback regulation due to unregulated tissue repair may drive already activated tumorigenesis. Heavy alcohol ingestion or high fat intake may disrupt the intestinal barrier functions, resulting in higher intake of endotoxins, which leads to activation of TLR signaling and the release of various cytokines.

In Study IV we showed for the first time that TLR5 expression is prognostic in hepatocellular carcinoma, with similar point estimate as previously known for p53, with good interobserver agreement. The role of TLR5 in tumor progression seems to be contradictory. In multiple studies, TLR5 has been observed to promote tumor progression and lack of MyD88 or TLR5 activity has enhanced antitumoral effects, whereas in other studies, lack of TLR5 activation has led to opposite responses; the reason behind these findings is speculative. One explanation could be differences in microbiome; for example, in gastric cancer and HCC, *Helicobacter Pylori* and *Fusobacterium Nucleatum* are known tumor promoting bacteria that induce innate immunity responses through TLR5 signaling via NF- κB dependent manner (Kostic

et al., 2013; Smith, 2014), and low TLR5 activation might lead to impaired recognition of these pathogens and promote carcinogenesis. Another explanation could be differences in tolerance and sensitivity to different doses of flagellin of different organs. It has been observed that high doses of flagellin cause liver damage via activation of TLR5, whereas smaller concentrations of flagellin can activate positive and protective effects of TLR5 signaling (Xiao et al., 2015). The accumulation of flagellin in the liver could potentially initiate inflammation and thus cause cancer progression over a long time period via disturbed regulation of inflammatory response through over-activation of TLR5.

The results of Study III suggest that TLR4 is an applicable biomarker in hepatocellular carcinoma, but also that TLR4 could be a useful target for immunotherapy in the future. Several clinical trials of TLR4-agonists are ongoing and nanotechnology might bring light to the treatment of hepatocellular carcinoma in the future (Shetab et al., 2018). TLR5 is also an interesting biomarker that could be used in predicting the outcome in hepatocellular carcinoma and similarly to TLR4, TLR5 could be a future target for immunotherapies (Zheng et al., 2017). However, replication studies are needed to examine the prognostic role of TLR4 and TLR5 in HCC. Optimal cut-offs need to be determined in order to use TLR in daily work.

In the future, tumor budding, TLR4 and TLR5 may be used as valuable prognostic biomarkers in daily practice for tailoring patient treatment. The benefit of these biomarkers is not only limited to predicting the survival of already treated patients from surgical resections, but they could also be used as preoperative markers to determine the intensity of the treatment. Of course, to make this possible, more studies are needed to determine the prognostic significance of tumor budding, TLR4 and TLR5 from core needle biopsies. By means of these biomarkers, patients with tumors that are currently non-resectable but have a benign biomarker panel could be precisely selected for aggressive treatment (liver transplantation, PVE/ALPPS and major liver resection). In practice, only patients within Milan criteria have been eligible for liver transplantation. In the future, biomarker panels may extend the criteria for liver transplantation for selected patients.

7 Summary and conclusions

In the present study, we showed that the surgical resection of hepatocellular carcinoma is the most effective treatment after adjustment for confounding factors. We also confirmed that tumor budding is an independent prognostic factor in hepatocellular carcinoma and that TLR4 and TLR5 are associated with poor prognosis in hepatocellular carcinoma. More specifically, the conclusions were as follows:

1. Surgical resection of hepatocellular carcinoma is the most effective treatment considering long-term survival after adjustment for confounding factors and could be used more in the Oulu University Hospital.
2. There is a significant rise in other treatment modalities than surgery.
3. Tumor budding can potentially be used in daily clinical practice.
4. Tumor budding is an independent prognostic factor in Western patients with surgically treated hepatocellular carcinoma. Also, more studies are needed to confirm the prognostic value of TSR in hepatocellular carcinoma.
5. TLR4 expression is a useful biomarker for poor prognosis in both surgically resected tissue samples and core biopsies with good interobserver agreement.
6. High TLR4 nuclei expression is associated with increased tumor recurrence and local recurrence. TLR2 expression was not associated with survival.
7. TLR5 expression is independently prognostic in hepatocellular carcinoma with similar point estimate as previously known p53. TLR8 expression was not associated with survival.
8. High Ki67 nuclei percentage was associated with poor survival.
9. Replication studies are needed in the future to examine the prognostic role of TLR2, TLR4, TLR5 and TLR8 in hepatocellular carcinoma.
10. Optimal cut-offs need to be determined in the future in order to use TLR in daily work.

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