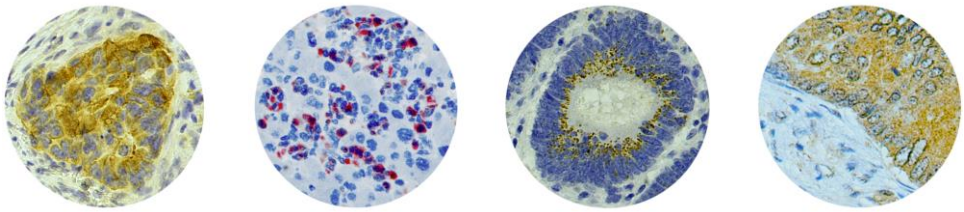
PROGNOSTIC BIOMARKERS IN COLORECTAL CANCER WITH RESECTABLE LIVER METASTASES

REETTA PELTONEN



Helsinki 2020

PROGNOSTIC BIOMARKERS IN COLORECTAL CANCER WITH RESECTABLE LIVER METASTASES

Reetta Peltonen

Transplantation and Liver Surgery, Abdominal Center
University of Helsinki and Helsinki University Hospital
&
Faculty of Medicine, Doctoral Programme in Clinical Research
University of Helsinki
Helsinki, Finland

ACADEMIC DISSERTATION

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Supervisor Professor Helena Isoniemi, M.D., Ph.D.
Transplantation and Liver Surgery, Abdominal Center
University of Helsinki and Helsinki University Hospital
Helsinki, Finland

Reviewers Professor Markus Mäkinen, M.D., Ph.D.
Cancer and Translational Medicine Research Unit
Department of Pathology
University of Oulu and Oulu University Hospital
Oulu, Finland

Docent Marja Hyöty, M.D., Ph.D.
Department of Gastroenterology
Tampere University Hospital
Tampere, Finland

Opponent Docent Arto Rantala, M.D., Ph.D.
Division of Digestive Surgery and Urology
Turku University Hospital and University of Turku
Turku, Finland

Custos Professor Caj Haglund, M.D., Ph.D.
Department of Gastrointestinal Surgery
Translational Cancer Medicine Program
University of Helsinki and Helsinki University Hospital
Helsinki, Finland

Faculty Rep. Professor Ari Ristimäki, M.D., Ph.D.
Department of Pathology and ATG Research Program
HUSLAB, HUS Diagnostic Center
University of Helsinki and Helsinki University Hospital
Helsinki, Finland

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Numbers don’t lie, but neither do they tell the whole story.

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Peltonen R, Österlund P, Lempinen M, Nordin A, Stenman UH, and Isoniemi H. Postoperative CEA is a better prognostic marker than CA19-9, hCGβ or TATI after resection of colorectal liver metastases.
Tumor Biology 2018 Jan;40(1):1010428317752944.
DOI: 10.1177/1010428317752944.

II Peltonen R*, Gramkow MH*, Dehlendorff C, Osterlund PJ**, Johansen JS**, and Isoniemi H**. Elevated serum YKL-40, IL-6, CRP, CEA, and CA19-9 combined as a prognostic biomarker panel after resection of colorectal liver metastases.
PLOS ONE 2020 Aug 5;15(8):e0236569. DOI: 10.1371/journal.pone.0236569.

III Peltonen R, Hagström J, Tervahartiala T, Sorsa T, Haglund C, and Isoniemi H. High expression of MMP-9 in primary tumors and preoperatively elevated MPO in serum predict prognosis in colorectal cancer with operable liver metastases.
Oncology 2020 Oct 7:1-17. DOI: 10.1159/000510609. Online ahead of print.

IV Peltonen R*, Ahopelto K*, Hagström J, Böckelman C**, Haglund C**, and Isoniemi H**. High TKTL1 expression as a sign of poor prognosis in colorectal cancer with synchronous rather than metachronous liver metastases.
Cancer Biology & Therapy 2020 Sep 1;21(9):826-831.
DOI: 10.1080/15384047.2020.1803008. Epub 2020 Aug 14.

* These authors contributed equally to the study.
** These senior authors contributed equally to the study.

Publication IV will also be included in the academic dissertation of Kaisa Ahopelto.

Some previously unpublished data are also presented.

ABSTRACT

Background and aims

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide. In Finland, 3,538 new cases were diagnosed in 2018. While the incidence of CRC is generally increasing, the mortality rates have been decreasing in many countries due to reduced risk factors, screening, and advances in pathological diagnostics, surgical techniques, and oncological treatments. Approximately half of all CRC patients develop metastatic disease, and up to 75% of the metastases are diagnosed in the liver. Contrary to many other cancers, even metastatic CRC may be treated curatively, if the metastases are limited and can be surgically removed. Currently, approximately 20–30% of the liver metastases can be resected, but over 50% of the patients develop recurrent disease afterwards. Estimating the prognosis after liver resection is of utmost importance, as identifying the patients with a high risk of recurrence enables adjusting the surgical and oncological treatments accordingly, and thus, improving postoperative survival.

The aim of this thesis was to evaluate the prognostic significance of 12 biomarkers measured in serum, plasma, and tissue samples of both the primary colorectal tumors and the liver metastases in patients undergoing curative-intent liver resection for colorectal metastases.

Materials and methods

Altogether 442 patients who underwent liver resection for colorectal metastases at the Helsinki University Hospital between the years 1998 and 2013 were included in this thesis. The four studies are based on the serum samples from all patients (I–III), the plasma samples from a subset of 168 patients (I), and the tumor tissue specimens from a subset of 111 patients who had both primary colorectal tumors and liver metastases operated on at the Helsinki and Uusimaa Hospital District (III and IV). Serum and plasma samples were drawn before liver resection and approximately 3 months afterwards. Tissue specimens included samples of both the primary colorectal tumors and the liver metastases.

The concentrations of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and C-reactive protein (CRP) were retrieved from clinical records (I and II). Those of human chorionic gonadotropin β (hCGβ) in plasma, tumor-associated trypsin inhibitor (TATI) in plasma, and matrix metalloproteinase-8 (MMP-8) in serum were measured by time-resolved immunofluorometric assay (IFMA) methods (I and III). YKL-40 (chitinase-3-like protein-1, CHI3L1), interleukin-6 (IL-6), matrix metalloproteinase-9 (MMP-9), and myeloperoxidase (MPO) were determined in the serum samples using commercially available enzyme-linked immunosorbent assay (ELISA) kits (II and III). The expressions of matrix metalloproteinase-2 (MMP-2), MMP-8, MMP-9, and transketolase-like protein 1 (TKTL1) were analyzed in the immunohistochemically stained tumor tissue samples. Clinical data were retrieved from patient records, and information about the dates of death was obtained from the Central Statistical Office of Finland. Survival analyses were performed using the Kaplan-Meier method and the Cox proportional hazards model.

Results

Postoperatively elevated CEA (>5.0 µg/l) was found to predict shorter disease-free/relapse-free survival (DFS/RFS) and overall survival (OS). Preoperatively elevated CEA associated only with shorter OS (I and II). Pre- and postoperatively elevated CA19-9 (>26 or >37 kU/l) indicated shorter DFS/RFS and OS, but postoperatively the additional value compared to CEA was limited (I and II).
Preoperatively elevated TATI (>13 µg/l) associated with poor 3-year DFS after liver resection in the whole patient cohort, and especially in patients with synchronous liver metastases. Postoperatively elevated hCGβ (>1.0 pmol/l) associated with poor 3-year OS in the whole cohort, and it was a sign of impaired prognosis especially among male patients and those with primary rectal tumors (I).
A biomarker panel comprising YKL-40, IL-6, CRP, CEA, and CA19-9 was found prognostic, as patients with 2–5 elevated biomarkers pre- or postoperatively were at an increased risk of recurrence and death after liver resection (II).
High expression of MMP-9 in primary colorectal tumors and high preoperative MPO in serum indicated improved prognosis after liver resection. Additionally, the prognostic significance of these biomarkers, as well as that of MMP-2 and MMP-8, were found to depend on the clinical characteristics of the patients (III).
High TKTL1 expression in the primary colorectal tumors associated with impaired prognosis after liver resection in patients with synchronous liver metastases, but with improved prognosis in those with metachronous metastases. Similar tendencies were observed concerning the expression in the liver metastases (IV).

Conclusions

In conclusion, CEA is a useful prognostic biomarker for most patients undergoing liver resection for colorectal metastases. However, about half of the patients do not have elevated serum levels of CEA despite of metastatic disease, and they may benefit from measuring other biomarkers. A biomarker panel comprising YKL-40, IL-6, CRP, CEA, and CA19-9 could be used to identify patients at a high risk of recurrence after liver resection already before the operation. Especially preoperative CA19-9 and YKL-40 also had independent prognostic value.
MMP-9 and TKTL1 in primary colorectal tumors may serve for assessing whether the patients benefit from liver resection or need more aggressive chemotherapy, but the synchronicity of the liver metastases should be taken into consideration. Elevated preoperative serum levels of MPO indicated improved prognosis, and low levels implied a high risk of recurrence. The prognostic value of MMP-2, MMP-8, MMP-9, and MPO varied according to clinical factors, possibly due to immunological or hormonal mechanisms.
The investigated biomarkers provide new information about CRC with liver metastases and increase our understanding of the disease. They help us define the prognosis after liver resection and adjust the individual patients' treatment accordingly. Thus, they contribute to enabling the best possible care.

TIIVISTELMÄ

Tausta ja tavoitteet

Paksu- ja peräsuolisyöpiä (kolorektaalisyöpiä) todetaan kolmanneksi eniten kaikista maailman syövistä. Suomessa uusia tautitapauksia todettiin vuonna 2018 yhteensä 3 538. Kolorektaalisyövän ilmaantuvuus on laajalti lisääntynyt, mutta toisaalta kuolleisuus on vähentynyt, minkä katsotaan johtuvan riskitekijöiden vähenemisestä, seulonnasta sekä patologisen diagnostiikan, kirurgisten menetelmien ja onkologisten hoitomahdollisuuksien kehittymisestä. Noin puolelle kolorektaalisyöpäpotilaista ilmaantuu etäpesäkkeitä (metastaaseja), joista jopa 75 % todetaan maksassa. Toisin kuin monissa muissa syövissä, kolorektaalisyövässä myös levinneestä taudista parantuminen on mahdollista, mikäli metastaaseja on rajallinen määrä ja ne on mahdollista poistaa kirurgisesti. Nykyisin jopa 20–30 % maksametastaaseista onnistutaan leikkaamaan (resekoimaan), mutta yli 50 %:lla potilaista tauti uusii myöhemmin. Leikkauksen jälkeisen ennusteen arvioiminen on huomattavan tärkeää, sillä tunnistamalla potilaat, joilla uusimisriski on suuri, kirurginen ja onkologinen hoito voidaan suunnitella yksilöllisesti ja siten parantaa ennustetta.
Tämän väitöskirjan tavoitteena oli arvioida 12:n merkkiaineen ennusteellista arvoa potilailla, joille oli tehty maksaresektio parantavalla hoitotavoitteella. Merkkiaineita tutkittiin seerumista, plasmasta sekä primääreistä suolikasvaimista ja maksametastaaseista otetuista kudosnäytteistä.

Aineisto ja menetelmät

Tämän väitöskirjan aineistoon kuuluu yhteensä 442 potilasta, joilta leikattiin kolorektaalisyövän maksametastaasit Helsingin yliopistollisessa keskussairaalassa vuosina 1998–2013. Kirjan neljän osatyön pohja-aineistona olivat seeruminäytteet, jotka otettiin kaikilta potilailta (I–III), plasmanäytteet 168:n potilaan alaryhmältä (I) sekä kasvainkudosnäytteet 111:ltä potilaalta, joilta oli leikattu sekä primääri suolikasvain että maksametastaasit Helsingin ja Uudenmaan sairaanhoitopiirissä (III ja IV). Seerumi- ja plasmanäytteet otettiin ennen maksaresektiota (preoperatiivisesti) ja noin kolme kuukautta sen jälkeen (postoperatiivisesti). Kudosnäytteet otettiin sekä suolikasvaimista että maksametastaaseista.
Tiedot karsinoembryonaalisen antigeenin (CEA), karbohydraattiantigeeni 19-9:n (CA19-9) ja C-reaktiivisen proteiinin (CRP) pitoisuuksista haettiin potilastietojärjestelmistä (I ja II). Plasman koriongonadotropiinin β-alayksikön (hCGβ) ja tuumoriin liittyvän trypsiinin estäjän (TATI) sekä seerumin matriksin metalloproteinaasin 8 (MMP-8) määritykset tehtiin käyttäen immunofluorometrisiä menetelmiä (IFMA) (I ja III). YKL-40:n (kitinaasi-3:n kaltainen proteiini 1, CHI3L1), interleukiini 6:n (IL-6), matriksin metalloproteinaasin 9 (MMP-9) ja myeloperoksidaasin (MPO) pitoisuudet määritettiin seeruminäytteistä entsyymivälitteisellä immunosorbenttimenetelmällä (ELISA) (II ja III). Matriksin metalloproteinaasin 2 (MMP-2), MMP-8:n, MMP-9:n ja transketolaasin kaltaisen proteiinin 1 (TKTL1) ilmentyminen analysoitiin kasvainkudosnäytteistä (III ja IV). Kliiniset tiedot haettiin potilastietojärjestelmistä ja tiedot kuolinpäivistä Tilastokeskukselta. Elossaoloanalyysit tehtiin käyttäen Kaplan–Meier-menetelmää ja Coxin regressiomallia.

Tulokset

Maksaresektion jälkeen suurentunut CEA-pitoisuus (> 5,0 µg/l) ennusti lyhentyneitä tautivapaata ja kokonaiselossaoloaikaa sekä leikkausta edeltävästi suurentunut CEA lyhentynyttä kokonaiselossaoloaikaa (I ja II). Resektiota ennen tai sen jälkeen suurentuneet CA19-9-pitoisuudet (> 26 tai > 37 kU/l) yhdistyivät lyhentyneisiin tautivapaaseen ja kokonaiselossaoloaikaan, mutta postoperatiivisesti CA19-9:n lisäarvo suhteessa CEA:han oli varsin vähäinen (I ja II).
Preoperatiivisesti suurentunut TATI (> 13 µg/l) yhdistyi huonontuneeseen tautivapaan elossaoloajan ennusteeseen kaikilla potilailla ja erityisesti niillä, joiden maksametastaasit todettiin enintään kuusi kuukautta suolileikkauksen jälkeen (synkroniset maksametastaasit). Postoperatiivisesti suurentunut hCGβ (> 1,0 pmol/l) ennusti huonontunutta kokonaiselossaoloaikaa kolmen vuoden aikana maksaresektion jälkeen kaikilla potilailla, ja lisäksi se oli huonon ennusteen merkki varsinkin miespotilailla ja peräsuolisyövässä (I).
YKL-40:n, IL-6:n, CRP:n, CEA:n ja CA19-9:n osalta todettiin, että taudin uusimisen ja kuoleman riski oli selvästi lisääntynyt potilailla, joilla kyseisistä merkkiaineista vähintään kahden pitoisuudet olivat suurentuneet pre- tai postoperatiivisesti (II).
MMP-9:n voimakas ilmentyminen suolikasvaimissa ja suuri MPO-pitoisuus seerumissa yhdistyivät hyvään ennusteeseen maksaresektion jälkeen. Lisäksi havaittiin, että näiden merkkiaineiden sekä MMP-2:n ja MMP-8:n ennusteelliseen arvoon vaikuttivat potilaiden kliiniset ominaisuudet ja taudinkuva (III).
TKTL1:n voimakas ilmentyminen suolikasvaimissa ja maksametastaaseissa yhdistyi huonontuneeseen ennusteeseen potilailla, joilla oli synkroniset maksametastaasit. Sen sijaan potilailla, joilla oli myöhemmin ilmaantuneet (metakroniset) metastaasit, voimakas ilmentyminen oli paremman ennusteen merkki (IV).

Yhteenveto

Yhteenvetona CEA:n todettiin olevan käyttökelpoinen merkkiaine suurella osalla potilaista, joilta leikataan kolorektaalisyövän maksametastaasit. Noin puolella potilaista kyseisen merkkiaineen pitoisuudet eivät kuitenkaan suurene edes levinneessä taudissa, ja heidän kohdallaan olisi hyvä harkita muiden merkkiaineiden käyttämistä. YKL-40:n, IL-6:n, CRP:n, CEA:n ja CA19-9:n muodostaman merkkiainepaneelin avulla voitaisiin tunnistaa jo ennen maksaresektiota potilaat, joilla taudin uusimisriski on lisääntynyt. Leikkausta edeltävästi varsinkin CA19-9 ja YKL-40 olivat ennusteellisia myös itsenäisesti.
Suolikasvainten MMP-9- ja TKTL1-ilmentymistä voitaisiin käyttää sen arvioimiseen, hyötyvätkö potilaat enemmän maksaresektiosta vai tehokkaammasta onkologisesta hoidosta. Tähän vaikuttaa maksametastaasien ilmaantumisaika. Seerumin suuren MPO-pitoisuuden havaittiin olevan hyvän ennusteen ja vähäisen pitoisuuden vastaavasti huonon ennusteen merkki. MMP-2:n, MMP-8:n, MMP-9:n ja MPO:n ennusteellinen arvo vaihteli potilaiden eri alaryhmissä, mikä saattaa liittyä immunologisiin ja hormonaalisiin säätelymekanismeihin.
Tutkitut merkkiaineet antavat uutta tietoa maksaan levinneestä kolorektaalisyövästä ja lisäävät ymmärrystämme kyseisestä taudista. Niiden avulla ennustetta maksaresektion jälkeen voidaan arvioida aiempaa paremmin, ja yksittäisten potilaiden hoitoa voidaan muokata sen mukaisesti. Täten nämä merkkiaineet edesauttavat parhaan mahdollisen hoidon toteutumista.

ABBREVIATIONS

5-FU	5-fluorouracil
18qLOH	Chromosome 18q loss of heterozygosity
AFAP	Attenuated familial adenomatous polyposis
ALPPS	Associating Liver Partition and Portal vein ligation for Staged hepatectomy
APC	Adenomatous Polyposis Coli (gene)
BRAF	Murine sarcoma viral (v-raf) oncogene homolog B1, B-Raf proto-oncogene
CA19-9	Carbohydrate antigen 19-9
CEA	Carcinoembryonic antigen
cfDNA	Cell-free DNA
CGI	CpG island
CHI3L1	Chitinase-like protein-1 (YKL-40)
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CME	Complete mesocolic excision
CRC	Colorectal cancer
CRM	Circumferential resection margin
CRP	C-reactive protein
CT	Computed tomography
CTC	Circulating tumor cell
ctDNA	Circulating tumor DNA (tumor-derived fragmented DNA)
CV	Coefficient of variation
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
DTPA	Diethylenetriaminepentaacetic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ENV	EnVision (DAKO Real EnVision / Real EnVision Flex)
FAP	Familial adenomatous polyposis
FCCTX	Familial colorectal cancer type X
FLR	Future liver remnant
FSH	Follicle-stimulating hormone
GTPase	Guanosine triphosphatase
HCC	Hepatocellular cancer
hCG	Human chorionic gonadotropin
hCGβ	Free β subunit of human chorionic gonadotropin
HCl	Hydrochloric acid
HE	Hematoxylin-eosin
HNPCC	Hereditary non-polyposis colorectal cancer
HOCl	Hypochlorous acid
HRP	Horseradish peroxidase
hsCRP	high-sensitivity CRP
IEC	Intestinal epithelial cell
IFMA	Immunofluorometric assay
IL-6	Interleukin-6
IQR	Interquartile range

JPS	Juvenile polyposis syndrome
HRAS	Harvey rat sarcoma viral oncogene
KRAS	Kirsten rat sarcoma viral oncogene
LH	Luteinizing hormone
LOH	Loss of heterozygosity
LS	Lynch syndrome
MAC	Mucinous adenocarcinoma
MAP	*MutYH*-associated polyposis
MAPK	Mitogen-activated protein kinase
MSI	Microsatellite instability
MDT	Multidisciplinary team
MMP-2	Matrix metalloproteinase-2 (gelatinase-A)
MMP-8	Matrix metalloproteinase-8 (collagenase-2, neutrophil collagenase)
MMP-9	Matrix metalloproteinase-9 (gelatinase-B)
MMR	Mismatch repair
MPO	Myeloperoxidase
MRF	Mesorectal fascia
MRI	Magnetic resonance imaging
NRAS	Neuroblastoma *RAS* viral oncogene homolog
NAP	*NTHL1*-associated polyposis
OS	Overall survival
PD1	Programmed cell death 1
PET	Positron emission tomography
PHTS	PTEN hamartoma tumor syndrome
PJS	Peutz-Jeghers syndrome
PlGF	Placental growth factor
PPP	Pentose phosphate pathway
PSTI	Pancreatic secretory trypsin-inhibitor (SPINK1, TATI)
RAGE	Receptor for advanced glycation end products
RAS	Rat sarcoma (referring to the *RAS* gene family)
RFA	Radiofrequency ablation
RFS	Relapse-free survival
RNA	Ribonucleic acid
ROC	Receiver-operating characteristic
SBRT	Stereotactic body radiotherapy
SI-CLP	Stabilin-1 interacting chitinase-like protein
SIRT	Selective internal radiation therapy
SPINK1	Serine peptidase inhibitor Kazal type 1 (PSTI, TATI)
SSL	Sessile serrated lesion
TACE	Transarterial chemoembolisation
TATI	Tumor-associated trypsin inhibitor (PSTI, SPINK1)
TGFβ	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinases
TKTL1	Transketolase-like protein 1
TMA	Tissue microarray
TME	Total mesorectal excision
TNM	Tumor Node Metastasis (classification of malignant tumors)
TSH^1	Two-stage hepatectomy
TSH^2	Thyroid-stimulating hormone
VEGF	Vascular endothelial growth factor
YKL-40	Chitinase-3-like protein-1 (CHI3L1)

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy in the world and the second-leading cause of cancer death, accounting for over 800,000 deaths every year (Bray et al., 2018). The incidence of CRC has been increasing over the last decades, which is linked to the rapid adoption of the Western lifestyle around the world. This includes changes in diet, obesity, and lack of physical activity, which all increase the risk of cancer. On the other hand, the treatment of CRC has improved substantially, partly due to significant research findings concerning the development and characteristics of the disease. Advances in screening, diagnostics, surgical techniques, and individualized oncological treatment options have led to an improved prognosis in the affected patients, also in metastatic stage IV cancer.

It has been estimated that approximately half of all CRC patients develop metastases, the majority of which are diagnosed in the liver (up to 75%) and the lungs (up to 30%) (Sorbye et al., 2007). Contradictory to several other cancers, the isolated metastases of CRC can nowadays quite often be operated on and even cure attained. As the liver is the most common metastatic site, interest is focused on the liver resections and their results. It has become obvious that patient selection according to specified characteristics is a major determiner of the outcome after resection. Despite of the advances in surgical techniques and more efficient perioperative chemotherapy, about half of the patients experience recurrence postoperatively. Thus, identifying the patients at a high risk of recurrence after liver resection has become a cornerstone of all treatment planning.

Several prognostic biomarkers have been studied over the years including antigens, enzymes, and hormones produced or induced by the tumor tissue. It is thought that the occurrence of these biomarkers in the circulation signals existing cancer that is not detectable by other means but may generate recurrent disease at a later time, and their expressions in the tumor tissue may reveal crucial information about the cancer's invasive and metastatic potential.

Currently, knowledge of the characteristics of the cancer itself and the cell-level mechanisms that enable tumor growth, invasion, and metastasis is continuously increasing. Six hallmarks of cancer have been identified: sustaining proliferative signaling; evading growth suppressors; activating invasion and metastasis; enabling replicative immortality; inducing angiogenesis; and resisting cell death (Hanahan and Weinberg, 2011). Inflammation creates a cellular environment that favors tumor invasion and metastasis through various mechanisms. Chronic inflammation has been linked to impaired prognosis in several malignancies including CRC (Nasr et al., 2018), and thus, it has been suggested that detecting

underlying inflammation in patients with colorectal liver metastases might help estimate the course of the disease.

Yet another aspect are the cell-level mechanisms contributing to the invasive ability of the cancer. Detecting tumor-related factors that signal more aggressive behavior would help adjust the patients' treatment accordingly. These factors include proteins that contribute to the metabolism of the cancer cells or their invasive capabilities, and hence, enable aggressive growth.

In this thesis, altogether 12 potentially prognostic biomarkers were analyzed in the serum, plasma, and tumor tissue of patients undergoing liver resection for colorectal metastases. The associations of the biomarkers with disease-free (DFS) or relapse-free survival (RFS) and overall survival (OS) after liver resection were studied in order to identify the patients who are at a high risk of recurrence or death. Important new information was also obtained concerning the concentrations and expressions of these biomarkers in CRC with liver metastases, and this increases knowledge of the characteristics of the disease. Estimating the risk of recurrence after liver resection will help adjust the oncological treatments individually and choose the optimal time point for the surgical interventions. In addition, when the characteristics of the cancer and the estimated course of the disease can be evaluated, the optimal treatment options may be identified and thus, the best possible care ensured for each patient.

REVIEW OF THE LITERATURE

1. EPIDEMIOLOGY

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy in the whole world (Torre et al., 2015) (**Table 1**). The deaths caused by CRC have been increasing during the last years: in 2012, it was fourth most common cause of cancer death worldwide, and in 2018, already the second (Bray et al., 2018). The new cases and deaths are expected to increase by as much as 60% by the year 2030, raising the global burden of this disease significantly. In Finland, CRC was the third most commonly diagnosed cancer and the second leading cause of cancer death in 2018, and its incidence has been increasing in accordance with the global trends: in 2004–2008, the incidence was 48.0/100,000/year, but in 2014–2018, already 60.9/100,000 (Syöpärekisteri, 2018) (Finnish Cancer Registry).

The incidence and mortality of CRC are strongly linked to the socioeconomic status of different countries and areas around the world. CRC was earlier considered a disease of the developed countries, but in the last years, the rates have been rapidly increasing especially in the less developed countries (Center et al., 2009, Arnold et al., 2017). These recent changes are supposedly due to the adoption of the Western lifestyle, as it brings along the generally known risk factors for CRC including obesity, low-fiber diet, physical inactivity, and smoking (Giovannucci, 2002, Botteri et al., 2008). On the other hand, in several developed countries, stabilizing or even declining trends in incidence and mortality have been reported (Torre et al., 2015). These declining trends presumably reflect the progress in early detection and prevention of CRC as well as the advances in oncological and surgical treatments.

2. ETIOLOGY

2.1 General risk factors

Colorectal cancer develops as a result of genetic, environmental, and lifestyle factors. Commonly known risk factors are physical inactivity, obesity, excess intake of red meat, low-fiber diet, smoking, and high consumption of alcohol (Giovannucci, 2002, Baena and Salinas, 2015, Cho et al., 2019).

Chronic inflammation has been identified as a significant cancer-promoting factor (Hanahan and Weinberg, 2011, Nasr et al., 2018). In the intestine, changes in the microbiome are suggested to influence the development of CRC through enhancing the release of bacterial toxins, the disruption of epithelial barrier, and the production of carcinogenic metabolites

(Lucas et al., 2017). Considering this, it is understandable that nutrition affects the risk of CRC by bringing along bacteria and other compounds into the intestine. The relationship of inflammation and cancer is, however, controversial: while the bacterial microbiota induces cancer-promoting low-grade inflammation, the innate immune activation is needed for anti-cancer immune responses (Schwabe and Jobin, 2013).

Table 1. The 5 most commonly diagnosed malignancies and the 5 leading causes of cancer death in the world and in Finland. Global estimates and Finnish statistics for the year 2018. Non-melanoma skin cancer excluded.

Cancer	**Total (N)**	**Males (n)**	**Females (n)**
Cancer incidence			
World	**18,078,957**	**9,456,418**	**8,622,539**
Lung	2,093,876	1,368,524	725,352
Breast	2,088,849	(not reported)	2,088,849
Colorectal	1,800,977	1,006,019	794,958
Prostate	1,276,106	1,276,106	-
Stomach	1,033,701	683,754	349,947
Finland	**34,372**	**17,836**	**16,536**
Prostate	5,016	5,016	-
Breast	4,934	33	4,967
Colorectal	3,538	1,865	1,673
Lung and trachea	2,745	1,710	1,035
Mature B-cell lymphomas	1,964	1,083	881
Cancer mortality			
World	**9,555,027**	**5,385,640**	**4,169,387**
Lung	1,761,007	1,184,947	576,060
Colorectal	861,663	474,606	387,057
Stomach	782,685	513,555	269,130
Liver	781,631	548,375	233,256
Breast	626,679	(not reported)	626,679
Finland	**12,730**	**6,803**	**5,927**
Lung and trachea	2,285	1,473	812
Colorectal	1,293	682	611
Pancreatic	1,268	633	635
Prostate	914	914	-
Breast	878	5	873

Adapted from (Bray et al., 2018, Pitkäniemi et al., 2020).

2.2 Hereditary colorectal cancer

Approximately 10% of colorectal cancers develop due to inherited mutations in cancer-predisposing genes (Lucas et al., 2017). The most common of these conditions is the Lynch syndrome (LS), earlier called hereditary non-polyposis colorectal cancer (HNPCC), which causes approximately 2–5% of all diagnosed CRCs (Patel and Ahnen, 2012, Mecklin, 1989). The prevalence of LS has been shown to be approximately 0.2% in Finland (Mecklin, 1987), 0.4% (1:226) in Iceland (Haraldsdottir et al., 2017), and in the United States, the estimated population incidence is 1:370 (Hampel et al., 2005).
LS is primarily caused by mutations in four genes of the DNA mismatch repair (MMR) system (*MLH1*, *MSH2*, *MSH6*, and *PMS2*), and it is inherited in an autosomal dominant pattern (Mecklin et al., 1995, Boland et al., 2018). However, LS may also be caused by mutations that affect a MMR gene, that is, those in adjacent genes or in the DNA methylation system (Peltomäki, 2016, Morak et al., 2011). The patients with LS have a 50–80% lifetime risk of developing CRC (Patel and Ahnen, 2012) depending on the precise mutational status. In addition, they have an increased risk of endometrial and ovarian cancer.

The second most common inherited syndrome is familial adenomatous polyposis (FAP), which lies behind approximately 1% of all CRCs. It is a rare condition with an estimated prevalence of 1–9/100,000. It is caused by mutations in the Adenomatous Polyposis Coli (*APC*) gene, and the inheritance pattern is autosomal dominant. Typically, patients with FAP develop hundreds of thousands of colonic adenomas already in adolescence, which then develop into cancer at an average age of 39 years. The lifetime risk for CRC is practically 100%, and the only effective way of preventing CRC is surgical colectomy (Patel and Ahnen, 2012). However, also extracolonic manifestations of the condition increase mortality. Attenuated FAP (AFAP) is a less severe form of FAP inherited in a similar manner.
In addition, there are other hereditary polyposis syndromes that are inherited in an autosomal recessive pattern. These include *MutYH*-associated polyposis (MAP), *NTHL1*-associated (NAP), and *MSH3*-associated polyposes (Terradas et al., 2019). Other syndromes include Peutz-Jeghers syndrome (PJS), juvenile polyposis syndrome (JPS), familial colorectal cancer type X (FCCTX), and PTEN hamartoma tumor syndrome (PHTS) (Patel and Ahnen, 2012, Boland et al., 2018).

3. PATHOLOGY

3.1 Development of colorectal cancer

The development of colorectal cancer occurs within the intestinal mucosa either via a conventional adenoma–carcinoma sequence or a serrated pathway (Pino and Chung, 2010, Mäkinen, 2007). The development begins with the transformation of the normal epithelial cells of the mucosa into hyperproliferative intestinal epithelial cells (IECs), which form pedunculated or serrated polyps (Simon, 2016). The former are so-called traditional adenomas, and the latter can be further divided into hyperplastic polyps, sessile serrated lesions (SSLs), and traditional serrated adenomas/polyps (Pai et al., 2019, Mäkinen et al., 2001, De Palma et al., 2019). CRCs originating from serrated lesions form a distinct subtype of the disease and constitute at least 15% of all cases.
The polyps themselves are benign, but as the IECs proliferate, they accumulate genetic mutations and, with time, acquire the ability to invade the submucosa. This series of events gradually leads to disseminating malignant tumors, in which cells are able to grow and divide without limitations (Lucas et al., 2017). The process usually takes years, but cancer can also develop in a significantly shorter time, especially in predisposing conditions such as hereditary polyposis syndromes.

The malignant transformation of the polyps requires at least seven distinct mutations in different genes (Fearon and Vogelstein, 1990). These include those in the *APC* gene, the *KRAS* gene, transforming growth factor β (*TGF-β*), *PIK3CA*, and *TP53* genes. It has been estimated that an average colorectal cancer comprises up to 80 mutations (Wood et al., 2007). Several proteins, metabolites, toxins, and other carcinogenic compounds can contribute to these or affect the normal cell cycle in other ways. In hereditary CRC, the predisposing mutations that inexorably lead to cancer are inherited.
Cells must have a certain degree of intrinsic genomic instability before they can acquire sufficiently of mutations to develop into cancer cells. In sporadic CRC, three major types of genetic instability pathways have been described: chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathway. These three pathways are not exclusive, and a tumor can exhibit overlapping features (Pino and Chung, 2010).

The CIN pathway accounts for an estimated 65–70% of the sporadic CRCs (Pino and Chung, 2010). In CIN-positive tumors, whole chromosomes or large portions of them are gained or lost at an accelerated rate, which leads to an imbalance in the chromosome number, genomic amplifications, and a high frequency of loss of heterozygosity (LOH) in the cells. These features are associated with the accumulation of mutations in tumor suppressor genes and oncogenes, which activate the pathways leading to the development of cancer.

MSI accounts for the development of approximately 15–20% of sporadic CRCs (Hu et al., 2020). Microsatellites are short, repetitive DNA sequences located in both the coding and non-coding regions of the human genome. These repetitive sequences are prone to replication errors, which are normally repaired by mismatch repair (MMR) genes. Deficiencies in the MMR system lead to increased additional mutations and a mutator phenotype, which is known as microsatellite instability (MSI). MSI is usually caused by methylation of the promoter region of the *MLH1* gene or, in 2–3% of CRCs, mutations in the MMR system genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* (Lee and Chu, 2018, Muzny et al., 2012).

CpG islands (CGIs) are short DNA sequences rich in the CpG dinucleotide (cytosine followed by guanine) and can be found in approximately half of all human genes. Aberrant methylation of cytosine within the CGIs causes loss of gene expression, including inactivation of the *MLH1* gene, which is the primary cause of MSI (Kane et al., 1997, Toyota et al., 1999). Thus, hypermethylation of the CGIs and MSI often occur concurrently. The CGI methylator phenotype is also closely associated with *BRAF* mutations (Weisenberger et al., 2006).

3.2 Histological subtypes

Of all diagnosed CRCs, the majority are adenocarcinomas, 10–15% serrated adenocarcinomas, 10–15% mucinous adenocarcinomas (MACs), and approximately 1% signet ring cell carcinomas and 1% medullary carcinomas (Hugen et al., 2014, Nitsche et al., 2013, Mäkinen, 2007). MAC is characterized by abundant extracellular mucin, which covers at least 50% of the tumor volume (Luo et al., 2019), and signet ring cell carcinoma is a MAC with more than 50% of a signet ring cell component (Song et al., 2019). The prognosis of MAC compared to adenocarcinoma is debatable, but signet ring cell carcinoma has been associated with worse clinical outcome (Song et al., 2019). In addition to these subtypes, less common forms of CRC exist, such as small cell carcinoma, squamous cell carcinoma, adenosquamous carcinoma, and undifferentiated carcinoma (WHO, 2019).

3.3 Differences between proximal and distal colon

Over the last years, several studies have shown that the tumors of the proximal (right) and distal (left) colon differ from each other in terms of biological and molecular characteristics. During the embryonic development, the proximal colon is derived from the midgut and the distal colorectum from the hindgut. These parts are joined together at the distal third of the transverse colon, and they have separate blood supplies, innervations, and lymphatic drainages (Boeckx et al., 2017, Lee et al., 2015, Missiaglia et al., 2014). Right-sided tumors are less frequent and more commonly diagnosed in females. They are typically serrated

adenocarcinomas, *BRAF*-mutated, microsatellite unstable, and of CGI methylator phenotype (Yamauchi et al., 2012). In addition, they are typically diagnosed at a later stage, associate with worse prognosis than left-sided tumors, and are insensitive to anti-epidermal growth factor receptor (EGFR) therapies (Tejpar et al., 2017).

3.4 Invasion and metastasis

The malignant colorectal tumors first invade through the layers of the intestinal wall into pericolic or perirectal tissues, and then further into the visceral peritoneum and adjacent tissues and organs (**Figure 1**). Submucosal lymphatic invasion has been shown to be the strongest predictor of lymph node metastasis (Ishii et al., 2009, Wada et al., 2015), while venous invasion, in particular, predicts distant metastasis (Suzuki et al., 2009). In addition, perineural invasion (Liebig et al., 2009) and tumor budding – the presence of single tumor cells or clusters of ≤4 tumor cells at the invasive margin of the tumor (Lugli et al., 2017) – indicate metastasis and impaired survival (Brockmoeller and West, 2019, Suzuki et al., 2009).

There are several factors that affect the tumor cells' ability to invade the adjacent tissues and to metastasize. In the last couple of decades, the role of tumor-associated inflammation has become more and more obvious. The infiltration of inflammatory cells has been found to promote tumor progression, as they contribute to the production of bioactive molecules that facilitate angiogenesis, invasion, and metastasis (Hanahan and Weinberg, 2011). Both tumor-antagonizing and tumor-promoting inflammatory cells can be found in basically all neoplastic lesions. However, the actions of those that are tumor-suppressing (cytotoxic) are typically inhibited by other immunosuppressive cells or by other mechanisms (Mougiakakos et al., 2010, Ostrand-Rosenberg and Sinha, 2009).

CRC metastasizes usually first to the liver and the lungs, which is most probably due to the venous circulation from the intestine to the liver via the portal vein and further into the lungs.

4. DIAGNOSIS AND STAGING

4.1 Symptoms

Approximately two-thirds of patients diagnosed with CRC have at least one abnormal symptom within the months before diagnosis (Cleary et al., 2007). The symptoms develop gradually, which typically leads to delayed examinations and diagnosis. The typical symptoms include rectal bleeding, diarrhea or constipation, abdominal pain, and loss of weight (Hamilton et al., 2005). Rectal bleeding may cause anemia and, subsequently, fatigue and cardiac symptoms. If the disease has metastasized, more distinctive symptoms may arise, such as loss of appetite, fever, and symptoms from the specific metastatic sites. However, even metastatic disease can be diagnosed by coincidence with no previous symptoms, or the symptoms can be inconspicuous for months.

4.2 Diagnosis and preoperative screening

Once CRC is suspected, the patient is usually directed to colonoscopy. In case a malignant tumor is found, biopsies from the tumor tissue can be taken directly for further immunohistochemical and genetic analyses. Another widely-used method is detecting hemoglobin in the stools, but the sensitivity of this test is only 33–75%, and it does not allow taking biopsies or removing lesions at the time of detection (Simon, 2016).

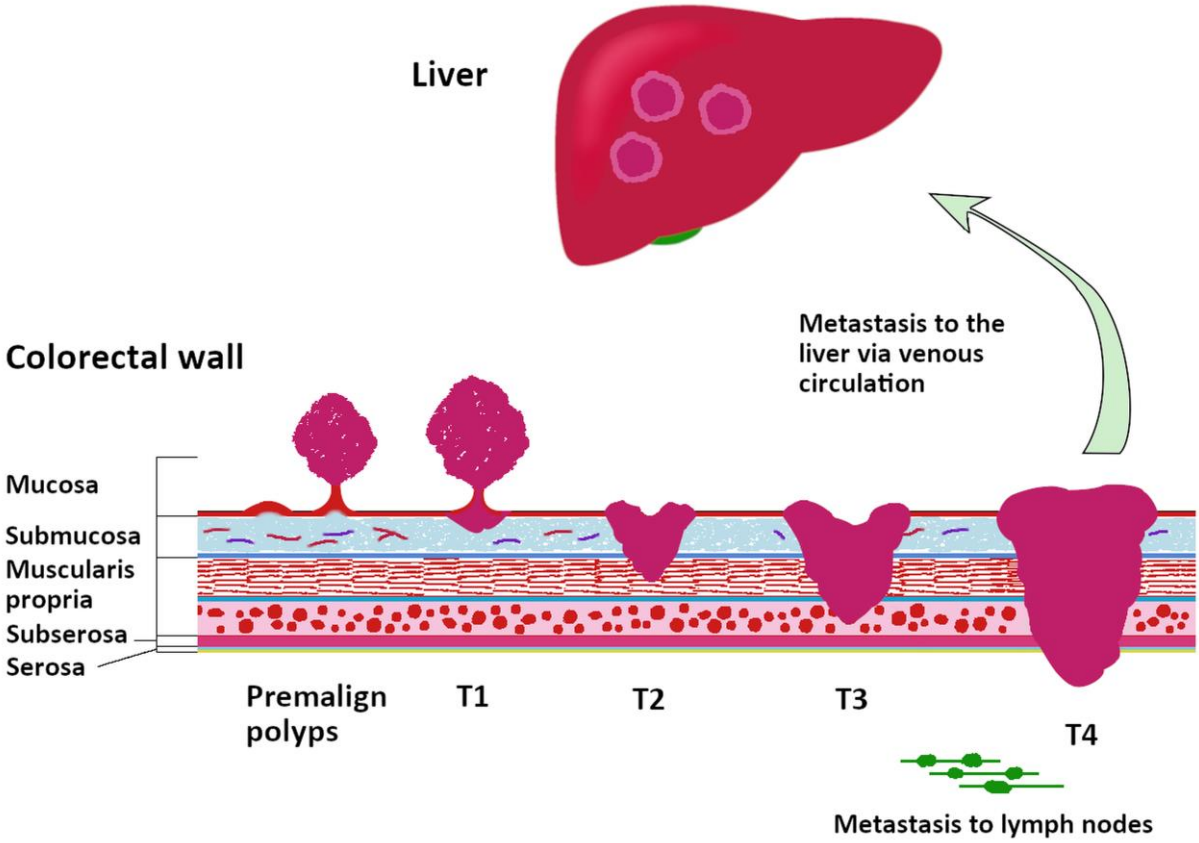
Magnetic resonance imaging (MRI) is primarily used in preoperative imaging of rectal cancer. It allows preoperative radiological T-staging (**Figure 1, Table 2**) and estimating the status between the tumor, mesorectal fascia, and other surrounding organs (Giusti et al., 2012). MRI is recommended for preoperative staging of all CRC patients (Dewhurst et al., 2012, Rollvén et al., 2013), but in clinical practice, computed tomography (CT) is more commonly used. In addition, endorectal ultrasound examination may provide useful information in the staging of superficial tumors (Taylor et al., 2008, Brown et al., 2004).

In recent years, other diagnostic imaging methods of primary CRC have also been widely investigated. These include CT colonography and positron emission tomography (PET)/CT colonography (Kijima et al., 2014). The merits of CT colonography are that it allows the evaluation of not only the colon, but also extracolonic findings, such as lymph node, liver, and lung metastases (Mainenti et al., 2006) with a low radiation dose (Yee et al., 2013). In addition, using 3D technique enables visualization of the vascular anatomy before laparoscopic surgery (Hirai et al., 2013).

PET is an imaging method based on visualizing enhanced metabolic processes, such as those within tumors, in the body. PET/CT colonography may be used for evaluation of recurrent CRC, but at present, it is recommended only for selected patients because of its expensiveness and limited resolution (Kijima et al., 2014).

Systemic disease is usually excluded with contrast-enhanced whole-body CT. If liver metastases are detected, the patient is further directed to high-resolution MRI imaging of the liver, which is especially beneficial in case of liver steatosis or contradictory CT results (Van Cutsem et al., 2016). PET with ^{18}F-fluorodeoxyglucose integrated with CT (^{18}F-FDG PET/CT) can be used for detecting possible extrahepatic spreading.

Figure 1. The development of colorectal cancer.



4.3 Classification and staging

Approximately 70% of CRCs are diagnosed in the colon and 30% in the rectum (Gaertner et al., 2015). Of the colon cancers, 22–44% have been reported to be right-sided and 56–78% left-sided (Boeckx et al., 2017, Lee et al., 2019a).
Approximately 20% of CRC patients have metastatic disease at the time of diagnosis (van der Geest et al., 2015, Yu and Cheung, 2018, Riihimäki et al., 2016), and at least 30% develop metastases later (Kanas et al., 2012). Up to 75% of the metastases are found in the liver (Sorbye et al., 2007). In recent European studies, 14.5–17.7% of the patients diagnosed with CRC have had synchronous liver metastases, and 7.1–12.8% have developed metachronous metastases after the operation on the primary tumor (Manfredi et al., 2006, Hackl et al., 2014, Engstrand et al., 2018). The majority of the liver metastases are diagnosed within three years after the diagnosis of the primary tumor, and they are found more often among males than among females and in lower age categories than higher (Hackl et al., 2014).

The staging of colorectal cancer at the time of diagnosis is the cornerstone of all treatment. The tumor/node/metastasis (TNM) classification and staging are presented in **Table 2**. Prior to the 21st century, disease stage was usually determined according to the Dukes' classification system (Akkoca et al., 2014, Sarma, 1986, Turnbull et al., 1967), which has since been largely replaced by the TNM classification. In this thesis, Dukes' classification was used in Study I, and TNM classifications 5–7 were used in Studies II–IV. There was some variation in the staging systems over the years according to the updates that were regularly made, but the changes did not affect the results presented in this thesis.

Table 2. TNM 8 classification and staging of colorectal cancer. The previously used Dukes' classification included.

TNM classification			**Definition**
T	X		The primary tumor cannot be defined
	0		No evidence of primary tumor
	1		Tumor grows into the submucosa
	2		Tumor grows into the muscularis propria
	3		Tumor grows into the subserosa or into pericolic or perirectal tissues
	4	a	Tumor grows through the wall of the bowel into the visceral peritoneum
		b	Tumor grows into or is attached to other tissues or organs
N	X		Lymph nodes cannot be evaluated
	0		No lymph node metastases
	1	a	1 lymph node metastasis
		b	2–3 lymph nodes metastases
		c	Tumor deposits without lymph node metastases
	2	a	4–6 positive lymph nodes
		b	≥7 positive lymph nodes
M	0		No distant metastases
	1	a	Cancer has spread to 1 distant organ
		b	Cancer has spread to >1 distant organs
		c	Cancer has spread to the peritoneum with or without other distant metastases

Staging			
TNM			**Dukes'**
I		T1-2N0M0	A
II	A	T3N0M0	B
	B	T4aN0M0	
	C	T4bN0M0	
III	A	T1-2N1/1cM0 T1N2aM0	C
	B	T1N2bM0 T2N2a-bM0 T3N1-2aM0 T4aN1M0	
	C	T3N2bM0 T4aN2a-bM0 T4bN1-2M0	
IV	A	Any T any N M1a	D
	B	Any T any N M1b	
	C	Any T any N M1c	

Adapted from the TNM Classification of Malignant Tumours (Brierley et al., 2017) and the modified Dukes' classification (Akkoca et al., 2014, Turnbull et al., 1967).

5. MANAGEMENT

5.1 Surgical treatment of primary colorectal tumors

Surgery for primary colorectal tumors is indicated in patients with local tumors, and in metastatic disease, if all the metastases are considered operable. The location of the primary tumor defines the surgical technique. Surgery can be either mini-invasive (laparoscopic or robotic) or open. In the former, perioperative recovery is usually faster, need for analgesics briefer, and incisions smaller. The rates of intra- and postoperative complications and postoperative mortality are similar, and no differences in the oncological outcomes have been observed (Fleshman et al., 2019, Liu et al., 2018, Baik et al., 2011, Buunen et al., 2009).

In colon cancer, the increasingly used surgical technique is complete mesocolic excision (CME). The procedure of choice depends on the location of the primary tumor, and the options are right hemicolectomy, extended right hemicolectomy or left hemicolectomy (Kim et al., 2016).
In rectal cancer, the standard technique is total mesorectal excision (TME). The most important surgical prognostic factors are the circumferential resection margin (CRM), which should be created along the mesorectal fascia (MRF), and removal of all mesorectal lymph nodes. Sphincter-sparing surgery is favored, if possible (Glynne-Jones et al., 2017).

Before surgery, most rectal cancer patients are directed to preoperative radiotherapy with or without sensitizing chemotherapy to reduce the risk of local recurrence. Only in the most favorable cases (cT1–2, cT3a/b, cN0–1), surgery alone is an option. In more locally advanced cases, either short-course radiotherapy or long-course chemoradiotherapy is recommended. The standard options are the following: a 25 Gy total dose at 5 Gy/fraction during 5–7 days, followed by surgery either within 5 days or after 5–9 weeks; or a 45–50 Gy total dose in 25–28 fractions, optionally followed by a 5.4 Gy boost in 3 fractions, if the CRM is threatened (van der Valk et al., 2020, Hyöty et al., 2019, Glynne-Jones et al., 2017). In inoperable cases, neoadjuvant therapy with (chemo)radiation and chemotherapy is also an option.

At the Helsinki University Hospital, the treatment of colon and rectal cancer patients is decided on at multidisciplinary team (MDT) meetings including gastrointestinal surgeons, oncologists, a radiologist, and a pathologist.

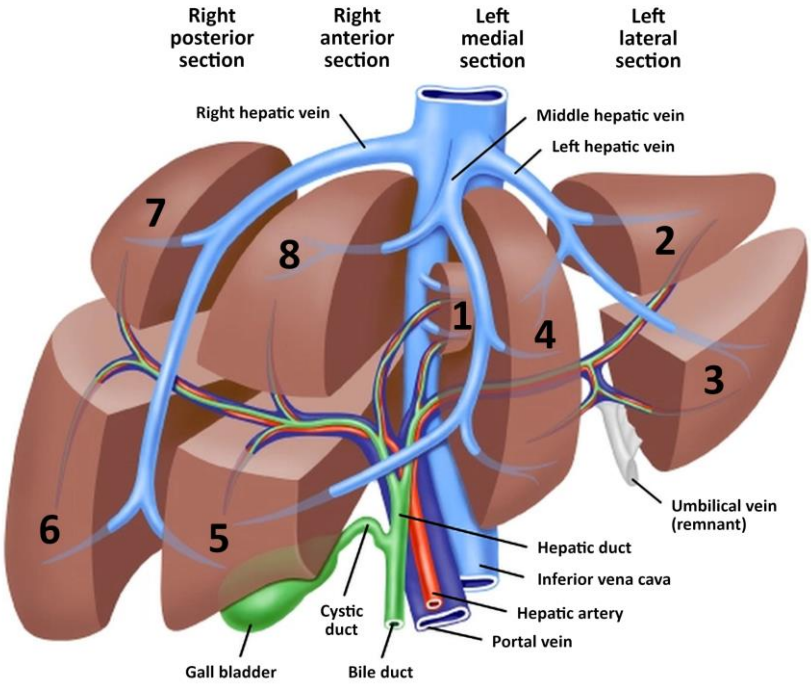
5.2 Surgical and ablative treatments of liver metastases

5.2.1 Liver resection

The possibilities of curative-intent surgery should always be assessed, when liver metastases are diagnosed. The treatment of liver metastases has evolved significantly in the last couple of decades, and the resection rates have increased correspondingly (Adam and Kitano, 2019, Hackl et al., 2014). At the Helsinki University Hospital, the number of resections for colorectal metastases has over doubled: altogether 133 resections were performed between the years 2000 and 2005; 214 in 2006–2010; 250 in 2011–2014; and 339 in 2015–2018 (Professor Helena Isoniemi, personal communication).

Earlier, liver metastases were considered resectable, if their number was 1–3, maximum diameter 5 cm, or potential resection margin at least 1 cm (Isoniemi and Osterlund, 2011). Nowadays, defining resectability has become more complicated, and the decisions on the liver resections are made at MDT meetings. The goal is to achieve complete negative resection margins while preserving at least 30% of the estimated total liver volume as a future liver remnant (FLR) and sparing at least two Couinaud's segments (**Figure 2**). In addition, portal and arterial inflow, venous outflow, and biliary drainage have to be maintained (Van Cutsem et al., 2016).

Figure 2. Couinaud's segments of the liver.



Original image from: Curley SA, Glazer ES. Hepatic resection techniques. In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA (http://www.uptodate.com). Accessed on October 15, 2020.

Advances in surgical techniques have enabled resecting multiple bilobar liver metastases that cannot be removed at a single operation. This can be accomplished by a technique called two-stage hepatectomy (TSH), which was first introduced by Adam et al. in 2000 (Adam et al., 2000) and comprises two separate interventions. At the first stage, the metastases in the left liver lobe are removed, and the contralateral portal vein branches are occluded either by surgical ligation or radiological embolization. This leads to hypertrophy of the FLR, and after 4–6 weeks, the remaining liver metastases in the right lobe can be resected at the second stage (Kabir et al., 2020). In addition, a variant of this technique called Associating Liver Partition and Portal vein ligation for Staged hepatectomy (ALPPS) has been developed. In ALPPS, an additional transection of the liver parenchyma along the falciform ligament is performed at the first stage, resulting in rapid hypertrophy of the FLR already within 1–2 weeks (Schnitzbauer et al., 2012, Kabir et al., 2020). These techniques can be applied to selected patients.
Before liver resection, all patients undergo CT and high-resolution MRI imaging, as described in **section 4.2**. ^{18}F-FDG PET/CT may be used for excluding extrahepatic spreading, and the liver metastases are further visualized with ultrasound during the operation (Van Cutsem et al., 2016).

At the Helsinki University Hospital, the follow-up after liver resection continues at the department of oncology, at first with an interval of three months. Laboratory tests and CT imaging are performed before every appointment. In addition, all patients have a three months' postoperative control at the clinic of liver surgery with laboratory tests, imaging, and clinical examination. High-resonance MRI imaging of the liver is performed in addition to CT, if necessary. The regular controls are continued for 5 years with gradually lengthening intervals, if no recurrence develops. In case a recurrence is detected, the oncological treatment is continued, and re-resection of the metastases may be considered with the same criteria as for primary liver resection (Hyöty et al., 2019).

5.2.2 Ablative treatments of liver metastases

For patients with oligometastatic disease – that is, 5 or more metastatic lesions at up to 3 different sites – curative-intent surgery combined with local ablative therapy may be an option. In addition, localized interventions can be considered in order to achieve long-term disease control. The treatment of choice depends on the site and volume of the metastases, and the options include radiofrequency ablation (RFA), microwave ablation, brachytherapy electroporation, stereotactic body radiotherapy (SBRT), selective internal radiation therapy (SIRT), and transarterial chemoembolisation (TACE), among others (Peltola et al., 2019, Van Cutsem et al., 2016). Currently, the possibilities of using these local interventions are carefully considered at MDT meetings according to patient-related factors and treatment goals.

5.3 Oncological treatment

At present, an increasing number of chemotherapy regimens are available, and their use is adjusted individually according to the molecular and pathological features of the cancer, clinical characteristics of the patient, and the results of the surgical procedures, not forgetting the preferences of the patients themselves. However, the basis of all present oncological treatment in CRC lies on a few chemotherapy agents (cytostatics) that continue to be widely used.

5.3.1 Chemotherapy agents

Fluoropyrimidines are a group of antimetabolite drugs that were found to have anticancer effects already in the 1950's (Heidelberger et al., 1957). Of these, 5-fluorouracil (5-FU), a synthetic analogue of the naturally occurring pyrimidine derivative uracil, has been used in the treatment of colon cancer for decades. *In vivo*, 5-FU is converted to several metabolites that incorporate into DNA and RNA, and to fluorodeoxyuridine monophosphate, which is an inhibitor of the enzyme called thymidylate synthase. All of these inhibit DNA synthesis and thus, tumor growth (Diasio and Harris, 1989). 5-FU is administered parenterally (intravenously, i.v.), and more than 80% of it is metabolized in the liver. Leucovorin (LV), a compound similar to folinic acid, is usually administered simultaneously to enhance the anticancer effects of the drug.
There are over 10 different forms of 5-FU, of which three orally (p.o.) administered prodrugs are commonly used in the treatment of CRC. Capecitabine is a fluoropyrimidine carbamate that passes unaltered through the intestine and is then metabolized via three steps to its active form, 5-FU, in the liver. Tegafur, on the other hand, is converted to 5-hydroxytegafur, which then breaks down spontaneously to form 5-FU (Thorn et al., 2011). The third option, S-1, is a drug consisting of three pharmacological agents: tegafur, gimeracil, and oteracil potassium (Kwakman et al., 2019).
Treatment with 5-FU can cause several side effects including diarrhea, nausea, mouth sores, and fatigue. It may also cause severe, dose-limiting or even lethal toxicity, especially in patients with dihydropyrimidine dehydrogenase (DPD) deficiency. This defect is caused by a mutation in the *DPYD* gene, and its prevalence in the general population is unknown. It is estimated that 2–8% of the patients may be vulnerable to toxic reactions to 5-FU (Boisdron-Celle et al., 2017), and routine testing for DPD deficiency before initiating fluoropyrimidine-based chemotherapy is currently generally recommended (Argiles et al., 2020).

Oxaliplatin is a platinum-based antineoplastic agent that belongs to the same family as cisplatin and carboplatin. It inhibits DNA synthesis selectively through binding to the guanine and cytosine moieties, which leads to cross-linking of DNA. Oxaliplatin is administered i.v., typically in combination with 5-FU/LV i.v. (FOLFOX) or capecitabine p.o. (CAPOX). In stage IV CRC, other drugs can also be added to the combination.

Oxaliplatin typically causes acute cold-induced neurotoxicity and chronic neuropathy, which affect basically all patients (Griffith et al., 2017). The chronic neuropathy develops cumulatively according to the administered doses, and it may not be regressive in all cases. Oxaliplatin can also cause other side effects, such as an anaphylactic reaction.

Irinotecan hydrochloride is a half-synthetic analogue of camptothecin, an extract from a Chinese tree *Camptotheca acuminate* (Fujita et al., 2015). It inhibits specifically the type I DNA topoisomerase, resulting in single-strand DNA breaks and irreversible inhibition of DNA synthesis. These events arrest the cell cycle in the S-G2 phase and lead to apoptosis. The use of irinotecan should be monitored carefully, as it may cause severe diarrhea. Other typical side effects include nausea, loss of appetite, chest pain, mucositis, and neutropenia. Irinotecan is administered i.v., usually in combination with 5-FU/LV i.v. (FOLFIRI) or capecitabine p.o. (CAPIRI), but it can be combined with other drugs too.

5.3.2 Targeted therapies

Targeted therapies have been used in metastatic (stage IV) colorectal cancer for more than 15 years, and the commonly used drugs include vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) inhibitors. These can be administered in combination with other chemotherapy drugs or as monotherapy (Van Cutsem et al., 2016).

VEGF, also known as VEGF-A, is a major angiogenic factor that promotes sprouting in tumors and contributes to tumor growth and metastasis. In addition, the other isoforms of VEGF (VEGF-B, -C, and -D) as well as placental growth factor (PlGF) also induce angiogenesis (Tammela et al., 2008, Tammela et al., 2005). EGFR, on the other hand, is a transmembrane tyrosine kinase protein that is involved in the RAS-RAF-MAPK signaling pathway, which mediates signals from outside a cell to the nucleus and regulates the growth, differentiation, proliferation, migration, and apoptosis of the cell. Activation of EGFRs also inhibits autophagy (Sigismund et al., 2018).

VEGF inhibitor bevacizumab is a recombinant humanized monoclonal antibody that binds to circulating VEGF-A, and thus, inhibits the activation of VEGF receptors (Ferrara et al., 2005). This leads to decreased sprouting and tumor growth. Bevacizumab is recommended for the treatment of metastatic CRC in combination with chemotherapy drugs, especially in *RAS*- or *BRAF*-mutated colon cancer and in right-sided disease. Other anti-angiogenic agents, such as regorafenib, aflibercept, and ramucirumab, have also been adopted to later-line clinical use in CRC (Tampellini et al., 2016, Van Cutsem et al., 2016).
EGFR inhibitors cetuximab and panitumumab are monoclonal antibodies that specifically bind to the EGFR, which results in the inhibition of the RAS-RAF-MAPK pathway (**section 7.3.1**) and, consequently, decrease in tumor proliferation and growth. EGFR inhibitors are recommended for the first-line treatment of metastatic CRC in left-sided *RAS*-wild-type

cancer in combination with chemotherapy drugs, and for later-line treatment either alone or combined (Van Cutsem et al., 2016) (**Table 3**).

Immunotherapies are an emerging treatment modality in solid cancers, but in CRC, they have not been of great benefit by far. However, the programmed cell death 1 (PD1) blocking antibodies pembrolizumab and nivolumab have shown promising efficacy in some patients with metastatic MSI-H/dMMR disease (Andre et al., 2020, Le et al., 2020, Ganesh et al., 2019) (**sections 6.1 and 7.3.2**).

5.3.3 Adjuvant chemotherapy in primary colorectal cancer

In colon cancer, the adjuvant treatment begins 3–6(–8) weeks after the operation on the primary tumor, and its duration is 3–6 months, depending on the stage of the disease, risk factors, and possible adverse effect profile (Argiles et al., 2020). In local stage I colon cancer, no adjuvant treatment is needed. In stage II, adjuvant treatment is considered for high-risk patients, and the options are fluoropyrimidines alone or in combination with oxaliplatin (CAPOX/FOLFOX). In stage III, adjuvant treatment is usually indicated, and the standard option is fluoropyrimidines with or without oxaliplatin.

The treatment of rectal cancer differs from that of colon cancer. Most rectal cancer patients receive radiotherapy with or without chemosenzitation therapy before the operation on the primary tumor, as described previously (**section 5.1**). After the operation, 5-FU alone or combined with oxaliplatin may be considered as adjuvant therapy (Benson et al., 2018b, Glynne-Jones et al., 2017).

5.3.4 Chemotherapy in patients with resectable liver metastases

The oncological treatment options in stage IV CRC have improved substantially in the last decades, and patient survival accordingly. The role of chemotherapy administered before (neoadjuvant) and/or after (adjuvant) liver resection has been evaluated in several studies (Wang et al., 2015, Nordlinger et al., 2013, Ciliberto et al., 2012, Mitry et al., 2008). Currently, perioperative – that is, both neoadjuvant and adjuvant – chemotherapy is recommended for most patients undergoing resection, but in selected cases with a good prognosis, the operation may be performed without preceding neoadjuvant therapy (Hyöty et al., 2019, Benson et al., 2018a, Benson et al., 2018b, Van Cutsem et al., 2016, Nordlinger et al., 2013). However, adjuvant therapy is usually indicated, as it has been shown to extend the disease-free survival time (Wang et al., 2015, Nordlinger et al., 2013, Ciliberto et al., 2012). The type of treatment is discussed and decided on at MDT meetings, in which a gastrointestinal surgeon, a liver and/or thoracic surgeon, an oncologist, a radiologist, and a pathologist are present.

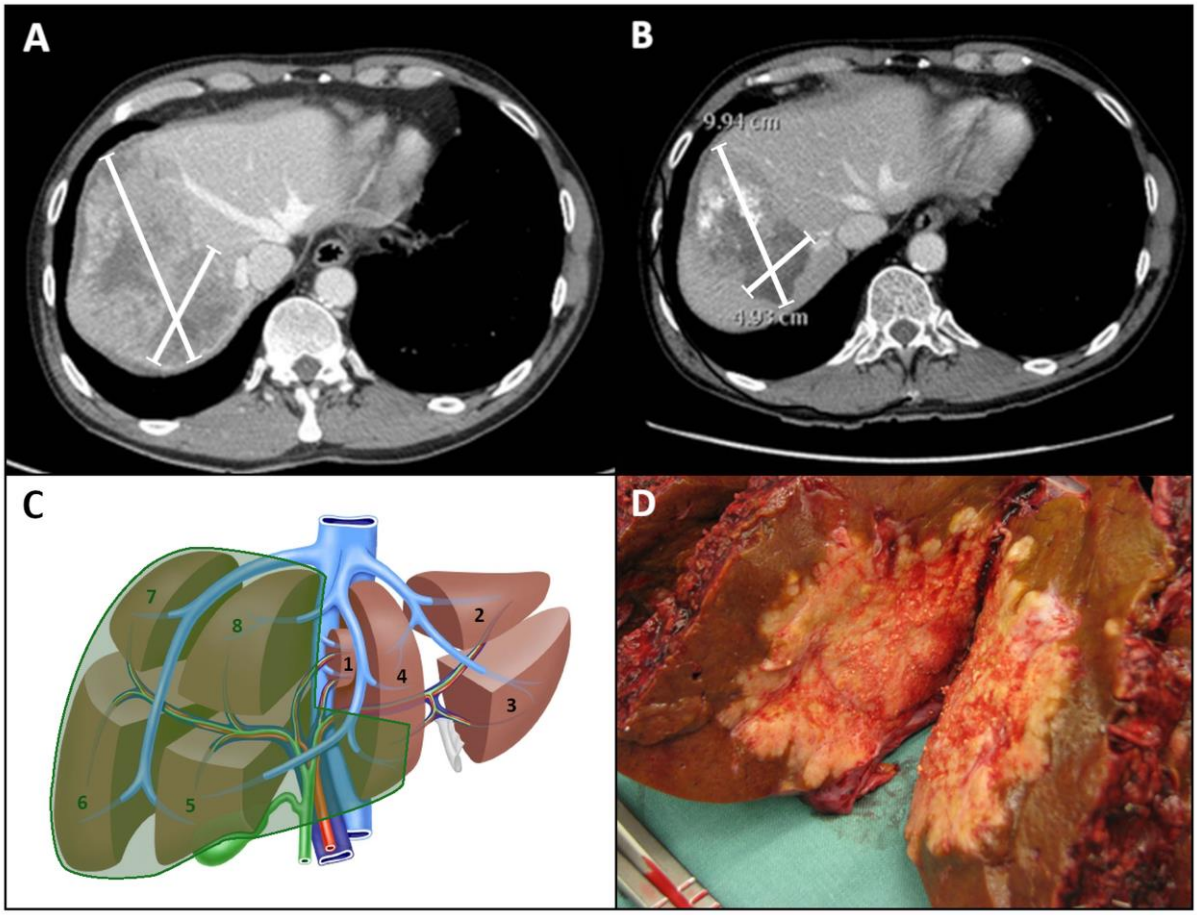
Liver resection may be performed directly in patients who have isolated liver metastases that are technically resectable, no extrahepatic disease, no other risk factors for poor prognosis, and who are at a sufficiently good physical condition. Only around 20% of the liver metastases are initially subject to curative-intent resection (Nordlinger et al., 2007, Khatri et al., 2005). In those cases, the recommended adjuvant chemotherapy is oxaliplatin-based (CAPOX/FOLFOX) and its duration 6 months (**Table 3**). If oxaliplatin is contraindicated, fluoropyrimidine alone is an option (Hyöty et al., 2019, Van Cutsem et al., 2016).

If the liver metastases are primarily easily resectable, but the patient has other risk factors for adverse prognosis or other operable metastases in addition to those in the liver, perioperative chemotherapy is indicated. The choice of neoadjuvant treatment depends on individual factors, but usually the recommended option is oxaliplatin-based (FOLFOX) (Van Cutsem et al., 2016) (**Table 3**). The duration of neoadjuvant chemotherapy is generally three months.
Adjuvant chemotherapy is started within 3–6 weeks after liver resection, and its duration should be at least three months, so that the combined duration of both neoadjuvant and adjuvant treatment is six months (Wang et al., 2015, Nordlinger et al., 2013, Ciliberto et al., 2012). The chosen treatment is preferably oxaliplatin-based as in neoadjuvant chemotherapy, unless contraindicating side effects have emerged.

5.3.5 Conversion chemotherapy

Nowadays even 30–61% of the initially non-resectable liver metastases can be rendered to resectable due to preoperative conversion chemotherapy (Stintzing et al., 2016, Gruenberger et al., 2015) (**Figure 3**). This therapy option may be considered for patients who have initially non-resectable liver metastases or metastases that are difficult to resect (borderline-resectable). The aim is to achieve maximal response rate and tumor shrinkage by using the most efficient chemotherapy regimens. If the patient's physical condition allows, targeted drugs are combined to the previously described baseline chemotherapy regimens as presented in **Table 3**. The treatment response is evaluated at MDT meetings at an interval of two months, and liver resection is performed as soon as a sufficient treatment response is attained, usually after 2–4 months.
After liver resection, adjuvant treatment is continued aiming at a total treatment duration of six months. However, targeted drugs are left out of the adjuvant treatment, as detriment has been observed in stage I–III disease (Van Cutsem et al., 2016, Hyöty et al., 2019).

Figure 3. Results of conversion chemotherapy in a 60-year-old patient with primarily non-resectable liver metastases of a maximum diameter of 12.6 cm (A). After 3 months of chemotherapy with 5-FU/LV, oxaliplatin, and bevacizumab, the metastases had become operable (B). Couinaud's segments 5–8 and part of 4b were resected (C). In the final pathological analysis, the maximum diameter of the metastases was only 6.8 cm (D).



Images 3 A, B and D courtesy of Professor Helena Isoniemi, Helsinki University Hospital, Helsinki, Finland. Image 3 C modified from Curley SA, Glazer ES. Hepatic resection techniques. In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA (http://www.uptodate.com). Copyright © 2020 UpToDate, Inc. Reproduced with permission.

5.3.6 Chemotherapy in non-operable metastatic disease

The oncological treatment of metastatic, non-curable CRC aims at cytoreduction or control of disease progression. Important factors determining the prognosis include the molecular characteristics of the disease and the clinical presentation of the metastases. The choice of treatment is discussed with the patient, and their expectations, comorbidities, and the possible toxicity of the treatments are taken into consideration.

Table 3. Oncological treatment options in conjunction with resection of colorectal liver metastases.

Liver metastases	**Type of disease**		**Neoadjuvant/conversion treatment**		**Adjuvant treatment**
Resectable	Favorable prognosis		-	**R E S E C T I O N**	1) Doublet 2) Fluoropyrimidine alone
	Unfavorable prognostic factors		1) Doublet 2) Fluoropyrimidine alone		
Borderline-resectable / initially non-resectable	*RAS* and *BRAF* wild-type	Left	1) Doublet + EGFR inhibitor 2) Triplet* + VEGF inhibitor 3) Doublet + VEGF inhibitor		
		Right	1) Triplet* + VEGF inhibitor 2) Doublet + VEGF inhibitor		
	RAS mutation		1) Triplet* + VEGF inhibitor 2) Doublet + VEGF inhibitor		
	BRAF mutation		1) Triplet* + VEGF inhibitor 2) Doublet + VEGF inhibitor		
Explanations					
Doublet	5-FU/LV or capecitabine + oxaliplatin or irinotecan (CAPOX/FOLFOX/CAPIRI/FOLFIRI) - EGFR inhibitors used only in combination with FOLFOX/FOLFIRI - VEGF inhibitors can be used in combination with all of the above-mentioned				
Triplet	5-FU/LV + oxaliplatin + irinotecan (FOLFOXIRI) * Considered only for physically fit and motivated patients				
VEGF inhibitor EGFR inhibitor	Bevacizumab Cetuximab or panitumumab				

Adapted from (Hyöty et al., 2019, Yoshino et al., 2018, Van Cutsem et al., 2016, Adam et al., 2015).

The primary choice for first-line treatment is made following the same principles as in conversion chemotherapy, if cytoreduction is the goal (**Table 3**). When aiming at disease control, the treatment is less intensive. The factors considered include the sidedness of the primary tumor, the mutational status, and the patient's medical condition and expectations. After a period of 4–6 months of induction therapy, either a treatment pause or maintenance therapy is commenced. In case of disease progression, the same systemic therapy is re-introduced or the regimen is changed to a second-line option. It is quite common that a patient undergoes 3–4 lines of treatment before the period of best supportive care (Van Cutsem et al., 2016, Yoshino et al., 2018).

6. PROGNOSIS

6.1 Prognostic factors

Several cancer- and patient-related, molecular, and genetic factors define the prognosis of CRC. Cancer-related risk factors for recurrence and poor prognosis include primary pT4 or pN2 stage, poor differentiation of the tumor(s), vascular and perineural invasion, obstruction or perforation of the bowel, as well as lymph node metastases after preoperative radio(chemo)therapy and insufficient resection margin in rectal cancer (Argiles et al., 2020). Patient-related risk factors include high age, physical frailty, comorbities, and liver insufficiency.
Molecular and genetic factors contribute to the characteristics of the malignant tumors. *BRAF* mutated tumors and either stable or low degree of microsatellite instability (MSS or MSI-L, **section 7.3.2**) signify worse prognosis, even though the prognosis seems to be somewhat better in *BRAF* mutated disease with a high degree of microsatellite instability (MSI-H) or mismatch repair deficiency (dMMR) (Ogino et al., 2012). In addition, *KRAS* mutations have been associated with an increased risk of recurrent disease (Hutchins et al., 2011), but the results have been controversial.

6.2 Overall survival in colorectal cancer

The prognosis of CRC is substantially affected by the location of the primary tumor, the mutational status, and the primary TNM status, among other clinical factors. In non-metastatic CRC, all diagnosed patients combined, 5-year overall survival (OS) has been 60–75% in recent European studies, and 10-year OS around 30% (Engstrand et al., 2018, Hackl et al., 2014).

When the location of the primary tumor is taken into account, the prognosis in stage II colon cancer is quite similar to that of stage I disease. In stage III, 5-year cancer-specific survival rates of 57% in left-sided cancer and almost 55% in right-sided cancer have been reported (Lee et al., 2019a). In stage IV disease, however, significant differences have been found in several studies. In *RAS*/*BRAF* wild-type patients, all oncological treatments combined, median OS has been around 33 months in left-sided cancer and 22 months in right-sided cancer (Boeckx et al., 2017).
In rectal cancer, 5-year OS is more than 90% in stage I disease, and declines to less than 10% in stage IV disease (Gaertner et al., 2015).

6.3 Survival in colorectal cancer with liver metastases

Survival rates after liver resection are constantly improving. In the last decade, 5-year survival rates of up to 53% (Stelzner et al., 2019, Engstrand et al., 2018, Hackl et al., 2014, Kanas et al., 2012) and 10-year cancer-specific survival of 23% (Rees et al., 2008) have been reported. For patients who undergo two-stage hepatectomy, 5-year OS has been 32–70% (Kabir et al., 2020). The patients who receive conversion chemotherapy before liver resection have slightly shorter postoperative survival than those who do not need it, but their prognosis is, in any case, significantly better than in case resection was not carried out (Van Cutsem et al., 2016, Adam et al., 2004).
Mortality in liver surgery is low, being currently close to 1% (Isoniemi and Osterlund, 2011). Despite of the improved treatments, up to 70% of the patients experience recurrence after curative-intent liver resection (House et al., 2010), usually within three years. However, re-resections are possible in selected cases with promising results.

The location of the primary tumor affects the prognosis also after liver resection, as the patients with right-sided primary tumors tend to have worse survival than those with left-sided tumors (Gasser et al., 2019) (**sections 3.3 and 7.3.1**). Median OS rates of 4.5 years in patients with right-sided primary tumors and 6.3 years in those with left-sided ones after liver resection have been reported (Elizabeth McCracken et al., 2019).

The prognosis of the patients not suitable for liver resection is poor: with palliative chemotherapy, one-year survival rate of up to 58.1% has been reported (Engstrand et al., 2018), but 5-year survival rates have been only 2.2–4.0% (Engstrand et al., 2018, Hackl et al., 2014, Manfredi et al., 2006). With best supportive care, one-year survival is around 8.2% and 5-year survival close to 0.0% (Engstrand et al., 2018, Manfredi et al., 2006).

7. PROGNOSTIC AND PREDICTIVE BIOMARKERS

Prognostic biomarkers help evaluate the patient's prognosis, for example, the risk of recurrence. They do not, however, predict the response to a specific treatment. Predictive biomarkers, on the other hand, aim to evaluate the patient's likelihood of benefit from specific clinical interventions, such as an operation or chemotherapy (Mehta et al., 2010). In this thesis, the investigated biomarkers can be considered both prognostic and predictive, as their concentrations or expressions may be prognostic of recurrence or death and predictive of the benefit of liver resection.

The serum/plasma biomarkers were determined principally in serum samples, and only the concentrations of tumor-associated trypsin inhibitor (TATI) and human chorionic gonadotropin β (hCGβ) were measured in plasma samples. Serum is a specimen obtained from full blood that has coagulated and from which fibrin clots, blood cells, and other coagulation factors are separated by centrifugation. Plasma is obtained, if an anticoagulant is added to the specimen before the removal of blood cells, and no time for coagulation is required. The sensitivity of the biomarker determinations may be better in serum because of higher metabolite concentrations, but on the other hand, plasma has demonstrated a better reproducibility, possibly due to the less complicated collecting procedure. In principle, serum and plasma are, however, considered to be equal for biomarker determinations (Yu et al., 2011, Lima-Oliveira et al., 2018).

7.1 Biomarkers in this thesis

7.1.1 Carcinoembryonic antigen (CEA)

CEA, first identified by Gold and Freedman in 1965 (Gold and Freedman, 1965), is the most commonly used biomarker in the detection and management of CRC. It is a glycoprotein functioning as an intracellular adhesion molecule, currently categorized as a member of the immunoglobulin superfamily. The production of CEA is normally minimal after birth, but elevated levels in serum have been detected in several malignancies, such as colorectal (Carpelan-Holmström et al., 1995), gastric (Shimada et al., 2014), pancreatic (Zhang et al., 2018b), breast (Uehara et al., 2008), and lung cancer (Molina et al., 2008). In addition, smoking (Alexander et al., 1976) and non-neoplastic conditions (Hao et al., 2019), such as alcoholic liver disease (Bell et al., 1979), diabetes (Chung et al., 2019), chronic renal failure (Filella et al., 1990), and inflammatory bowel diseases (Gardner et al., 1978), can cause CEA levels to increase.

In local CRC, elevated serum levels have been associated with recurrent disease after the operation on the primary tumor (Ramphal et al., 2019, Konishi et al., 2018, Baqar et al., 2019, Carpelan-Holmstrom et al., 2004), and also in CRC with liver metastases, elevated CEA

levels before or after liver resection have indicated recurrence (Bredt and Rachid, 2014, Okazaki et al., 2017).

At present, CEA is the only biomarker recommended for monitoring CRC. It should be measured for all the newly diagnosed CRC patients as well as in the follow-up after curative-intent surgery (Sturgeon et al., 2008, Duffy et al., 2007, Locker et al., 2006, Duffy et al., 2003). However, it has been reported in several studies that approximately 30–40% of CRCs do not produce elevated levels of CEA despite of metastatic disease (Saito et al., 2018, Thomsen et al., 2018). Thus, there is an obvious need for more reliable biomarkers.

7.1.2 Carbohydrate antigen 19-9 (CA19-9)

CA19-9 (sialyl Lewis A) is a tetrasaccharide characterized as sialylated lacto-N-fucopentaose II (Magnani et al., 1981, Magnani et al., 1982). It is normally produced by pancreatic and biliary ductal cells as well as by gastric, colonic, endometrial, and salivary epithelia. It was originally discovered in 1979 and, subsequently, identified as a tumor marker in 1981 (Koprowski et al., 1981). Soon afterwards, CA19-9 in serum was found to be elevated in gastrointestinal malignancies, such as pancreatic cancer (Del Villano et al., 1983, Jalanko et al., 1984), cholangiocarcinoma (Jalanko et al., 1984), colorectal cancer (Koprowski et al., 1981, Sears et al., 1982, Kuusela et al., 1984), and gastric cancer (Yang et al., 2014, Liang et al., 2016). In pancreatic adenocarcinoma, serum CA19-9 has recently been found to be useful for estimating the response to neoadjuvant chemotherapy and survival (Al Abbas et al., 2020). In CRC, CA19-9 is currently not recommended in the routine follow-up, as its sensitivity has been found to be inferior to that of CEA in detecting recurrences (Okamura et al., 2017). However, it is considered an emerging prognostic biomarker (Duffy et al., 2014, Yakabe et al., 2010). In addition, it has been suggested that CA19-9 might be a target for therapeutic antibodies in the treatment of gastrointestinal malignancies (Weitzenfeld et al., 2019, Sawada et al., 2011, Ragupathi et al., 2009).

7.1.3 Human chorionic gonadotropin β (hCGβ)

Human chorionic gonadotropin (hCG) belongs to a family of glycoprotein hormones together with luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). These consist of two distinct subunits, α and β. The α subunits are identical, while the β subunits are specific and account for the biological characteristics of the individual hormones.

HCG is normally produced by syncytiotrophoblastic cells of the placenta during pregnancy, and it is a specific marker for malignant tumors of trophoblastic origin. HCG has also been suggested to act as an angiogenic factor in tumor development (Zygmunt et al., 2002). The free β subunit (hCGβ) can be detected in tissue, serum, and urine. Elevated serum

concentrations of hCGβ have been observed in several non-trophoblastic malignancies including ovarian (Marcillac et al., 1992, Vartiainen et al., 2001), gastric (Louhimo et al., 2004), colorectal (Lundin et al., 2001, Louhimo et al., 2002), biliary (Alfthan et al., 1992), pancreatic (Alfthan et al., 1992, Syrigos et al., 1998), and hepatocellular cancer (HCC) (Lyytinen et al., 2013), and they have associated with adverse prognosis.

In CRC, the prognostic significance of elevated hCGβ concentrations in serum has been controversial. It has been considered inferior to CEA in detecting recurrent disease (Carpelan-Holmstrom et al., 2004), but on the other hand, it has been found to predict adverse outcome in women (Birgisson et al., 2012).

7.1.4 Tumor-associated trypsin inhibitor (TATI)

TATI is a 6-kDA peptide that was originally identified in the urine of a patient with ovarian cancer (Stenman et al., 1982, Huhtala et al., 1982). It was later found to be identical to pancreatic secretory trypsin-inhibitor (PSTI / serine peptidase inhibitor Kazal type 1 [SPINK1]), which had been previously isolated from the bovine pancreas (Greene and Giordano, 1969). TATI is normally produced by pancreas (Eddeland and Ohlsson, 1978, Hedström et al., 2001) as well as by several other tissues (Lasson et al., 1986, Halila et al., 1985, Jönsson et al., 1996), and it can be detected in tissue samples, serum, and urine. TATI is a protease inhibitor that can inhibit the degradation of extracellular matrix by tumor cells (Koivunen et al., 1991). On the other hand, it has been shown to be involved in angiogenesis and to promote tumor growth (Gouyer et al., 2008). TATI is structurally similar to epidermal growth factor (EGF) (Scheving, 1983), and it has been shown to stimulate EGF receptors, and thus, to increase tumor invasiveness (Ozaki et al., 2009). In addition, it acts as an acute phase reactant (Ogawa et al., 1985).

Increased serum levels of TATI have been observed in several malignancies, and they have been shown to have prognostic value in ovarian (Venesmaa et al., 1998, Paju et al., 2001), renal (Paju et al., 2004), bladder (Kelloniemi et al., 2003), gastric (Kasurinen et al., 2020), and colorectal cancer (Gaber et al., 2010, Gaber et al., 2011) as well as in HCC (Lyytinen et al., 2013). Studies concerning the prognostic value of serum TATI in CRC have been few, but in the studies by Gaber et al., TATI was found to be an independent prognostic biomarker and possibly even of stronger prognostic value than CEA. The prognostic role of TATI in CRC, however, remains to be established.

7.1.5 YKL-40, interleukin-6 (IL-6), and C-reactive protein (CRP)

Systemic inflammation has been associated with impaired prognosis in several malignancies (Hanahan and Weinberg, 2011, Nasr et al., 2018). YKL-40 (also called chitinase-3-like protein-1 [CHI3L1]), IL-6, and CRP are established biomarkers of systemic inflammation, and their potential role as prognostic biomarkers in cancer has raised growing interest during the last decades.

YKL-40 is a glycoprotein that was identified in a human osteoblastoma cell line in 1992 (Johansen et al., 1992). It belongs to the family of chitinase-like proteins that includes also YKL-39 and stabilin-1 interacting chitinase-like protein (SI-CLP). These proteins are secreted by cancer cells and various other cell types including macrophages, neutrophils, synoviocytes, and chondrocytes, among others (Kzhyshkowska et al., 2016). The function of YKL-40 is not completely known, but it promotes tumor invasion and metastasis via several mechanisms, mainly by contributing to angiogenesis independently and by inducing VEGF expression (Francescone et al., 2011). In addition, YKL-40 upregulates proinflammatory mediators (Libreros et al., 2012) and activates the *TGFβ* pathway (Qiu et al., 2018) as well as the Akt signaling pathway in colonic epithelial cells (Chen et al., 2011). It interacts with several receptors, such as IL-13Rα2 and the receptor for advanced glycation end products (RAGE) (Yeo et al., 2019).
Soon after discovery, elevated serum levels of YKL-40 were found to associate with poor overall survival in patients with recurrent breast cancer (Johansen et al., 1995). Since then, elevated serum or plasma YKL-40 has also been shown to indicate poor prognosis in several other malignancies including colorectal (Cintin et al., 1999, Cintin et al., 2002, Fuksiewicz et al., 2018), ovarian (Høgdall et al., 2003, Dupont et al., 2004), hepatocellular (Zhu et al., 2012), pancreatic (Palmquist et al., 2020), and lung cancer (Xu et al., 2014) as well as melanoma (Ismail et al., 2019).

IL-6 is a proinflammatory cytokine, more specifically a 21–28 kDa 4-helix bundled glycoprotein (Rossi et al., 2015), which is secreted by macrophages and various cancer cells (Tanaka et al., 2016, Yeo et al., 2019). It was first shown to induce B lymphocytes to produce immunoglobulin and to stimulate hepatocytes (Hirano et al., 1986). IL-6 is involved in several biological processes including autoimmune diseases (Jones et al., 2018) and cancer (Yeo et al., 2019, Vainer et al., 2018). IL-6 stimulates the production of YKL-40 and CRP (Tanaka and Kishimoto, 2012, Nielsen et al., 2011, Nishikawa et al., 2008), and it has been shown to induce tumor growth and metastasis (Zhang et al., 2018a, Jayatilaka et al., 2017). In addition, IL-6 is involved in the formation of a pro-metastatic niche in the liver, and thus, liver metastasis (Lee et al., 2019b). In pancreatic cancer, elevated serum IL-6 alone and in combination with YKL-40 has been associated with shorter overall survival (Chen et al., 2020).

CRP is the first identified acute-phase protein (Abernethy and Avery, 1941), and it was originally named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae*. CRP is produced by hepatocytes (Uete et al., 1971), even though other sites of synthesis have also been suggested. It has been widely used in the clinic for decades as a biomarker of systemic inflammation and tissue damage (Pepys and Baltz, 1983, Pepys and Hirschfield, 2003). At present, growing interest is focused on high-sensitivity CRP (hsCRP), which is more precise than standard CRP and can be measured at very low concentrations by high-sensitivity assays (Pearson et al., 2003).

Recently, the interest in CRP as a possible cancer biomarker has grown. Elevated serum and plasma levels of CRP have been associated with impaired prognosis in several cancers, such as prostate (Liao et al., 2020, Stikbakke et al., 2020), cervical (Wang et al., 2020), lung (Pastorino et al., 2019), and colorectal cancer (Matsubara et al., 2020, Kim et al., 2018, Thomsen et al., 2016). In CRC with liver metastases, elevated CRP before liver resection has been found to be a strong predictor of impaired prognosis (Kostner et al., 2016).

7.1.6 Matrix metalloproteinases 2, 8, and 9 (MMP-2, -8, and -9)

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are structurally related but genetically distinct. They are enzymes capable of degrading almost all proteins in the extracellular matrix (ECM). The first metalloproteinase, collagenase, was found in 1962 (Gross and Lapiere, 1962), and since then, altogether 28 members in vertebrates have been identified. Of these, at least 23 are expressed in human tissues. MMPs can be divided into six categories according to substrate specificity, sequential similarity, and domain organization (Laronha and Caldeira, 2020, Wang and Khalil, 2018) (**Table 4**). They are secreted by various cells and tissues as inactive (latent) proMMPs, which contain a cysteine switch. When the switch is cleaved by other proteolytic enzymes, such as other MMPs, they become activated (Nagase et al., 2006, Cui et al., 2017).

MMPs have several important functions in tissue remodeling, inflammation, and pathogenesis. By degrading ECM and non-matrix proteins, they facilitate malignant tumor growth and invasion. They can also regulate immune responses by processing different bioactive substances including growth factors, cytokines, chemokines, and serum proteins (Sorsa et al., 2006). The activity of MMPs increases significantly in inflammatory diseases and malignancies (Sorsa et al., 2004).

The proteolytic activity of the MMPs is regulated via mRNA expression, activation of the proenzymes, and by endogenous proteins (Visse and Nagase, 2003, Cui et al., 2017). It has been demonstrated that trypsin-2, the major isoenzyme of human tumor-associated trypsinogens, can directly activate collagenolytic MMPs (Moilanen et al., 2003). TATI and tissue inhibitors of metalloproteinases (TIMPs), in turn, regulate and inhibit MMPs (Chirco et al., 2006, Herszényi et al., 2012).

MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are both type IV collagenases, and they are involved in cancer invasion and metastasis. MMP-2 is mainly produced by dermal fibroblasts, leukocytes, and platelets (Saito et al., 2001, Seizer and May, 2013). MMP-9 is produced by various cells including epithelial cells, fibroblasts, macrophages, granulocytes, and T-cells (Cui et al., 2017, Wang and Khalil, 2018).

Increased tumor expression of both MMP-2 and MMP-9 has been associated with poor prognosis in recurrent glioblastoma (Zhou et al., 2019) and gastric cancer (Yao et al., 2017, Mrena et al., 2006, Zhang et al., 2003). MMP-2 expression has indicated impaired prognosis also in several other malignancies including colorectal (Deng et al., 2017), breast (Talvensaari-Mattila et al., 2003), esophageal (Ao et al., 2013), and endometrial cancer (Honkavuori-Toivola et al., 2013) as well as osteosarcoma (Zhang and Zhang, 2015). MMP-9 expression in tumor tissue, on the other hand, has been associated with improved prognosis in extrahepatic bile duct cancer (Park et al., 2018) and HCC (Chen et al., 2012). In CRC, interestingly, positive expression has indicated both impaired prognosis (Chu et al., 2012) and improved prognosis (Koskensalo et al., 2012b).

Contrary to the tissue expression of MMP-9, its serum or plasma concentrations have not had significant prognostic value despite of active investigation. By far, this has been noted in CRC (Liang and Chang, 2018), lung cancer (Gong et al., 2016), and HCC (Lempinen et al., 2013).

MMP-8 (collagenase-2, neutrophil collagenase) is produced mainly by neutrophils and macrophages (Wang and Khalil, 2018), but also by several other cell types (Van Lint and Libert, 2006). Besides degrading the ECM, MMP-8 has been found to impair migration of the tumor cells via transforming growth factor-β1 (TGF-β1) and vascular endothelial growth factor-C (VEGF-C) in oral squamous cell cancer (Åström et al., 2017). As the expressions of other MMPs, those of MMP-8 in tumor tissue and serum have been widely investigated in different malignancies. In CRC patients, the serum levels have been shown to be significantly increased compared to healthy subjects (Väyrynen et al., 2012).

The results concerning the prognostic value of the tissue expression of MMP-8 in malignant tumors have been variable (Juurikka et al., 2019), and MMP-8 has primarily been reported to have tumor-suppressing qualities. In breast cancer models, MMP-8 has prevented invasion and metastasis (Agarwal et al., 2003, Montel et al., 2004), and the expression in breast cancer tumors has correlated with lower incidence of lymph node metastases (Gutiérrez-Fernández et al., 2008). MMP-8 has also been shown to protect against the development of skin tumors in mice (Balbín et al., 2003), and its expression has associated with improved survival in squamous cell cancer of the mobile tongue (Korpi et al., 2008).

On the contrary, elevated serum concentrations of MMP-8 have been associated with impaired prognosis in CRC (Sirniö et al., 2018, Böckelman et al., 2018, Väyrynen et al., 2012) and HCC (Lempinen et al., 2013). In gastric cancer, both elevated and low serum concentrations have indicated poor survival (Laitinen et al., 2018). Based on these findings, it seems possible that the tissue expression and serum concentrations of MMP-8 have distinct

prognostic value. Sirniö et al. found that there was no correlation between the density of tumor-infiltrating neutrophils and serum MMP-8 levels, suggesting that the neutrophils in circulation might be a source of serum MMP-8 (Sirniö et al., 2018). In addition, it is worth mentioning that Linkov et al. observed that MMP-8 levels in serum are unique to each person, indicating that knowing the individual baseline for every patient might help using MMP-8 as a prognostic biomarker (Linkov et al., 2009).

Table 4. The human matrix metalloproteinase (MMP) family grouped according to substrate specificity, sequential similarity, and domain organization.

Group	**MMPs included**
Collagenases	1, 8, 13
Gelatinases	2, 9
Stromelysins	3, 10, 11
Matrilysins	7, 26
Membrane-type MMPs (MT-MMPs)	14 (MT 1), 15 (MT 2), 16 (MT 3), 17 (MT 4), 24 (MT 5), 25 (MT 6)
Other MMPs	12, 19, 20, 21, 23, 27, 28

Adapted from (Laronha and Caldeira, 2020, Wang and Khalil, 2018, Visse and Nagase, 2003).

7.1.7 Myeloperoxidase (MPO)

MPO is a heme-containing lysosomal enzyme that is found mainly in neutrophilic granulocytes and in smaller quantities also in monocytes and macrophages (Krawisz et al., 1984). Inflammation induces its production by those cells. When activated, MPO uses H_2O_2 to oxidize chloride ions and other halides, which leads to the formation of hypochlorous acid (HOCl), among others (Gomez-Mejiba et al., 2010, Klebanoff et al., 2013). HOCl can subsequently oxidize DNA in inflammatory cells and surrounding epithelial cells. During this process, DNA-centered free radicals are formed, which leads to the production of several oxidation products. DNA damage causes increased mutations and alters the function of enzymes and proteins that subsequently contribute to carcinogenesis (Ohshima et al., 2003). In addition, MPO can activate latent MMPs via the production of HOCl (Weiss et al., 1985, Saari et al., 1990).

On the other hand, MPO is thought to be needed for the defensive immune responses to function optimally. MPO can oxidatively inactivate pathogenic microbes as well as tissue inhibitor of metalloproteinases-1 (TIMP-1) (Sorsa et al., 2006), and it is known that patients with genetic MPO deficiency can have increased number of infections of variant degree of severity (Parry et al., 1981).

In cancer, MPO is an emerging biomarker, but there are by far only a few studies on its prognostic value. High infiltration of MPO-expressing neutrophilic granulocytes in tumors has been associated with favorable prognosis in both colorectal and breast cancer (Droeser et al., 2013, Zeindler et al., 2019). In a study by Coelho et al., serum levels of MPO were found to be higher in breast cancer patients than in healthy volunteers, and they associated with lymphovascular invasion negativity (Coelho et al., 2014). Thus, it seems possible that MPO has a protective role in cancer, but its prognostic value remains to be established.

7.1.8 Transketolase-like protein 1 (TKTL1)

Tumor cells require energy for survival and proliferation. Unlike cells in healthy tissues, they are able to consume glucose and to convert it to lactate even in the presence of oxygen. This phenomenon is known as the Warburg effect, and it was originally observed by Otto Warburg already in 1924 (Warburg et al., 1924). Normally, degradation of glucose happens via two main pathways: the Embden-Meyerhof pathway, and the pentose phosphate pathway (PPP). The non-oxidative part of the PPP is catalyzed by transketolase (TKT) enzyme reactions, which enable oxygen-independent glucose degradation.
Three human transketolase genes encoding corresponding enzymes have been identified: *TKT*, transketolase-like 1 (*TKTL1*), and transketolase-like 2 (*TKTL2*) (Coy et al., 2005, Langbein et al., 2006). Of the three enzymes, TKTL1 is suggested to account for the tumor-specific effects of transketolase enzyme reactions (Langbein et al., 2006), and its role in tumor cell metabolism and cancer progression has been under active investigation in recent years.

By far, the expression of TKTL1 in tumor tissue has been associated with impaired survival in CRC (Ahopelto et al., 2016, Schwaab et al., 2011, Langbein et al., 2006), laryngeal squamous cell carcinoma (Völker et al., 2007), non-small cell lung cancer (Kayser et al., 2011), and gastric cancer (Ahopelto et al., 2020, Song et al., 2015). In addition, Diaz-Moralli et al. (Diaz-Moralli et al., 2011) found that the expression of TKTL1 in primary colorectal tumors increased progressively in disease stages I to III, but in stage IV, there was a strong decrease, suggesting that the expression pattern changes along with metastasis formation.

7.2 Other potential biomarkers in CRC

Several biomarkers have raised interest as potential predictors of the outcome of CRC patients in the past years. Carbohydrate antigens CA12-5, CA19-5, CA242, CA50, and CA72-4 have been reported to be inferior to CEA and CA19-9. In some cases, however, CA242 has shown superior prognostic value compared to CA19-9 in predicting 5-year relapse-free survival (Yang et al., 2012).

Some studies have evaluated the prognostic value of matrix metalloproteinases 7 (Vočka et al., 2019), 13, and 21 (Huang et al., 2011) as well as TIMP-1 (Böckelman et al., 2018, Vočka et al., 2019), and the results have been promising. Other biomarkers of interest include, for example, vascular endothelial growth factor (VEGF) (Mohamed et al., 2019) and inflammatory cells, mainly neutrophils, T-cells, and macrophages (Governa et al., 2017, Wikberg et al., 2017).

7.3 Genetic alterations

7.3.1 Mutations

The most important genetic mutations observed in CRC are those in the *RAS* (*KRAS*, *NRAS*, and *HRAS*), *BRAF*, *p53*, and *PIK3CA* genes, and the most common of these are the *KRAS* and *BRAF* mutations. KRAS and BRAF are both proteins that belong to the RAS-RAF-MAPK signaling pathway, which mediates signals from outside a cell to the nucleus and, thus, regulates the growth, differentiation, proliferation, migration, and apoptosis of the cell. The *KRAS* gene encodes a guanosine triphosphatase (GTPase) protein called KRAS, and the *BRAF* gene a serine/threonine kinase, which is a protein belonging to the RAF family. KRAS can be activated by stimuli from the transmembrane EGF receptor, and the activation of KRAS, in turn, activates the BRAF kinase. Mutations in either the *KRAS* or *BRAF* gene cause a continuous activation of the corresponding protein, leading to uncontrolled dividing and growth of tumor cells via the signaling cascade (Santarpia et al., 2012).

RAS mutations are present in 43–58% of CRCs, most typically in right-sided tumors (Serebriiskii et al., 2019, Rimbert et al., 2018, Loree et al., 2018), and they have been shown to predict non-responsiveness to EGFR inhibitor therapy (Di Fiore et al., 2007, Lièvre et al., 2008). *BRAF* mutations occur in 10–22% of CRCs, also more often in right-sided disease. They are predictive of particularly poor prognosis and resistance to EGFR inhibitors unless the carcinoma shows microsatellite instability (Phipps et al., 2015).
Other mutations that associate with impaired prognosis include *TP53* mutations, which have been reported to be present in approximately 50% of CRCs, and *PIK3CA* mutations that occur in 10–30% of the cases (Therachiyil et al., 2020, Tejpar et al., 2010, Ogino et al., 2009). In addition, chromosome 18q loss of heterozygosity (18qLOH) (Sarli et al., 2004) has been associated with impaired overall survival. Other genes that are under active studying include *PTEN*, *UGT1A1*, *CTNNB1*, *FGFR3*, *GNAS*, *HER2*, and *SMAD4*. The prevalence of the mutations depends on the location of the primary tumor (Loree et al., 2018).

The research on these genes and their prognostic as well as predictive significance continues. At the Helsinki University Hospital, the mutation status has been routinely tested in all patients with metastatic CRC since 2013.

7.3.2 Microsatellite instability (MSI)

Microsatellites are repeated DNA sequences, as described in the previous **section 3.1**, and the MSI status in colorectal tumors is a defining factor of prognosis and response to treatment. In addition, it has importance as a screening tool for hereditary CRC.
The MSI status is classified as follows: either 1) high degree of instability (MSI-H), or 2) low degree of instability (MSI-L) or microsatellite stable (MSS), together referred to as non-MSI-H. In the latter, the tumor has no characteristics of MSI. In addition, the presence or absence of a functional MMR system, which indirectly demonstrates the MSI status, can be classified as 1) deficient MMR (dMMR), leading to a mutator phenotype, or 2) proficient MMR (pMMR), meaning that small errors during DNA replication are repaired normally. Approximately 15% of sporadic CRCs are defined as MSI-H/dMMR tumors (Lee and Chu, 2018, Lech et al., 2016).

MSI-H/dMMR status is predictive of a good prognosis, independently of the stage of CRC, and non-MSI-H of a poor outcome (De' Angelis et al., 2018). This is thought to be caused by a strong antitumoral response in the patient. As the mutations accumulate in the MSI-H/dMMR tumors, the immune system is activated, and an increased cytolytic response in the patient is triggered, leading to elimination of the tumor cells (Drescher et al., 2009). MSI-H/dMMR CRCs have been found to be insensitive to 5-FU (Argiles et al., 2020), which affects the choice of oncological treatment.

At the Helsinki University Hospital, the MSI status has been tested in all patients with metastatic CRC since 2018.

7.4 Emerging technologies

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs. MiRNA genes are prone to damage because of their chromosomal location. In CRC, miRNAs have been shown to be frequently deregulated, and this can lead to silencing tumor suppressor genes or activating oncogenes and, consequently, cancer progression (Lin and Gregory, 2015, Yiu and Yiu, 2016). MiRNAs can be detected in serum or plasma, various body fluids, and tissue samples, and they are considered potential markers for screening, prognosis, and response to treatment in CRC.

Liquid biopsies constitute an emerging technology, which has great potential in the detection and management of CRC and is likely to be applied to clinical use shortly. Liquid biopsy testing means analyzing different biomarkers in any body fluid, for example, blood, urine, or cerebrospinal fluid. Through liquid biopsies, circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), cell-free DNA (cfDNA), RNA, miRNA, and several different proteins can be analyzed (Normanno et al., 2018).

AIMS OF THE STUDY

The aim of this thesis was to evaluate the prognostic significance of selected biomarkers in colorectal cancer with resectable liver metastases. The study material consisted of patients undergoing curative-intent liver resection.

The specific aims were to evaluate the associations of the following biomarkers with disease-free/relapse-free survival and overall survival after liver resection:

1. Pre- and postoperative CEA and CA19-9 in serum (I and II)

2. Pre- and postoperative hCGβ and TATI in plasma (I)

3. Pre- and postoperative YKL-40, IL-6, and CRP in serum combined with CEA and CA19-9 in serum (II)

4. Tissue expressions of MMP-2, MMP-8, and MMP-9 in primary colorectal tumors and liver metastases as well as pre- and postoperative MMP-8, MMP-9, and MPO in serum (III)

5. Tissue expression of TKTL1 in primary colorectal tumors and liver metastases (IV).

MATERIALS AND METHODS

1. PATIENTS (I–IV)

Altogether 462 patients with colorectal cancer metastasized to the liver underwent their first liver resection with a curative intent at the Helsinki University Hospital between the years 1998 and 2013. The primary colorectal tumors were operated on at different hospitals in Finland between 1988 and 2013. Of all patients, 20 were excluded from these studies because of either extrahepatic metastases that could not be operated on or non-curative liver resection.
These studies are based on the serum samples from all patients (I–III), the plasma samples from a subset of 168 patients (I), and the tissue samples from a subset of 111 patients (III and IV) who had both their primary colorectal tumors and liver metastases operated on at the Helsinki and Uusimaa Hospital District between 1988 and 2007. The designs of the four studies are shown in **Table 5**.

Liver metastases were defined as synchronous, if they were diagnosed at the same time with the primary colorectal tumor or within six months after the operation on the primary tumor. Liver resection was considered major, if more than two Couinaud's segments were removed.

Clinical data were retrieved from patient records, and information about the dates of death was obtained from the Central Statistical Office of Finland (TK-53-1004-9). The studies were approved by the Ethics Committee at the Helsinki University Hospital (IRB99/07/01; HUS531/E6/01; HUS460/E6/05; HUS323/13/3/2008; HUS242/13/03/02/2011; Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013). Collection and analysis of blood and tissue samples were approved by the National Supervisory Authority for Welfare and Health (Valvira) (STM Dno 4858/04/047/08; Valvira Dnro 10041/06.01.03.01/2012).

2. SERUM AND PLASMA SAMPLES (I–III)

The serum and plasma samples (I–III) were drawn in conjunction with the liver resection, before and approximately 3 months after the operation. The median time between preoperative sampling and the liver resection was 17 days (IQR 9–29 days), and the median time between the resection and postoperative sampling 94 days (IQR 89–98 days). The samples of CEA, CA19-9, and CRP were analyzed when taken. The samples of hCGβ, TATI, YKL-40, IL-6, MMP-8, MMP-9, and MPO were centrifuged within ½ to 2 hours, and they were stored at -80° Celsius until analyzed.

Table 5. Study periods, numbers of patients included, and biomarkers investigated.

Study	Patients included (N)	Primary tumors operated (years)	Liver resections performed (years)	Data cut-off date	Biomarkers investigated	Biomarkers determined in
I	168	1988–2007	1998–2007	June 17th, 2015	CEA CA19-9 hCGβ TATI	- Serum (CEA, CA19-9) - Plasma (TATI, hCGβ)
II	441	1988–2013	1998–2013	October 31st, 2017	CEA CA19-9 YKL-40 IL-6 CRP	- Serum
III	111	1988–2007	1997–2007	January 11th, 2019	MMP-2 MMP-8 MMP-9 MPO	- Tissue samples of primary colorectal tumors and liver metastases (MMP-2, MMP-8, MMP-9) - Serum (MMP-8, MMP-9, MPO)
IV	111	1988–2007	1997–2007	October 31st, 2017	TKTL1	- Tissue samples of primary colorectal tumors and liver metastases

The serum concentrations of CEA and CA19-9 were determined with either an immunoenzymatic assay, Bayer Immuno 1 (CEA: 1998–10/2005; and CA19-9: 1998–1/2006), or an immunochemiluminometric assay, Abbott Architect (CEA: 10/2005–2013; and CA19-9: 1/2006–2013), and those of CRP with an immunoturbidimetric method. The samples were analyzed at HUSLAB laboratories, Helsinki University Hospital, and the results were retrieved from clinical records. The serum levels of CEA were expressed as µg/l, those of CA19-9 as kU/l, and those of CRP as mg/l.

The plasma concentrations of hCGβ were quantified by an immunofluorometric assay (IFMA) based on monoclonal antibodies (Stenman et al., 2004), and those of TATI by a time-resolved IFMA (Janeiro et al., 2012). The detection limits were 0.5 pmol/l for hCGβ and 0.15 µg/l for TATI.

The serum concentrations of YKL-40 and IL-6 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (YKL-40: MicroVue YKL-40 ELISA [Catalog #8020], Quidel Corporation, San Diego, CA, USA; and IL-6: Quantikine HS600B, R&D Systems, Abingdon, OX, UK). The detection limit for YKL-40 was 20 ng/ml, and the intra- and

interassay CVs <5% and <6%, respectively (Bojesen et al., 2011). The detection limit for IL-6 was 0.01 pg/ml, and the intra- and interassay CVs ≤8 and ≤11%, respectively (Knudsen et al., 2008).

The serum concentrations of MMP-8 were measured by a time-resolved IFMA (Mauramo et al., 2018). The monoclonal MMP-8-specific antibodies 8708 and 8706 (Oy Medix Biochemica Ab, Espoo, Finland) were used as a catching and a tracer antibody, respectively. The tracer antibody was labeled using europium chelate (Hemmilä et al., 1984). The assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM $CaCl_2$, 50 µM $ZnCl_2$, 0.5% BSA, 0.05% sodium azide, and 20 mg/l DTPA. Samples were first diluted in assay buffer and incubated for 1 hour, and then they were incubated for 1 hour with the tracer antibody. Enhancement solution was added, and after 5 minutes, fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The detection limit for MMP-8 was 0.08 ng/ml and the CV 7.3%.
The serum concentrations of MMP-9 and MPO were determined using commercially available ELISA kits according to the manufacturers' instructions (MMP-9: Amersham BioSciences UK Ltd., Buckinghamshire, UK; and MPO: Immundiagnostik AG, Bensheim, Germany). The detection limit for MMP-9 was 0.6 ng/ml (Mäkitalo et al., 2012) and that for MPO 1.6 ng/ml (Pradhan-Palikhe et al., 2010).

3. TUMOR TISSUE SPECIMENS (III and IV)

The tissue samples of primary colorectal tumors and liver metastases were fixed in formalin and embedded into paraffin immediately after the operations. The paraffin-embedded specimens and the hematoxylin-eosin stained slides made from each block were obtained from the Department of Pathology of the Helsinki University Hospital. One block of primary tumor tissue and one of liver metastasis tissue were chosen for each patient. In case of several tumor samples, the blocks with the largest amount of viable (non-necrotic) tumor tissue were chosen. Representative tumor areas and spots of healthy tissue were marked on the corresponding slides (RP) and verified by an experienced pathologist (JH). Six punches of 1,000 µm in diameter were taken from the tumor areas and two from the healthy tissue areas of each block. The punches were taken with a semiautomatic tissue microarray (TMA) Grand Master 3D instrument (Histech Ltd, Budapest, Hungary) and mounted on TMA paraffin blocks. Two series of blocks were subsequently constructed, both containing three samples of the patients' colorectal tumor tissue, three samples of liver metastasis tissue, and one sample of both healthy colorectal and liver tissue.

4. IMMUNOHISTOCHEMISTRY (III and IV)

TMA blocks were cut into 4-µm sections for immunohistochemistry and dried on the slides for 12–24 hours in 37° Celsius. The sections were then deparaffinized in xylene and rehydrated through a graded series of ethanol and distilled water. For antigen retrieval, Tris-EDTA (pH 9) (MMP-2 and MMP-8) or Tris–HCl buffer (pH 8.5) (MMP-9 and TKTL1) was used, and the samples were heated in the PreTreatment module (Agilent Dako, Lab Vision Corp., Fremont, CA, USA) for 20 minutes at 98°C.

Staining process was performed with the Lab Vision Autostainer 480 (LabVision Corp., Fremont, CA, USA) using Dako Real EnVision (ENV). First, the samples were incubated for 5 minutes to inactivate endogenous peroxidases. Second, the slides were incubated with the chosen primary antibodies (**Table 6**) at room temperature using the Dako REAL Antibody Diluent S2022 (Dako). Third, the slides were incubated with the secondary antibody (Dako Real ENV rabbit/mouse HRP antibody) for 30 minutes, and then visualized with Dako Real ENV Dab chromogen kept on glass for 10 minutes. Between each step of the staining procedure, the slides were washed with PBS containing 0.04% Tween 20. The slides were counterstained with Dako Mayer's Hematoxylin S3309, dehydrated through a graded series of water, ethanol, and xylene (aqua–70% ethanol–96% ethanol–absolute alcohol–xylene), and finally mounted using Pertex® Histolab mounting medium.
Melanoma and tongue cancer specimens were used as positive controls for MMP-2, MMP-8, and MMP-9, and gastric and colon cancer as those for TKTL1.

Table 6. Antibodies used in the immunohistochemical stainings.

Biomarker	Antibody	Company	Dilution (incubation)	Staining	Study
MMP-2	Mouse monoclonal anti-MMP-2 (Clone CA-4001)	Lab Vision Corporation, Fremont, CA, USA	1:50 (O/N)	Cytoplasmic	III
MMP-8	In-house (Sorsa et al., 1994), polyclonal		1:400 (O/N)	Inflammatory cells	III
MMP-9	Mouse monoclonal anti-MMP-9 (MS-817-PO)	NeoMarkers, Fremont, CA, USA	1:1500 (1 hour)	Granular cytoplasmic	III
TKTL1	Mouse monoclonal anti-human TKTL1 (RIDA PentoCheck IHC, clone JFC12T10)	R-Biopharm AG, Darmstadt, Germany	1:200 (O/N)	Cytoplasmic	IV

O/N: overnight.

5. SCORING (III and IV)

The immunohistochemical staining intensities of colorectal tumor tissue and liver metastasis tissue were scored independently by two researchers (study III: RP and JH; study IV: KA and JH) without knowledge of the clinical data. In case of different scoring results, the samples were re-evaluated until consensus was reached. The highest score was chosen to represent each sample. Tissue spots without tumor cells or with too few cells for adequate judging were excluded.
The maximum number of tumor tissue spots per slide was 56, and the area of each spot was 0,79 mm^2. As there were altogether 6 spots of both colorectal tumor and liver metastasis tissue per patient, the analyzed area was approximately 4.7 mm^2 of colorectal tumor and 4.7 mm^2 of liver metastasis tissue per patient.

The scoring of MMP-2 (III) was based on the intensity of cytoplasmic staining in tumor cells as follows: 0: no positivity in any cancer cells; 1: mild staining in all or some cancer cells; 2: moderate staining in most cancer cells; and 3: strong staining in all cancer cells (**Figure 4**, page 59).

The expression of MMP-8 (III) was graded depending on the amount of stained inflammatory cells in immediate proximity to the tumor cells as follows: 0: no staining or only one stained granulocyte; 1: some stained granulocytes; 2: several stained granulocytes; and 3: abundantly of stained granulocytes (**Figure 4**).

The expression of MMP-9 (III) was scored based on the intensity of granular cytoplasmic staining in tumor cells as follows: 0: no staining; 1: mild granulary staining in some cancer cells; 2: moderate granulary staining in all or some cancer cells; and 3: strong granulary staining in all cancer cells (**Figure 4**). In liver tissue, no staining or only mild granulary staining was seen as well as some stained inflammatory cells.

The scoring of TKTL1 expression (IV) was based on the intensity of cytoplasmic staining in tumor cells as follows: 0: no staining; 1: mild staining; 2: moderate staining; and 3: strong staining (**Figure 4**). In normal liver tissue, TKTL1 expression was visible in epithelial cells, in inflammatory cells, and in the stroma.

6. STATISTICAL ANALYSES (I–IV)

The distributions of the biomarkers in different subgroups of patients were assessed using the Mann-Whitney U test and the Kruskal-Wallis H test. Correlations between the biomarkers or the biomarkers and clinicopathological variables were estimated with the Spearman's correlation test (I–IV), Wilcoxon signed-rank test (II), and the Fisher's exact test (III). Cut point analyses for CEA, CA19-9, hCGβ, TATI, YKL-40, IL-6, CRP, MMP-8, MMP-9, and MPO (I–III) were performed using ROC curves and calculating the area under the curve.

Overall survival (OS) was calculated from the date of the liver resection to the date of death from any cause, and disease-free/relapse-free survival (DFS [I, III, and IV] / RFS [II]) was defined as the time from the liver resection to the first recurrence of CRC or death. Patients alive without recurrence were censored at the end of follow-up. Univariate and multivariate survival analyses were performed using Cox proportional hazards model. Survival curves were created according to the Kaplan-Meier method and compared with the log-rank test.
The variables included in the multivariate Cox regression analyses (I and II) were the following: study I: serum or plasma concentrations of the investigated biomarkers (CEA, CA19-9, hCGβ, and TATI), type of liver metastases (synchronous/metachronous), and adjuvant chemotherapy (yes/no); and study II: serum concentrations of the biomarkers (YKL-40, IL-6, CRP, CEA and CA19-9), age, gender, location of the primary tumor, type of liver metastases (synchronous/metachronous), type of liver resection (minor/major), the number and size of the liver metastases, and the resection margins (R0/1/2). In studies III and IV, multivariate analyses were not performed, because it was estimated that their statistical reliability would be controversial due to the quite small patient cohort.

Statistical analyses were carried out and survival curves were created using SPSS Statistics versions 22 (I) and 25 (III and IV) (SPSS Inc., Chicago, IL, USA) or the statistical software R version 3.3.3 (R Core Team, Vienna, Austria) (II). In studies I, III, and IV, the biomarkers were included in the survival analyses using specified cut-off values. In studies II and IV, they were also included as $\log_2$-transformed continuous variables. A p-value of less than 0.05 was considered significant, and statistical trend was defined as $p<0.10$.

RESULTS

1. PATIENT CHARACTERISTICS (I–IV)

The clinicopathological characteristics of the patients included in the four studies are presented in **Table 7**. The median age of all patients at the time of the operation on the primary tumor was 63.3 years (min. 31.3–max. 82.1) and at the time of the liver resection 64.8 years (min. 33.0–max. 84.0). Altogether 58.8% (260/442) of the patients were male, and 49.1% (217/442) of them were over 65 years old at the time of the liver resection. The primary tumor was located in the rectum in 41.6% (184/442), in the left colon or rectosigmoid junction in 39.4% (174/442), and in the right or transversal colon in 19.0% (84/442) of the cases. The median time between the operation on the primary tumor and the liver resection was 11.4 months (interquartile range [IQR] 5.9–25.4).
Within the follow-up until January 11th, 2019, disease-free survival for all patients was 41.4% (183/442) at three years and 34.4% (152/442) at five years after liver resection. The 5-year overall survival rate was 56.8% (251/442). The median time to recurrence after liver resection was 9.8 months (IQR 3.5–17.7).

2. CONCENTRATIONS AND EXPRESSIONS OF THE BIOMARKERS (I–IV)

2.1 Biomarkers in serum and plasma (I–III)

The median concentrations and the numbers of elevated biomarkers in studies I–III are presented in **Table 8**. In the statistical analyses, the same cut-off values were used for both pre- and postoperative biomarker concentrations, except for those of MMP-8 (study III).

2.1.1 CEA and CA19-9 (I and II)

In study I (N=168), the cut-off value for CEA was 5.0 µg/l and that for CA19-9 26 kU/l according to the reference limits that are routinely used in Finland. In study II (N=441), the cut-off values were calculated using ROC curve analyses in relation to 3-year RFS and 3-year OS, and the cut points were 5.0 µg/l for CEA and 37 kU/l for CA19-9.

Table 7. Characteristics of the patients included in the four studies.

Characteristic	n (%) or median (min.–max.)		
	Study I	**Study II**	**Studies III and IV**
N	168	441	111
Gender			
Male	101 (60.1)	260 (59.0)	64 (57.7)
Female	67 (39.9)	181 (41.0)	47 (42.3)
Age at the liver resection			
Median (years)	64.3 (36.3–81.5)	64.9 (33.0–84.0)	64.4 (36.3–81.5)
>65 years	80 (47.6)	217 (49.2)	53 (47.7)
Primary tumor			
Rectum	67 (39.9)	183 (41.5)	48 (43.2)
Left colon or rectosigmoid junction	Left and right:	174 (39.5)	44 (39.6)
Right or transversal colon	101 (60.1)	84 (19.0)	19 (17.1)
Primary TNM staging			
Dukes' A–B[1]	40 (23.8)	-	-
Dukes' C–D[1]	122 (72.6)	-	-
Missing data	6 (3.6)	-	-
T1–2	17 (10.1)	49 (11.1)	16 (14.4)
T3–4	109 (64.9)	339 (76.9)	87 (78.4)
Missing data	42 (25.0)	53 (12.0)	8 (7.2)
N0	45 (26.8)	121 (27.4)	35 (31.5)
N1	51 (30.4)	112 (25.4)	41 (36.9)
N2	28 (16.7)	106 (24.0)	28 (25.2)
Missing data	44 (26.2)	102 (23.1)	7 (6.3)
Liver metastases			
Synchronous[2]	89 (53.0)	254 (57.6)	66 (59.5)
Metachronous	79 (47.0)	187 (42.4)	45 (40.5)
Number, median (max.)	1 (8)	1 (16)	1 (8)
Largest diameter, median (max.)	2.6 cm (10.0)	2.5 cm (15.0)	2.5 cm (9.0)
Resection margins[3]			
R0	157 (93.5)	412 (93.4)	102 (91.9)
R1	11 (6.5)	19 (4.3)	8 (7.2)
R2	0	9 (2.0)	0
Missing data	0	1 (0.2)	1 (0.9)
Follow-up time, living patients (years)	10.8 (8.1–16.9)	8.6 (4.7–19.7)	15.2 (11.5–20.3)
5-year disease-free survival	51 (30.4)	152 (34.5)	33 (29.7)
5-year overall survival	83 (49.4)	252 (57.1)	66 (59.5)
10-year overall survival	57 (33.9)	-	41 (36.9)
Recurrence or death within follow-up			
Yes	127 (75.6)	318 (72.1)	88 (79.3)
No	41 (24.4)	123 (27.9)	23 (20.7)

[1] Dukes' classification used only in Study I. TNM classification presented according to availability.
[2] Synchronous: diagnosed within 6 months after the operation on the primary tumor.
[3] R0: resection margin histologically free; R1: histologic neoplastic infiltration; R2: macroscopic neoplastic infiltration.

Of all patients combined, 49.7% (219/441, missing 1) had a normal CEA level (≤5.0 µg/l) preoperatively. Of these CEA-negative patients, 31 (14.2%) had a CA19-9 level of >26 kU/l and 17 (7.8%) that of >37 kU/l. On the other hand, altogether 71.0% (311/438, missing 4) of the patients had a normal CA19-9 level (≤26 kU/l) preoperatively, and of those patients, 39.9% (124/311) had elevated CEA. In total, 22.0% (96/437, missing 5) of the patients had elevated CEA (>5.0 µg/l) and a CA19-9 level of >26 kU/l preoperatively, and 19.0% (83/437) elevated CEA and a CA19-9 level of >37 kU/l.

After liver resection, 83.2% (367/441, missing 1) of the patients had a normal CEA level (≤5.0 µg/l), and of those, 36 (9.8%) had a CA19-9 level of >26 kU/l and 19 (5.2%) that of >37 kU/l. Altogether 85.6% (374/437, missing 5) of the patients had a CA19-9 level of ≤26 kU/l postoperatively, and of those, 12.6% (47/374) had elevated CEA. Only 6.2% (27/437, missing 5) of the patients had postoperatively elevated CEA and CA19-9 >26 kU/l, and 5.5% (24/437) elevated CEA and CA19-9 >37 kU/l.

2.1.2 HCGβ and TATI (I)

The cut-off value for hCGβ was based on the 75th percentiles and that for TATI on the medians of both pre- and postoperative plasma concentrations, as the ROC curve analyses did not provide statistically significant cut points. The cut-off value for hCGβ was 1.0 pmol/l, and that for TATI 13 µg/l.

2.1.3 YKL-40, IL-6, and CRP (II)

The cut-off values for YKL-40, IL-6, and CRP were chosen based on the cut points with a sensitivity closest to 80% in the ROC curve analyses in relation to 3-year RFS and 3-year OS. An age-corrected 95th percentile in healthy individuals was chosen for YKL-40, and it was calculated as suggested by Bojesen et al. (Bojesen et al., 2011). The YKL-40 percentiles were derived as follows: percentile=100/(1+(YKL-40^-3)*(1.062^age)*5000).
The cut-off value for IL-6 was 4.95 pg/ml, and that for CRP 5 mg/l.

2.1.4 MMP-8, MMP-9, and MPO (III)

For MMP-8, the tertiles of the preoperative values were used as cut points, as the ROC curve analyses lacked statistically significant results. The cut-off values were the following: preoperative MMP-8: 29.6 and 76.2 ng/ml; and postoperative MMP-8: 20.8 and 56.1 ng/ml.
The cut-off levels for MMP-9 and MPO were chosen based on the ROC curve analyses in relation to 5-year DFS, and they were the following: MMP-9: 77.7 ng/ml; and MPO: 218.6 ng/ml.

Table 8. Studies I–III: Concentrations of the biomarkers measured in serum/plasma before liver resection and three months after the operation.

Study	Serum biomarkers	Conc.	Preoperative	Postoperative
I	CEA (N)	µg/l	168	168
	Median (IQR)		7.2 (3.0–31.8)	2.4 (1.5–3.9)
	>5.0 µg/l, n (%)		94 (56.0)	27 (16.1)
II	CEA (N)		440	440
	Median (IQR)		5.2 (2.5–18.8)	2.4 (1.4–4.1)
	>5.0 µg/l, n (%)		221 (50.2)	74 (16.8)
I	CA19-9 (N)	kU/l	168	168
	Median (IQR)		16 (5–59)	7 (<5–18)
II	>26 kU/l, n (%)		60 (35.7)	24 (14.3)
	CA19-9 (N)		437	436
	Median (IQR)		13 (5–32)	9 (4–16)
	>37 kU/l, n (%)		99 (22.7)	43 (9.9)
I	hCGβ (N)	pmol/l	168	168
	Median (IQR)		0.6 (0.4–1.0)	0.6 (0.4–0.9)
	>1.0 pmol/l, n (%)		41 (24.4)	38 (22.6)
I	TATI (N)	µg/l	168	168
	Median (IQR)		12.7 (10.6–16.3)	12.7 (10.1–16.2)
	>13.0 µg/l, n (%)		80 (47.6)	78 (46.4)
II	YKL-40 (N)	ng/ml	413	413
	Median (IQR)		74 (46–124)	87 (51–146)
	Elevated*, n (%)		57 (13.8)	78 (18.9)
II	IL-6 (N)	pg/ml	413	413
	Median (IQR)		3.4 (2.1–6.0)	5.1 (2.9–8.4)
	>4.95 pg/ml, n (%)		142 (34.4)	214 (51.8)
II	CRP (N)	mg/l	429	434
	Median (IQR)		<5 (<5–4)	<5 (<5–7)
	>5 mg/l, n (%)		86 (20.0)	129 (29.7)
III	MMP-8 (N)	ng/ml	108	111
	Median (IQR)		48.3 (22.9–96.5)	33.2 (16.8–86.8)
	Preoperative:			
	29.7–76.2 ng/ml, n (%)		35 (32.4)	
	>76.2 ng/ml, n (%)		36 (33.3)	
	Postoperative:			
	20.9–56.1 ng/ml, n (%)			36 (32.4)
	>56.1 ng/ml, n (%)			37 (33.3)
III	MMP-9 (N)	ng/ml	108	110
	Median (IQR)		79.6 (50.8–111.0)	75.9 (39.8–114.5)
	>77.7 ng/ml, n (%)		56 (51.9)	54 (49.1)
III	MPO (N)	ng/ml	108	110
	Median (IQR)		196.7 (132.7–341.1)	186.3 (103.4–286.0)
	>218.6 ng/ml, n (%)		48 (44.4)	43 (39.1)

* >age-corrected 95th percentile in healthy individuals (Bojesen et al., 2011).
IQR: interquartile range.

2.2 Biomarkers in tissue specimens (III and IV)

The expressions of all the investigated biomarkers in tissue specimens of the primary colorectal tumors and the liver metastases are presented in **Table 9**.

2.2.1 MMP-2, MMP-8, and MMP-9 (III)

Staining of MMP-2 was visible in the cytoplasm and cell membranes of the tumor cells. In the statistical analyses, the negative and mildly positive samples (score 0–1) were grouped as "low", and moderately and strongly stained samples as "high" expression (**Figure 4**).
MMP-8 expression was seen in the granulocytes in close proximity to the tumor cells. Samples with zero or only a few stained granulocytes (score 0–1) were grouped as "low", and samples with several or abundantly of stained cells (score 2–3) as "high" expression (**Figure 4**).
Granulary staining of MMP-9 was seen in the cytoplasm of the tumor cells. The samples with no staining or only mild granulary staining in some tumor cells (score 0–1) were grouped as "low", and those with moderate or strong granulary staining in several or all tumor cells (score 2–3) as "high" expression (**Figure 4**).

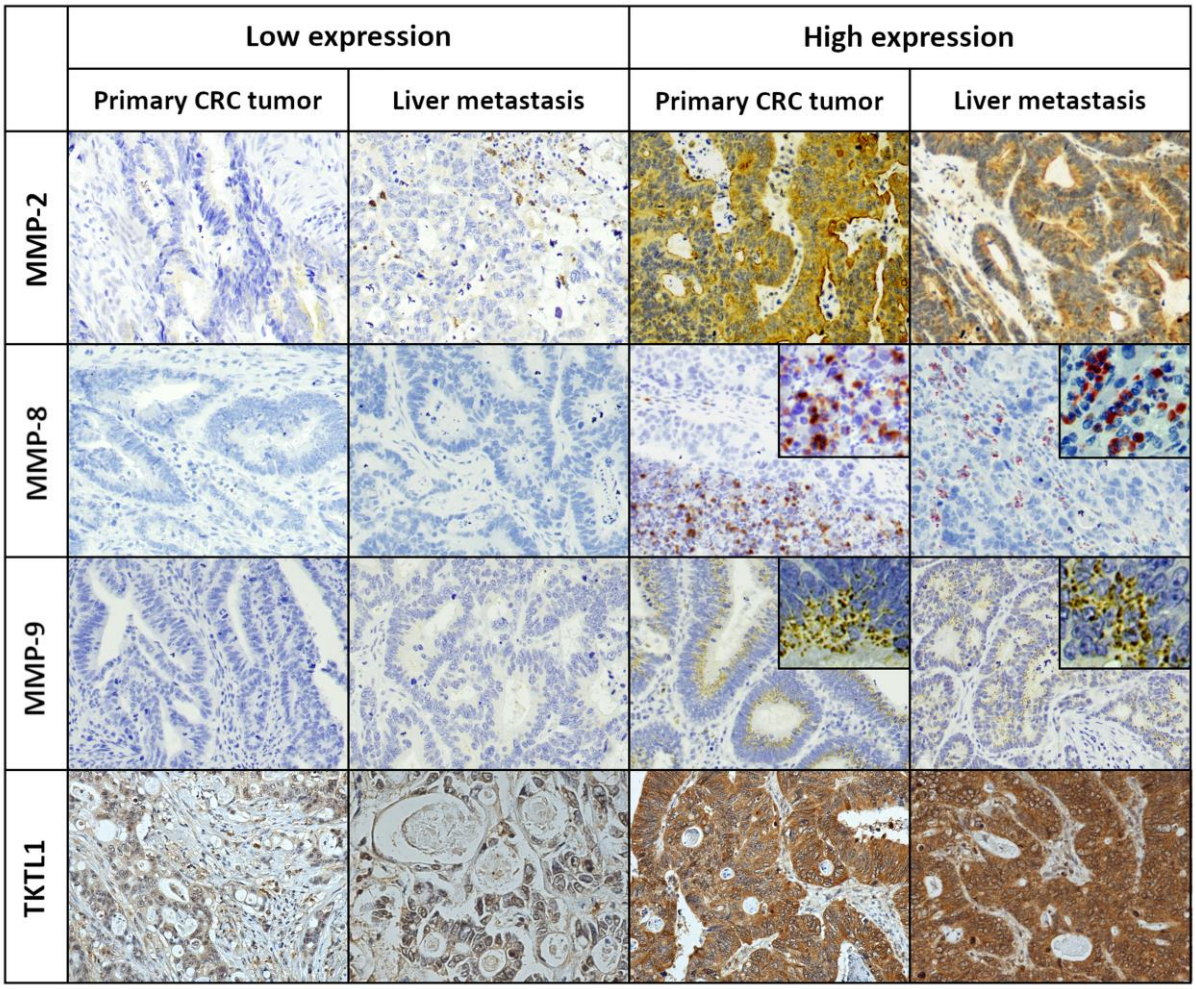
Table 9. Studies III and IV: Expressions of the biomarkers in tissue samples of the primary colorectal tumors and the liver metastases.

Study	**Tissue biomarkers' expressions**	**n (%)**	
		Colorectal tumors	**Liver metastases**
III	MMP-2 (N) 0 1 2 3	81 1 (1.2) 9 (11.1) 49 (60.5) 22 (27.2)	88 7 (8.0) 41 (46.6) 30 (34.1) 10 (11.4)
III	MMP-8 (N) 0 1 2 3	81 6 (7.4) 32 (39.5) 36 (44.4) 7 (8.6)	88 8 (9.1) 47 (53.4) 31 (35.2) 2 (2.3)
III	MMP-9 (N) 0 1 2 3	81 23 (28.4) 28 (34.6) 26 (32.1) 4 (4.9)	88 26 (29.5) 42 (47.7) 18 (20.5) 2 (2.3)
IV	TKTL1 (N) 0 1 2 3	81 0 8 (9.9) 48 (59.3) 25 (30.9)	89 5 (5.6) 15 (16.9) 42 (47.2) 27 (30.3)

2.2.2 TKTL1 (IV)

Staining of TKTL1 was visible in the cytoplasm of the tumor cells. For the statistical analyses, the samples with negative, mild or moderate staining (score 0–2) were grouped as “low”, and those with strong staining (score 3) as “high” expression (**Figure 4**).

Figure 4. Studies III and IV: Immunohistochemical stainings of MMP-2, MMP-8, MMP-9, and TKTL1 grouped according to the expression level. Magnification x200.



MMP-2, -8, and -9: low: score 0–1, high: score 2–3; TKTL1: low: score 0–2, high: score 3.
Original images taken by Professor Jaana Hagström.

3. CORRELATIONS (I–IV)

3.1 The biomarkers' serum and plasma concentrations (I–III)

In study I, preoperative CEA correlated with preoperative CA19-9 (rho 0.40, p<0.001). Postoperative CEA correlated with postoperative CA19-9 (rho 0.18, p=0.019) and postoperative TATI (rho 0.16, p=0.033). Postoperative CA19-9 correlated with postoperative hCGβ (rho 0.21, p=0.008) in addition to CEA. Postoperative hCGβ correlated with pre- and postoperative TATI (rho 0.19, p=0.015; and rho 0.18, p=0.019, respectively).

In study II, preoperative CEA correlated with preoperative CA19-9 (rho=0.41, p<0.001), preoperative YKL-40 (rho=0.11, p=0.027), and preoperative CRP (rho=0.22, p<0.001). Postoperative CEA correlated with postoperative CA19-9 (rho=0.22, p<0.001) and postoperative YKL-40 (rho=0.15, p=0.002).
Pre- and postoperative CA19-9 correlated with CEA as mentioned above. Postoperative CA19-9 also correlated with postoperative IL-6 (rho=0.13, p=0.008) and CRP (rho 0.13, p=0.007).
Pre- and postoperative YKL-40 correlated with CEA as mentioned above. In addition, preoperative YKL-40 correlated with preoperative IL-6 (rho=0.39, p<0.001) and preoperative CRP (rho 0.10, p<0.001), and postoperative YKL-40 with postoperative IL-6 (rho=0.28, p<0.001) and postoperative CRP (rho 0.18, p<0.001).
IL-6 and CRP correlated with the other biomarkers as mentioned above. In addition, preoperative IL-6 correlated with preoperative CRP (rho=0.33, p<0.001), and postoperative IL-6 with postoperative CRP (rho 0.55, p<0.001).

In study III, the serum values of MMP-8, MMP-9, and MPO all correlated with each other pre- and postoperatively (rho 0.20–0.70, p<0.001–0.001).

3.2 The biomarkers' expression in tissue specimens (III and IV)

In study III, MMP-2 expression in the primary colorectal tumors correlated with that of MMP-8 in the primary tumors (rho -0.30, p=0.007), but not with the expression of MMP-9. No statistically significant correlations were found concerning MMP-2 expression in the liver metastases.
MMP-8 expression in the primary tumors correlated only with that of MMP-2, as mentioned above. No correlations were found concerning the tissue expression of MMP-9 in the primary tumors or liver metastases.
In study IV, TKTL1 expression in the primary colorectal tumors did not correlate with the expression in the liver metastases.

3.3 MMP-2, MMP-8, and MMP-9 expression in tissue specimens and MMP-8, MMP-9, and MPO in serum (III)

The expression of MMP-2 in the primary colorectal tumors or the liver metastases did not correlate with the serum concentrations of MMP-8, MMP-9 or MPO, and neither did that of MMP-8 in the primary colorectal tumors. Instead, MMP-8 expression in the liver metastases correlated with both pre- and postoperative MMP-8 concentrations in serum (rho 0.33, p=0.002; and rho 0.24, p=0.027, respectively) as well as with preoperative MPO in serum (rho 0.22, p=0.044). MMP-9 expression in the primary colorectal tumors correlated with postoperative MMP-8 in serum (rho -0.43, p=0.002), when only patients with synchronous liver metastases were included. No correlations were found concerning the expression of MMP-9 in the liver metastases and the serum concentrations.

4. SURVIVAL ANALYSES (I–IV)

4.1 Serum and plasma concentrations and survival (I–III)

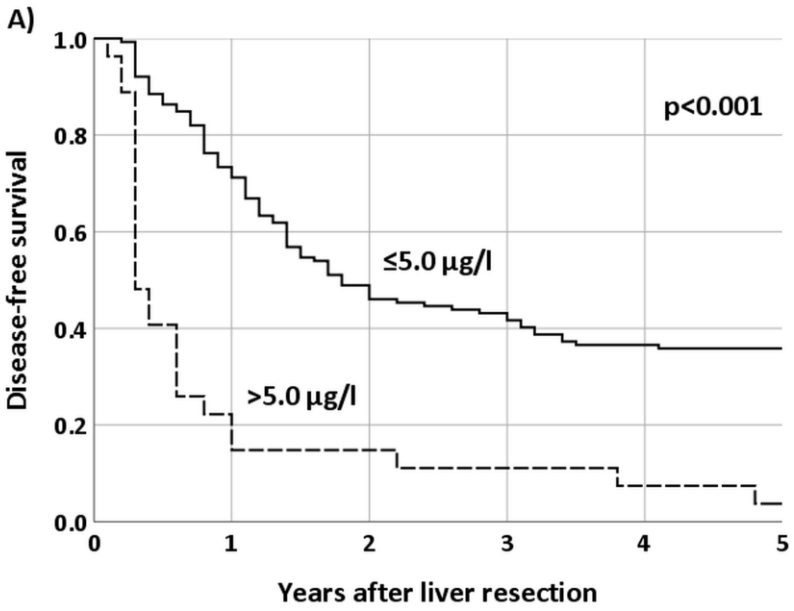
The results of the univariate Cox regression analyses according to specified cut-off levels in relation to DFS/RFS and OS are shown in **Tables 10 and 11**.

4.1.1 CEA and CA19-9 and survival (I and II)

In study I, Kaplan-Meier analyses showed that preoperatively elevated concentrations of CEA (>5.0 μg/l) associated with poor OS within three years after liver resection (p=0.010), but not within the whole follow-up time. There was no association with DFS. Postoperatively elevated CEA associated with shorter DFS and OS (both: p<0.001), and this finding remained significant in the multivariate analysis (3-year DFS: HR 3.78, p<0.001; OS: HR 3.68, p<0.001). Of the patients that experienced recurrence after liver resection, 78% (90/115) had a normal CEA level (≤5.0 μg/l) postoperatively, and these patients were found to develop recurrence later than those with elevated CEA postoperatively. Altogether 93% (107/115) of all recurrences were detected within 3 years after resection.
Preoperatively elevated CA19-9 (>26 kU/l) associated with shorter OS (p=0.006), but not with DFS. Postoperatively elevated values associated with both shorter DFS and OS (p=0.011 and p=0.002, respectively).
Only 14.9% (11/74) of the patients with normal CEA had elevated CA19-9 preoperatively and 9.9% (14/141) postoperatively. In those patients, CA19-9 was not prognostic.
The postoperative concentrations of CEA in relation to 5-year DFS and 10-year OS, and the pre- and postoperative concentrations of CA19-9 in relation to 10-year OS are shown in **Figures 5 and 6** with corresponding p-values.

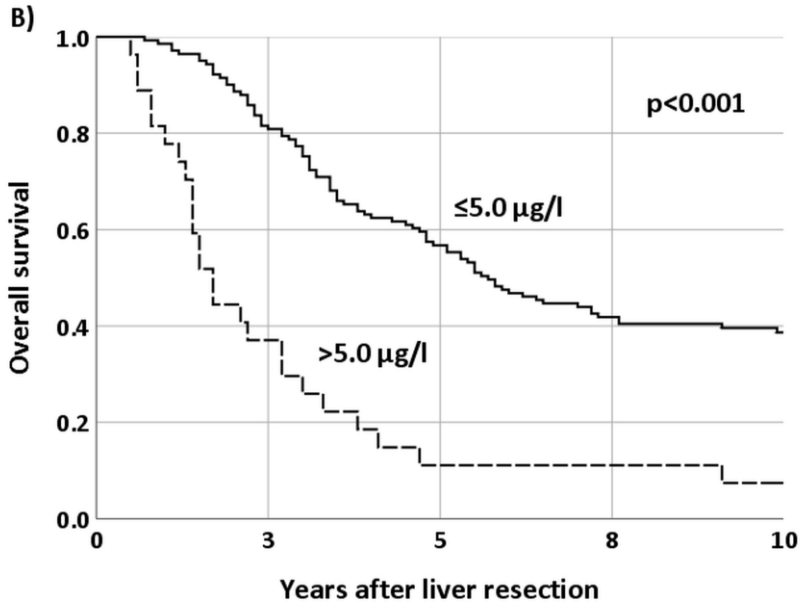
In study II, Cox regression method was used for the survival analyses, and the biomarkers were included in the analyses as $\log_2$-transformed continuous variables (**Tables 12 and 13**). Preoperative concentrations of CEA associated with poor OS (HR 1.07), but not with RFS. Postoperative concentrations associated with poor RFS (HR 1.24) and OS (HR 1.33), and the postoperative values remained significant in multivariate analysis (RFS: HR 1.17; OS: HR 1.24).
Concentrations of CA19-9 associated with both poor RFS and OS preoperatively (RFS: HR 1.08; OS: HR 1.12) as well as postoperatively (RFS: HR 1.12; OS: HR 1.22). Pre- and postoperative CA19-9 remained significant also in the multivariate analyses in relation to RFS (HR 1.12 and HR 1.09, respectively) and OS (HR 1.13 and HR 1.17, respectively).
CA19-9 was elevated (>37 kU/l) in 7.8% (17/219) of the patients with normal CEA (≤5.0 μg/l) before liver resection, and in 5.2% (19/366) of those with normal CEA postoperatively. In those cases, CA19-9 did not provide additional prognostic information regarding RFS and OS.

Figure 5. Study I: Postoperative serum concentrations of CEA in relation to A) 5-year disease-free survival, and B) 10-year overall survival. P-values from log-rank test.



Patients at risk (n)

CEA | years	0	1	2	3	5
≤5.0 µg/l	141	100	65	58	50
>5.0 µg/l	27	5	4	3	1



Patients at risk (n)

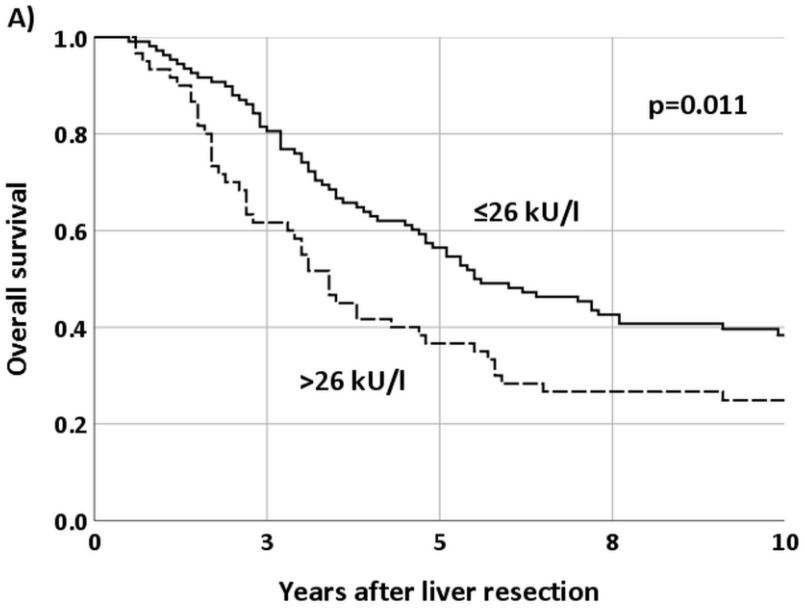
CEA | years	0	3	5	8	10
≤5.0 µg/l	141	109	80	57	55
>5.0 µg/l	27	8	3	3	2

4.1.2 HCGβ and TATI and survival (I)

In the analyses according to the Kaplan-Meier method, preoperatively elevated hCGβ (>1.0 pmol/l) did not associate with DFS or OS, and postoperatively an association was found only with poor OS within three years after liver resection (p=0.017).
Instead, preoperatively elevated TATI (>13 µg/l) associated with shorter DFS within three years after liver resection (p=0.017) and also within the whole follow-up time nearly significantly (p=0.051), but not with shorter OS. Postoperatively measured TATI did not associate with DFS or OS.
These findings were not significant in the multivariate analyses.

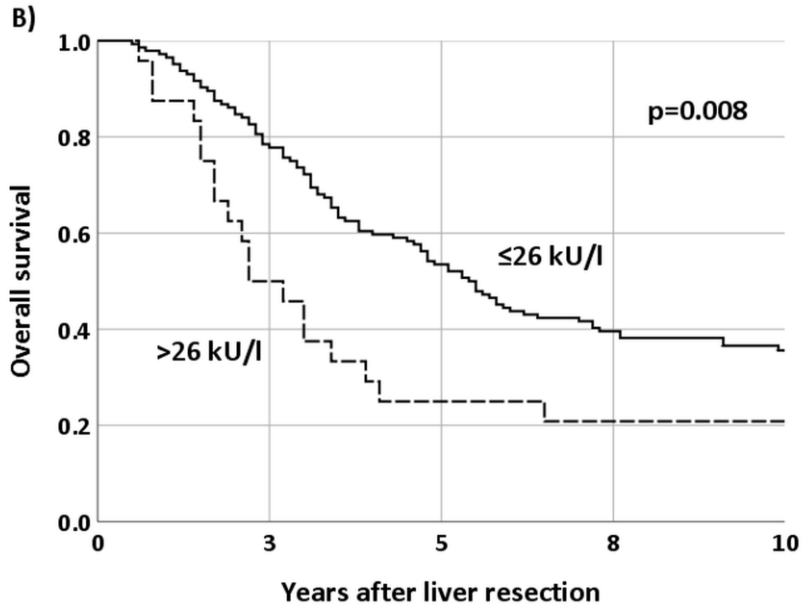
For this thesis, the prognostic significance of hCGβ and TATI was further examined in specified subgroups of patients. These were created according to gender (male/female), age (under/over 65 years at the time of the liver resection), location of the primary tumor (rectum/colon), and the type of liver metastases (synchronous/metachronous). It was found that postoperatively elevated hCGβ (>1.0 pmol/l) indicated shorter OS among males (p=0.032), but not among females, and shorter DFS and OS in patients with primary rectal tumors (DFS: p=0.038; OS: p=0.003). In addition, preoperatively elevated TATI (>13 µg/l) associated with worse DFS among patients with synchronous liver metastases (p=0.029), but not among those with metachronous ones. Otherwise, no statistically significant associations were found in these subgroups.

Figure 6. Study I: Serum concentrations of CA19-9 A) preoperatively, and B) postoperatively in relation to 10-year overall survival. P-values from log-rank test.



Patients at risk (n)

CA19-9 | years	0	3	5	8	10
≤26 kU/l	108	82	61	44	42
>26 kU/l	60	35	22	16	15



Patients at risk (n)

CA19-9 | years	0	3	5	8	10
≤26 kU/l	144	106	77	55	52
>26 kU/l	24	11	6	5	5

Table 10. Studies I–III: Results of the univariate Cox regression analyses of the serum/plasma biomarkers in relation to disease-free/relapse-free survival. Reference values are those below the (lower) cut-off limit. Table contains previously unpublished data (R.P. 2020).

Study	Biomarker	Cut-off value(s)	n	HR (95% CI)	p-value
Preoperative biomarkers in serum/plasma					
I II	CEA CEA	>5.0 µg/l >5.0 µg/l	94/168 221/440	0.99 (0.70–1.41) 1.01 (0.81–1.26)	0.969 0.927
I II	CA19-9 CA19-9	>26 kU/l >37 kU/l	60/168 99/437	1.28 (0.90–1.83) 1.63 (1.27–2.10)	0.174 <0.001
I	hCGβ	>1.0 pmol/l	41/168	0.95 (0.63–1.44)	0.802
I	TATI	>13 µg/l	80/168	1.41 (1.00–2.00)	0.053
II	YKL-40	>95th percentile[1]	57/413	1.49 (1.10–2.03)	0.011
II	IL-6	>4.95 pg/ml	142/413	1.25 (0.98–1.58)	0.069
II	CRP	>5 mg/l	86/429	1.16 (0.88–1.52)	0.289
III	MMP-8	29.7–76.2 ng/ml[2] >76.2 ng/ml	35/108 36/108	0.58 (0.34–0.99) 0.98 (0.59–1.62)	0.047 0.929
III	MMP-9	>77.7 ng/ml	56/108	0.76 (0.49–1.16)	0.200
III	MPO	>218.6 ng/ml	48/108	0.45 (0.29–0.71)	0.001
Postoperative biomarkers in serum/plasma					
I II	CEA CEA	>5.0 µg/l >5.0 µg/l	27/168 74/440	3.75 (2.41–5.83) 2.03 (1.54–2.68)	<0.001 <0.001
I II	CA19-9 CA19-9	>26 kU/l >37 kU/l	24/168 43/436	1.80 (1.13–2.85) 1.77 (1.25–2.51)	0.013 0.001
I	hCGβ	>1.0 pmol/l	38/168	1.15 (0.76–1.73)	0.523
I	TATI	>13 µg/l	78/168	1.10 (0.78–1.56)	0.581
II	YKL-40	>95th percentile[1]	78/413	1.44 (1.09–1.89)	0.010
II	IL-6	>4.95 pg/ml	214/413	1.28 (1.02–1.61)	0.031
II	CRP	>5 mg/l	129/434	1.35 (1.06–1.71)	0.014
III	MMP-8	20.9–56.1 ng/ml[3] >56.1 ng/ml	36/111 37/111	1.40 (0.84–2.34) 1.22 (0.74–2.04)	0.195 0.437
III	MMP-9	>77.7 ng/ml	54/110	1.05 (0.69–1.60)	0.829
III	MPO	>218.6 ng/ml	43/110	0.80 (0.51–1.24)	0.318

[1] >age-corrected 95th percentile in healthy individuals.

[2] Cut-off values according to the tertiles of the preoperative serum concentrations.

[3] Cut-off values according to the tertiles of the postoperative serum concentrations.

Table 11. Studies I–III: Results of the univariate Cox regression analyses of the serum/plasma biomarkers in relation to overall survival. Reference values are those below the (lower) cut-off limit. Table contains previously unpublished data (R.P. 2020).

Study	Biomarker	Cut-off value(s)	n	HR (95% CI)	p-value
Preoperative biomarkers in serum/plasma					
I II	CEA CEA	>5.0 µg/l >5.0 µg/l	94/168 221/440	1.30 (0.90–1.89) 1.28 (1.01–1.63)	0.168 0.045
I II	CA19-9 CA19-9	>26 kU/l >37 kU/l	60/168 99/437	1.69 (1.16–2.46) 1.82 (1.39–2.38)	0.006 <0.001
I	hCGβ	>1.0 pmol/l	41/168	1.18 (0.77–1.80)	0.444
I	TATI	>13 µg/l	80/168	1.21 (0.84–1.75)	0.302
II	YKL-40	>95th percentile[1]	57/413	1.77 (1.28–2.43)	<0.001
II	IL-6	>4.95 pg/ml	142/413	1.30 (1.00–1.67)	0.048
II	CRP	>5 mg/l	86/429	1.16 (0.87–1.56)	0.316
III	MMP-8	29.7–76.2 ng/ml[2] >76.2 ng/ml	35/108 36/108	0.51 (0.29–0.91) 1.09 (0.65–1.84)	0.024 0.747
III	MMP-9	>77.7 ng/ml	56/108	0.89 (0.56–1.39)	0.600
III	MPO	>218.6 ng/ml	48/108	0.56 (0.35–0.89)	0.015
Postoperative biomarkers in serum/plasma					
I II	CEA CEA	>5.0 µg/l >5.0 µg/l	27/168 74/440	3.68 (2.35–5.79) 2.24 (1.67–3.00)	<0.001 <0.001
I II	CA19-9 CA19-9	>26 kU/l >37 kU/l	24/168 43/436	2.11 (1.31–3.40) 2.28 (1.60–3.26)	0.002 <0.001
I	hCGβ	>1.0 pmol/l	38/168	1.48 (0.97–2.25)	0.071
I	TATI	>13 µg/l	78/168	1.11 (0.77–1.60)	0.586
II	YKL-40	>95th percentile[1]	78/413	1.72 (1.29–2.31)	<0.001
II	IL-6	>4.95 pg/ml	214/413	1.31 (1.02–1.68)	0.032
II	CRP	>5 mg/l	129/434	1.57 (1.22–2.02)	<0.001
III	MMP-8	20.9–56.1 ng/ml[3] >56.1 ng/ml	36/111 37/111	1.78 (1.02–3.11) 1.65 (0.95–2.89)	0.043 0.077
III	MMP-9	>77.7 ng/ml	54/110	1.30 (0.83–2.04)	0.250
III	MPO	>218.6 ng/ml	43/110	0.92 (0.58–1.47)	0.731

[1] >age-corrected 95th percentile in healthy individuals.
[2] Cut-off values according to the tertiles of the preoperative serum concentrations.
[3] Cut-off values according to the tertiles of the postoperative serum concentrations.

4.1.3 YKL-40, IL-6, CRP and survival (II)

In the Cox regression analyses, the concentrations of YKL-40 associated with shorter RFS and OS, when measured before liver resection (RFS: HR 1.19; OS: HR 1.29) and three months after it (RFS: HR 1.21; OS: HR 1.26) (**Table 12**). In the multivariate analyses, however, only preoperative YKL-40 concentrations in relation to OS remained significant (HR 1.19) (**Table 13**).

IL-6 associated with shorter RFS (HR 1.15) and OS (HR 1.16) preoperatively and postoperatively (RFS: HR 1.11; OS: HR 1.13) (**Table 12**). These findings were not significant in the multivariate analyses.

Elevated CRP (>5 mg/l) associated only with shorter OS postoperatively (HR 1.11), and only in the univariate analysis (**Table 12**).

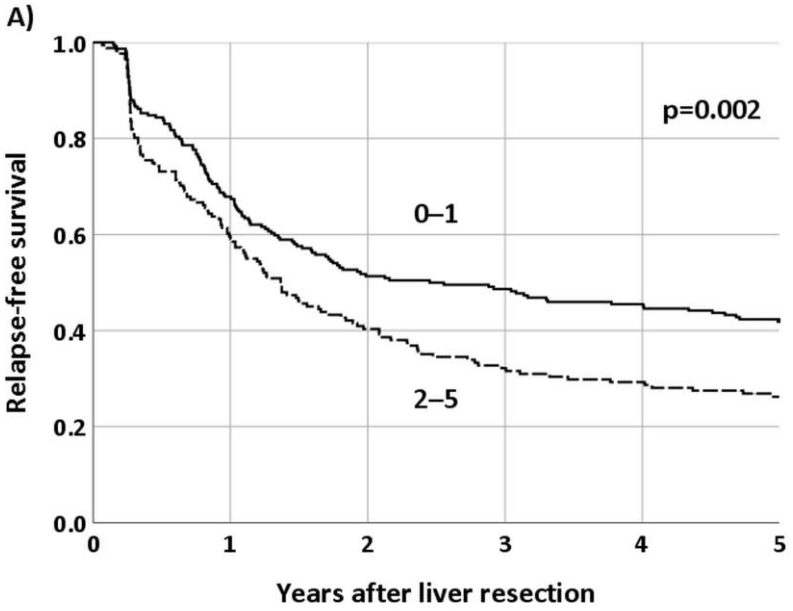
4.1.4 Combined prognostic value of serum YKL-40, IL-6, CRP, CEA, and CA19-9 (II)

For further analyses, the serum concentrations of the inflammatory biomarkers YKL-40, IL-6, and CRP, and the cancer biomarkers CEA and CA19-9 were combined. Altogether 43.9% (175/399) of the patients had 2–5 biomarkers elevated preoperatively, and 41.4% (167/403) postoperatively.

It was found that patients with 2–5 elevated biomarkers preoperatively had significantly shorter RFS (HR 1.37) and OS (HR 1.76) compared to those with 0–1 elevated biomarkers (**Figure 7; Table 12**), and this finding was significant also in the multivariate analyses (RFS: HR 1.40; OS: HR 1.71) (**Table 13**). The same was true for postoperatively elevated values, as 2–5 elevated biomarkers indicated worse RFS and OS in both the univariate (RFS: HR 1.54; OS: HR 1.83) (**Figure 8; Table 12**) and the multivariate analyses (RFS: HR 1.57; OS: HR 1.84) (**Table 13**).

When compared to no elevated biomarker concentrations, having CEA and/or CA19-9 elevated postoperatively associated with shorter RFS and OS (RFS: HR 1.87, 95% CI 1.36–2.58, $p<0.001$; OS: HR 2.15, 95% CI 1.52–3.02, $p<0.001$), while having YKL-40, IL-6 and/or CA19-9 elevated did not. Concerning the preoperative values, no such associations were observed.

Figure 7. Study II: Kaplan-Meier curves of preoperative YKL-40, IL-6, CRP, CEA, and CA19-9 in serum showing the difference between 0–1 and 2–5 elevated biomarkers in relation to A) 5-year relapse-free survival, and B) 10-year overall survival. P-values from log-rank test.



Patients at risk (n)

Biomarkers | years	0	1	2	3	5
0–1 elevated	224	152	115	108	89
2–5 elevated	175	105	72	58	43

Patients at risk (n)

Biomarkers | years	0	3	5	8	10
0–1 elevated	224	189	140	77	43
2–5 elevated	175	110	76	36	23

Table 12. Study II: Results of the univariate Cox regression analyses in relation to relapse-free and overall survival. Biomarkers are presented as log_2-transformed continuous variables.

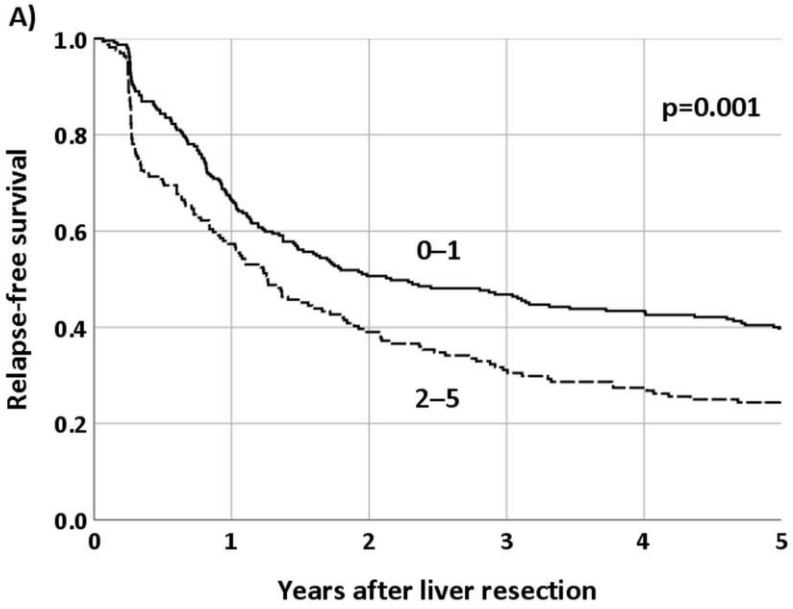
	n	Relapse-free survival		Overall survival	
		HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Preoperative biomarkers					
CEA	440	1.01 (0.96–1.07)	0.583	1.07 (1.01–1.12)	0.019
CA19-9	437	1.08 (1.03–1.14)	<0.001	1.12 (1.06–1.18)	<0.001
YKL-40	413	1.19 (1.07–1.32)	<0.001	1.29 (1.16–1.44)	<0.001
IL-6	413	1.15 (1.03–1.28)	0.010	1.16 (1.03–1.30)	0.011
CRP	429	1.08 (0.96–1.21)	0.219	1.07 (0.95–1.21)	0.240
0 elevated	111/399	Reference		Reference	
1 elevated	113/399	0.81 (0.58–1.12)	0.202	0.86 (0.60–1.24)	0.433
2 elevated	101/399	1.13 (0.83–1.56)	0.438	1.63 (1.16–2.30)	0.005
3 elevated	44/399	1.60 (1.08–2.36)	0.018	1.82 (1.19–2.79)	0.006
4 elevated	29/399	1.57 (0.99–2.49)	0.055	1.78 (1.08–2.93)	0.023
5 elevated	1/399	2.86 (0.40–20.7)	0.298	3.24 (0.45–23.5)	0.245
0–1 elevated	224/399	Reference		Reference	
2–5 elevated	175/399	1.37 (1.10–1.72)	0.005	1.76 (1.39–2.24)	<0.001
Postoperative biomarkers					
CEA	440	1.24 (1.14–1.34)	<0.001	1.33 (1.22–1.46)	<0.001
CA19-9	436	1.12 (1.04–1.20)	0.002	1.22 (1.13–1.31)	<0.001
YKL-40	413	1.21 (1.09–1.34)	<0.001	1.26 (1.13–1.41)	<0.001
IL-6	413	1.11 (1.01–1.23)	0.033	1.13 (1.02–1.26)	0.022
CRP	434	1.07 (0.99–1.15)	0.075	1.11 (1.02–1.20)	0.011
0 elevated	137	Reference		Reference	
1 elevated	99/403	1.18 (0.87–1.62)	0.290	1.03 (0.72–1.47)	0.882
2 elevated	106/403	1.41 (1.05–1.91)	0.023	1.57 (1.13–2.17)	0.007
3 elevated	48/403	1.98 (1.37–2.86)	<0.001	2.42 (1.64–3.56)	<0.001
4 elevated	10/403	2.94 (1.48–5.86)	0.002	4.45 (2.21–8.96)	<0.001
5 elevated	3/403	3.67 (1.16–11.7)	0.028	10.2 (3.16–32.9)	<0.001
0–1 elevated	236/403	Reference		Reference	
2–5 elevated	167/403	1.54 (1.23–1.92)	<0.001	1.83 (1.44–2.33)	<0.001

Table 13. Study II: Results of the multivariate Cox regression analyses in relation to relapse-free and overall survival. Biomarkers are presented as log_2-transformed continuous variables.

	n	Relapse-free survival		Overall survival	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Preoperative biomarkers					
CEA	440	1.00 (0.93–1.07)	0.952	1.02 (0.95–1.09)	0.602
CA19-9	437	1.12 (1.06–1.19)	<0.001	1.13 (1.06–1.20)	<0.001
YKL-40	413	1.08 (0.96–1.22)	0.212	1.19 (1.04–1.35)	0.010
IL-6	413	1.04 (0.91–1.18)	0.566	1.03 (0.90–1.18)	0.685
CRP	429	1.03 (0.88–1.20)	0.756	0.91 (0.77–1.06)	0.215
0 elevated	111/399	Reference		Reference	
1 elevated	113/399	0.81 (0.58–1.12)	0.202	0.82 (0.57–1.18)	0.284
2 elevated	101/399	1.21 (0.87–1.68)	0.252	1.58 (1.11–2.24)	0.011
3 elevated	44/399	1.63 (1.08–2.47)	0.021	1.60 (1.02–2.50)	0.042
4 elevated	29/399	1.88 (1.12–3.16)	0.016	1.75 (1.01–3.04)	0.046
5 elevated	1/399	3.60 (0.48–26.8)	0.211	2.90 (0.39–21.7)	0.299
0–1 elevated	224/399	Reference		Reference	
2–5 elevated	175/399	1.40 (1.11–1.78)	0.005	1.71 (1.33–2.21)	<0.001
Postoperative biomarkers					
CEA	440	1.17 (1.07–1.29)	<0.001	1.24 (1.12–1.36)	<0.001
CA19-9	436	1.09 (1.01–1.17)	0.029	1.17 (1.08–1.28)	<0.001
YKL-40	413	1.06 (0.94–1.19)	0.323	1.10 (0.97–1.24)	0.131
IL-6	413	0.98 (0.87–1.11)	0.755	0.95 (0.83–1.09)	0.498
CRP	434	1.02 (0.92–1.12)	0.741	1.09 (0.99–1.20)	0.096
0 elevated	137	Reference		Reference	
1 elevated	99/403	1.00 (0.73–1.38)	0.993	0.88 (0.61–1.27)	0.488
2 elevated	106/403	1.30 (0.95–1.78)	0.102	1.40 (0.99–1.97)	0.055
3 elevated	48/403	1.84 (1.25–2.71)	0.002	2.33 (1.56–3.48)	<0.001
4 elevated	10/403	2.50 (1.22–5.12)	0.012	4.42 (2.16–9.05)	<0.001
5 elevated	3/403	3.14 (0.97–10.1)	0.055	8.91 (2.71–29.2)	<0.001
0–1 elevated	236/403	Reference		Reference	
2–5 elevated	167/403	1.57 (1.24–1.98)	<0.001	1.84 (1.43–2.37)	<0.001

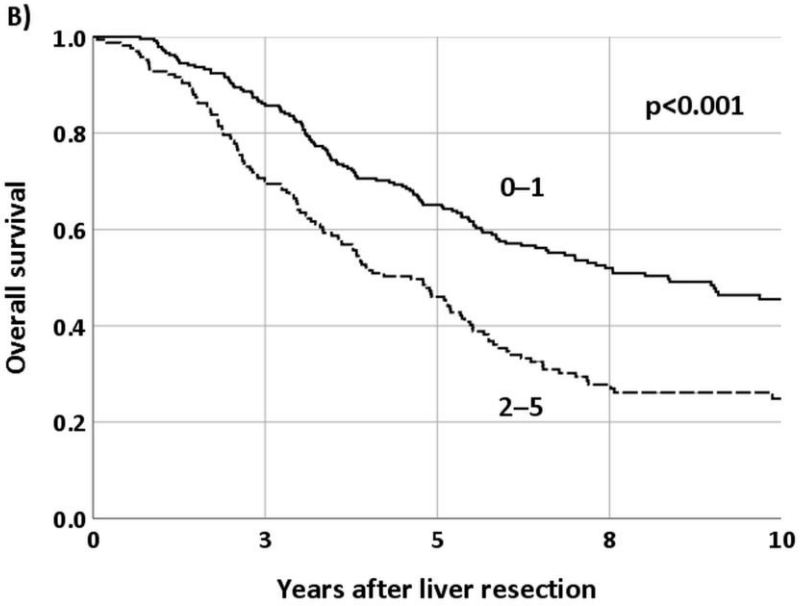
Clinical variables included in the multivariate analyses: age; gender; location of the primary colorectal tumor; type of liver metastases (synchronous/metachronous); type of liver resection (minor/major); the number and size of the liver metastases; and the resection margins (R0/1/2).

Figure 8. Study II: Kaplan-Meier curves of postoperative YKL-40, IL-6, CRP, CEA, and CA19-9 in serum showing the difference between 0–1 and 2–5 elevated biomarkers in relation to A) 5-year relapse-free survival, and B) 10-year overall survival. P-values from log-rank test.



Patients at risk (n)

Biomarkers | years	0	1	2	3	5
0–1 elevated	236	157	119	109	93
2–5 elevated	167	96	66	53	38



Patients at risk (n)

Biomarkers | years	0	3	5	8	10
0–1 elevated	236	194	151	86	50
2–5 elevated	167	107	72	28	20

4.1.5 MMP-8, MMP-9, MPO and survival (III)

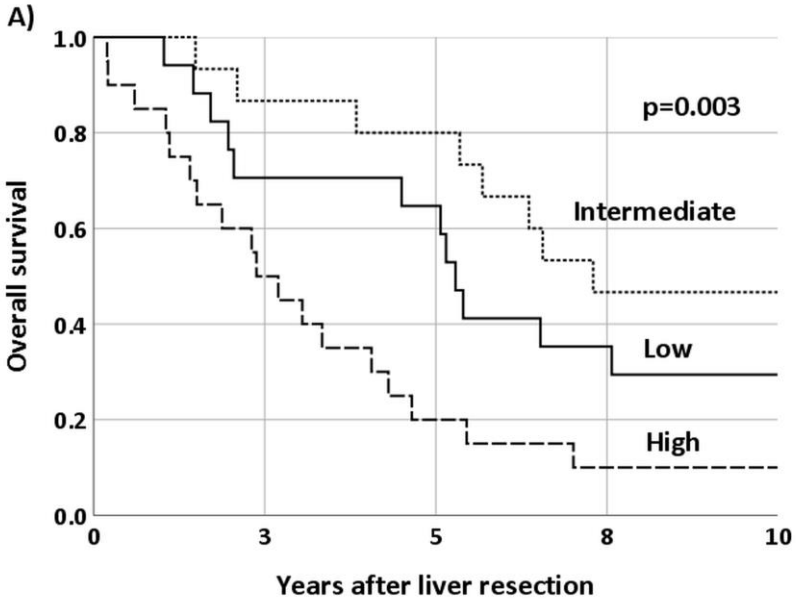
In Kaplan-Meier log-rank and Cox regression analyses (p-values from the log-rank tests presented here), both elevated and low serum concentrations of MMP-8 (≤29.6 and >76.2 ng/ml) before liver resection associated with poor OS (p=0.023). This was especially obvious among males (OS: p=0.026), in synchronous disease (OS: p=0.051), and among patients who had received neoadjuvant chemotherapy before liver resection (DFS: p=0.026; OS: p=0.043). Preoperatively high MMP-8 (>76.2 ng/ml), but not low, associated with worse survival among over 65-year-old patients (OS: p=0.005) (**Figure 9 A**) and among patients with primary colon tumors (DFS: p=0.020; OS: p=0.002).
Postoperative MMP-8 was not prognostic in the whole patient cohort, but intermediate and high values (both >20.8 ng/ml) associated with impaired prognosis among over 65-year-old patients (OS: p=0.045), in colon cancer (OS: p=0.020), and in synchronous disease (DFS: p=0.020; OS: p=0.002).

The serum values of MMP-9 were not prognostic in the whole patient cohort, but postoperatively elevated values (>77.7 ng/ml) associated with poor survival among over 65-year-old patients (DFS: p=0.003; OS: p<0.001) (**Figure 9 B**). In other subgroups of patients, no statistically significant associations were found.

Preoperatively elevated MPO (>218.6 ng/ml) associated with improved DFS and OS in the whole patient cohort (DFS: p<0.001; OS: p=0.014) (**Figure 10**), and this was most evident among females (DFS: p=0.001; OS: p=0.013), under 65-year-old patients (DFS: p<0.001; OS: p=0.004), and those with synchronous liver metastases (DFS: p=0.001; OS: p=0.005). In addition, among patients who had received neoadjuvant chemotherapy, low preoperative MPO associated with poor prognosis (DFS: p<0.001; OS: p=0.020), but the association was non-significant among those who had not received neoadjuvant treatment.
Postoperatively elevated MPO (>218.6 ng/ml) indicated poor survival only among over 65-year-old patients (OS: p=0.037).

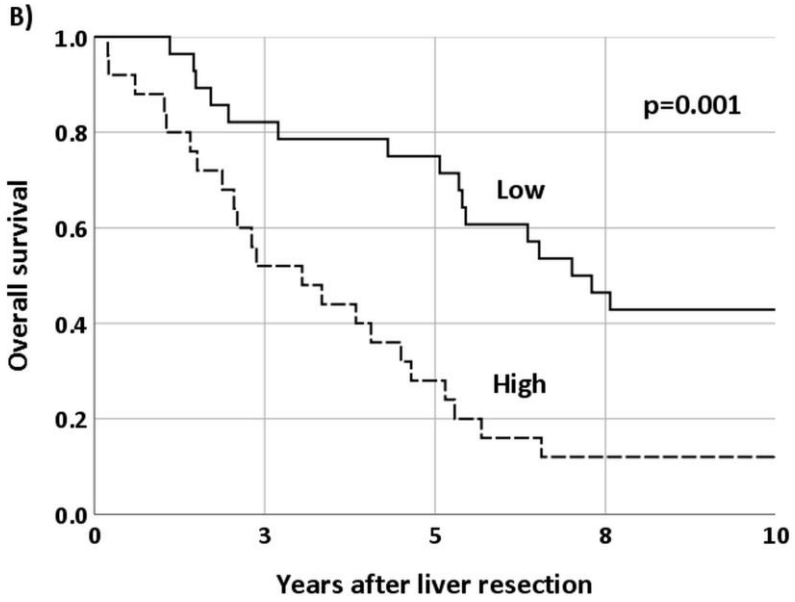
Neither postoperative increase nor decrease in the serum concentrations of these biomarkers associated with prognosis after liver resection in the whole patient cohort.

Figure 9. Study III: A) Preoperative serum concentrations of MMP-8, and B) postoperative serum concentrations of MMP-9 in over 65-year-old patients in relation to 10-year overall survival. P-values from log-rank test.



Patients at risk (n)

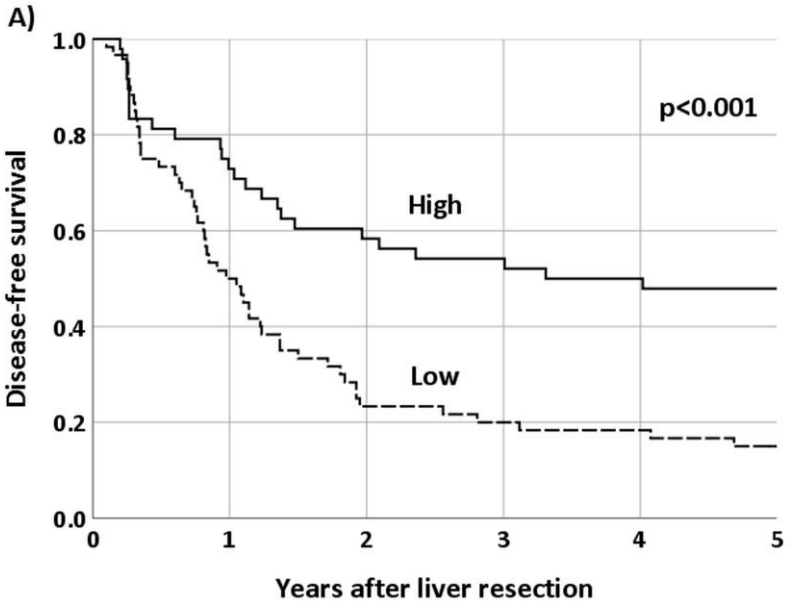
MMP-8 | years	0	3	5	8	10
Low (≤29.6 ng/ml)	17	12	11	5	5
Intermediate	15	13	12	7	7
High (>76.2 ng/ml)	20	9	4	2	2



Patients at risk (n)

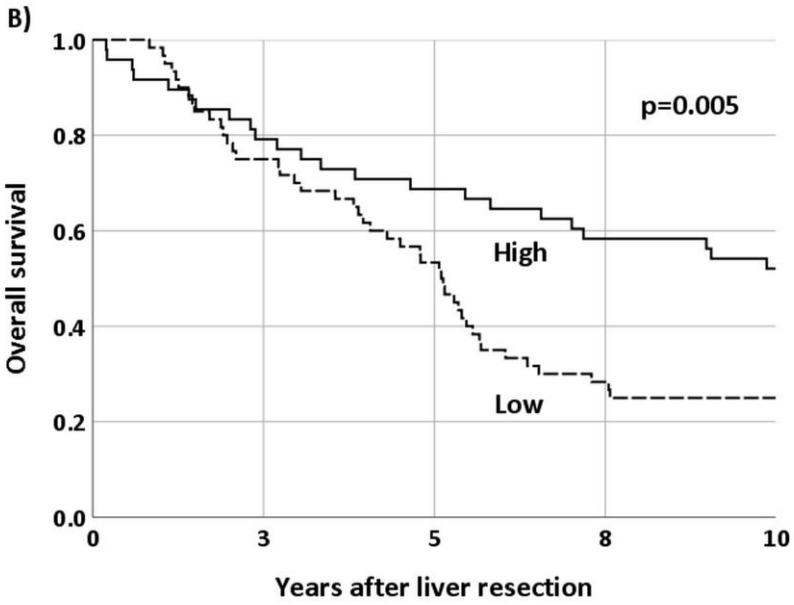
MMP-9 | years	0	3	5	8	10
Low (≤77.7 ng/ml)	28	22	21	12	12
High (>77.7 ng/ml)	25	13	7	3	3

Figure 10. Study III: Preoperative serum concentrations of MPO in relation to A) 5-year disease-free survival, and B) 10-year overall survival. P-values from log-rank test.



Patients at risk (n)

MPO | years	0	1	2	3	5
High (>218.6 ng/ml)	48	35	28	26	23
Low (≤218.6 ng/ml)	60	30	14	12	9



Patients at risk (n)

MPO | years	0	3	5	8	10
High (>218.6 ng/ml)	48	37	33	28	25
Low (≤218.6 ng/ml)	60	42	32	15	15

4.2 Tumor tissue specimens and survival (III and IV)

The results of the univariate Cox regression analyses in relation to disease-free and overall survival are shown in **Table 14**.

4.2.1 MMP-2, MMP-8, MMP-9 and survival (III)

In the survival analyses (p-values of the log-rank tests presented here), the expression of MMP-2 and MMP-8 in the primary colorectal tumors or the liver metastases did not associate with DFS or OS, when the whole patient cohort was studied. However, low expression of MMP-2 in the liver metastases indicated worse DFS among female patients (p=0.032), and high expression of MMP-8 in the liver metastases worse OS in patients with primary colon tumors (p=0.046). In addition, low MMP-8 expression in the primary tumors associated with impaired prognosis (DFS: p=0.005; OS: p=0.097) among patients who had not received neoadjuvant chemotherapy before liver resection (n=40/111; 36.0%), while no association was found among those who had (n=66/111; 59.5%).

High expression of MMP-9 in the primary colorectal tumors associated with better DFS (p=0.010) and also with better OS nearly significantly (p=0.067) (**Figure 11**). The association was most obvious among female patients (DFS: p=0.005; OS: p=0.014), in patients with primary colon tumors (DFS: p=0.011; OS: p=0.037), in metachronous disease (DFS: p=0.005; OS: p=0.032), and among patients who had not received neoadjuvant therapy (DFS: p=0.006; OS: p=0.062). Among those who had received neoadjuvant therapy, no difference between the low and high expression groups was observed. The expression of MMP-9 in the liver metastases did not associate with either DFS or OS.

4.2.2 TKTL1 and survival (IV)

The expression of TKTL1 did not associate with DFS or OS across the entire patient cohort (N=111). However, in patients with synchronous liver metastases (n=66/111; 59.5%), high TKTL1 expression in the primary colorectal tumors associated with shorter DFS compared to patients with low TKTL1 expression (HR 2.14, p=0.030). Concerning TKTL1 expression in the liver metastases, no statistically significant associations were found, but only 12% (2/17) of the patients with high expression were alive without recurrence three years after liver resection compared to 30% (10/33) of those with low expression.

In metachronous disease (n=45/111; 40.5%), patients with high TKTL1 expression in the primary tumors had a better DFS compared to those with low expression, even though the finding was not statistically significant (HR 0.41, 95% CI 0.15–1.13, p=0.084). In addition, 70% (7/10) of the patients with high expression in the liver metastases were alive without recurrence after three years compared to 32% (9/28) of those with low expression.

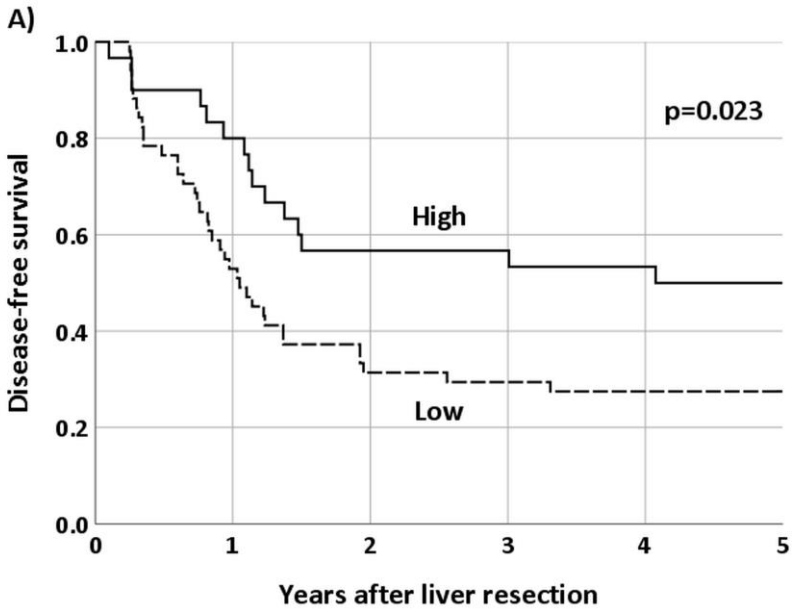
In the subgroups of patients with high TKTL1 expression in either the primary tumors (n=25) or the liver metastases (n=27), patients with synchronous metastases had a worse DFS compared to those with metachronous ones (primary tumors: HR 4.66, 95% CI 1.57–13.8, p=0.005; liver metastases: HR 3.55, 95% CI 1.23–10.2, p=0.019) (**Figure 12**). For the patients with high expression of TKTL1 in the primary tumors, DFS at three years after liver resection was only 7% (1/14) in synchronous disease compared to 73% (8/11) in metachronous disease, and for those with high expression in the liver metastases, 12% (2/17) in synchronous compared to 70% (7/10) in metachronous disease.

Table 14. Studies III and IV: Results of the univariate Cox regression analysis of the biomarker expressions in tumor tissue in relation to disease-free and overall survival. Low expression groups are used as a reference. Table contains previously unpublished data (R.P. 2020).

Study	Biomarker	Score	n	Disease-free survival		Overall survival	
				HR (95% CI)	p-value	HR (95% CI)	p-value
Biomarkers in colorectal tumor tissue							
III	MMP-2	High	71/81	1.03 (0.49–2.18)	0.929	0.79 (0.37–1.69)	0.549
III	MMP-8	High	43/81	0.69 (0.42–1.14)	0.144	0.74 (0.43–1.28)	0.281
III	MMP-9	High	30/81	0.50 (0.29–0.86)	0.012	0.59 (0.33–1.05)	0.070
IV	TKTL1	High	25/81	1.00 (0.58–1.73)	0.998	0.90 (0.50–1.62)	0.729
Biomarkers in liver metastasis tissue							
III	MMP-2	High	40/88	0.77 (0.48–1.23)	0.271	0.75 (0.45–1.25)	0.267
III	MMP-8	High	33/88	1.21 (0.75–1.95)	0.431	1.29 (0.77–2.14)	0.334
III	MMP-9	High	20/88	0.99 (0.57–1.71)	0.969	0.79 (0.43–1.45)	0.442
IV	TKTL1	High	27/89	1.03 (0.62–1.72)	0.897	1.12 (0.65–1.92)	0.686

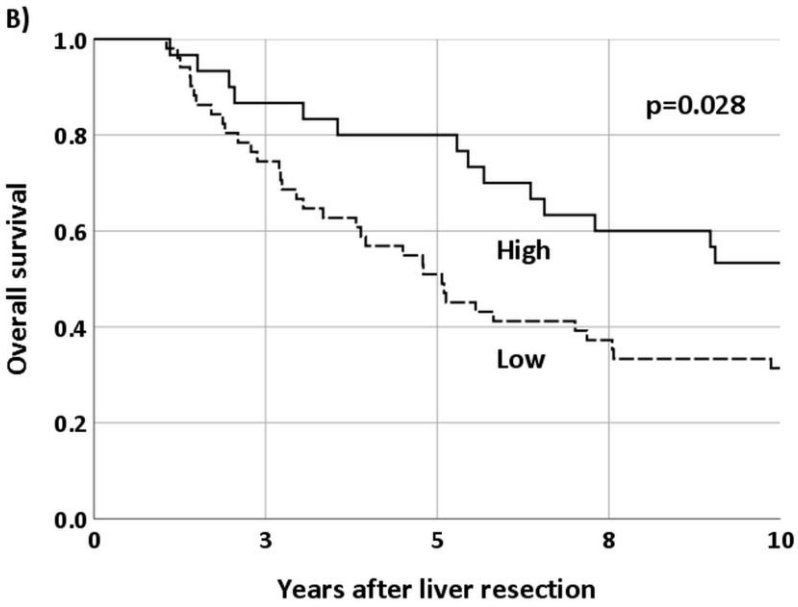
MMP-2, -8, and -9: low: score 0–1, high: score 2-3; TKTL1: low: score 0–2, high: score 3.

Figure 11. Study III: The expression of MMP-9 in primary colorectal tumors in relation to A) 5-year disease-free survival, and B) 10-year overall survival. P-values from log-rank test.



Patients at risk (n)

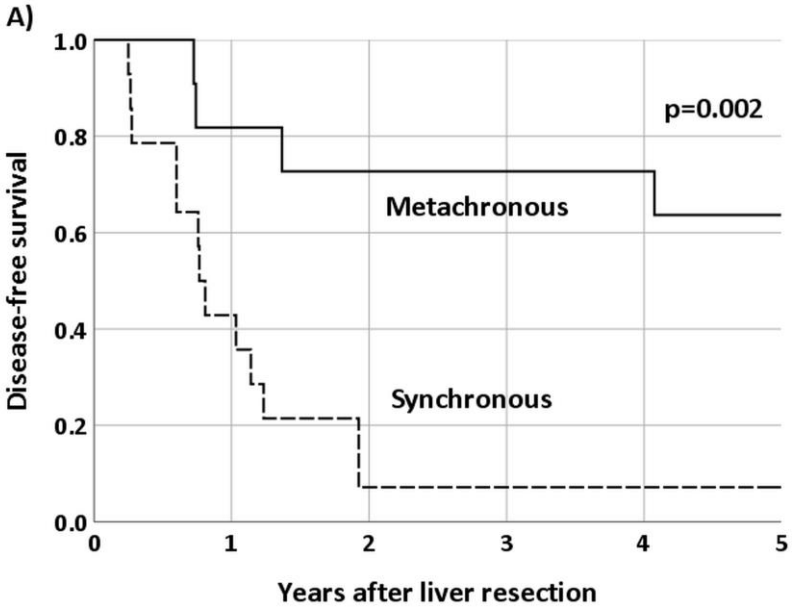
MMP-9 | years	0	1	2	3	5
High (score 2–3)	30	24	17	17	15
Low (score 0–1)	51	27	16	15	14



Patients at risk (n)

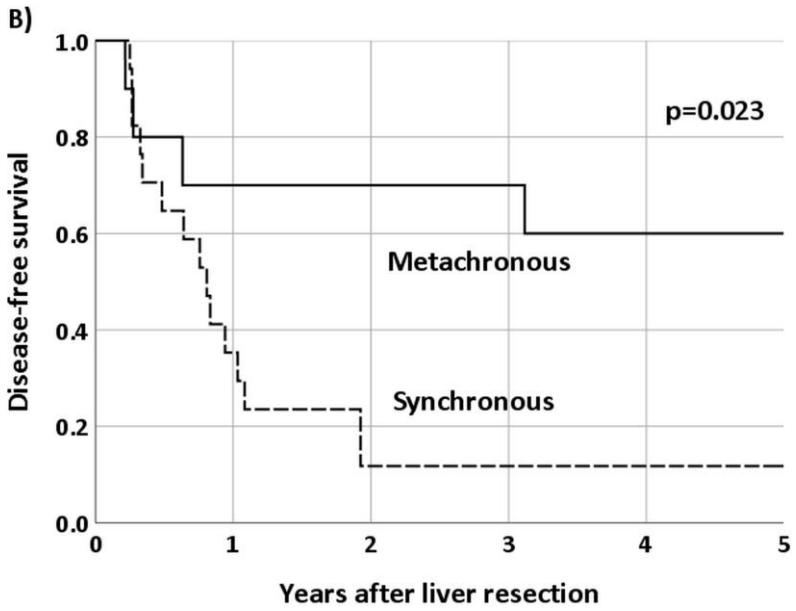
MMP-9 | years	0	3	5	8	10
High (score 2–3)	30	26	24	18	16
Low (score 0–1)	51	34	26	17	16

Figure 12. Study IV: Patients with high expression of TKTL1 (score 3) in A) colorectal tumors, and B) liver metastases. Kaplan-Meier curves presenting the difference in 5-year disease-free survival between synchronous and metachronous disease. P-values from log-rank test.



Patients at risk (n)

Years	0	1	2	3	5
Metachronous	11	9	8	8	7
Synchronous	14	6	1	1	1



Patients at risk (n)

Years	0	1	2	3	5
Metachronous	10	7	7	7	6
Synchronous	17	6	2	2	2

DISCUSSION

In this thesis, the prognostic value of 12 different biomarkers was evaluated in patients undergoing curative-intent liver resection for colorectal metastases. Of these, 10 were determined in serum or plasma, and four in tissue specimens of the primary colorectal tumors and the liver metastases. Altogether 442 patients were included in the four studies.
The results confirmed the prognostic value of serum CEA and CA19-9, but also identified their limitations. Novel information was obtained concerning the inflammatory serum biomarkers YKL-40 and IL-6 combined with CRP. A biomarker panel comprising these three biomarkers together with CEA and CA19-9 was presented as a clinically useful tool for estimating the risk of relapse and death after liver resection.
Serum MPO was identified as a prognostic biomarker in metastatic CRC for the first time. In addition, valuable new information about the concentrations of MMP-8 and MMP-9 in serum as well as the expressions of MMP-2, MMP-8, MMP-9, and TKTL1 in tumor tissue was obtained, and differences in their prognostic significance depending on the clinical characteristics of the patients were observed. All the investigated biomarkers were shown to have some prognostic value in CRC with resectable liver metastases.

1. CEA and CA19-9 (I and II)

In this thesis, elevated CEA (>5.0 µg/l) in serum associated with shorter DFS/RFS and OS, when measured three months after liver resection. Preoperatively elevated values associated only with shorter OS.

CEA is produced by tumor cells in several malignancies (Carpelan-Holmström et al., 1995, Molina et al., 2008, Shimada et al., 2014, Uehara et al., 2008, Zhang et al., 2018b), which leads to the assumption that any increase above the normal serum levels might be indicative of existing cancer in the body. However, there are various confounding factors – such as smoking and non-neoplastic diseases – that also affect the serum levels (Alexander et al., 1976, Hao et al., 2019). In our studies, all patients had diagnosed metastatic colorectal cancer, which decreases the likelihood of CEA increase due to non-malignant reasons.

The fact that the elevated levels of CEA before liver resection only associated with overall survival seems logical, as they reflect the already-existing liver metastases, which were then resected. In a previous study by Kawahara et al. (Kawahara et al., 2018), however, elevated preoperative CEA levels were indicative of future recurrence. This is contradictory to our results, but the difference may be due to distinct cut-off levels. Kawahara et al. used a cut-off level of 50.0 µg/l, which is significantly higher than that of 5.0 µg/l used in the studies

presented here. It seems possible that identifying the patients at a high risk of postoperative recurrence would require a higher cut point for preoperative CEA.
The postoperative values most probably reflect either residual or newly formed cancer, and thus, have a direct association with prognosis. Earlier, elevated CEA within one month after liver resection has been found to predict rapid recurrence (Takamoto et al., 2016), and postoperative increase has also associated with recurrence during the years following liver resection (Hara et al., 2013). Our results are in line with these, as nearly all patients with elevated postoperative CEA experienced recurrence, and most of the recurrences were detected within three years after resection.
The dilemma with CEA is that only 30–40% of the CRC tumors are reported to produce it (Saito et al., 2018, Thomsen et al., 2018). In this thesis, CEA was elevated preoperatively in 50.2% (222/442) of the patients, although all of them had diagnosed metastases at that point. In study I, the patients who had a normal CEA level postoperatively were found to develop recurrence later than those with elevated levels. The connection between elevated CEA seems to be in direct connection with existing cancer, with or without other clinical findings, but normal CEA after liver resection does not exclude recurrence at a later time.

Interestingly, both pre- and postoperatively elevated levels of CA19-9 associated with shorter DFS/RFS and OS, although the association between the preoperative values and RFS was only found in study II. Among patients with normal CEA pre- or postoperatively, CA19-9 did not provide any additional prognostic information. However, it should be noted that preoperatively elevated CEA was only mildly prognostic in itself, and thus, preoperative CA19-9 should be evaluated independently. Approximately one-third of all patients had a CA19-9 level of >26 kU/l before liver resection, and the elevated values indicated an increased risk of early recurrence and death. Based on the results presented in studies I and II, CA19-9 is an important prognostic biomarker, when measured before liver resection. Postoperatively, however, CEA seems to be more useful than CA19-9 for most patients.

Based on the results of this thesis, CEA is a valuable prognostic biomarker after liver resection, but it should be measured regularly in order to detect recurrence. More than 50% of all patients included in this study had elevated levels before liver resection as a sign of CEA-producing carcinomas, and postoperatively increased values indicated recurrence with high accuracy. Thus, any postoperative increase should lead to further examinations and treatment. CEA is currently the only biomarker recommended to be measured regularly in the follow-up of CRC (Argiles et al., 2020), and our findings confirm its usefulness. However, in patients who do not have elevated CEA levels, other biomarkers could be considered instead.
In the future, higher cut-off levels for CEA could be tested before liver resection. Based on earlier publications (Kawahara et al., 2018, Bredt and Rachid, 2014), it seems possible that in case of existing liver metastases, the prognostic significance of preoperative CEA would be stronger with cut-off levels higher than the routinely used 5.0 µg/l.

Quite surprisingly, CA19-9 seemed have more prognostic significance than CEA, when measured before liver resection. Instead, postoperatively its additional value compared to CEA remained marginal. CA19-9 is currently not recommended for the routine follow-up of CRC (Duffy et al., 2014), but our results suggest that it is a promising biomarker for the evaluation of future prognosis before proceeding to liver resection.

An additional aspect to be considered is the individual variation in the biomarker levels. Changes in the concentrations of CEA and CA19-9 within reference limits in consecutive samplings should also be studied, as increase or decrease might reveal recurrent disease among some individuals, even though the concentrations remained normal according to the cut-off limits.

At the Helsinki University Hospital, both CEA and CA19-9 are measured before every control appointment at the department of oncology after the operation on the primary colorectal tumor or the liver metastases. In case these biomarkers have not been elevated despite of metastatic disease, using other biomarkers instead should be considered. In addition, the significance of increasing concentrations within the reference limits should be noted, as it is possible that they reflect disease progression.

2. HCGβ and TATI (I)

Both hCGβ and TATI are promising biomarkers in cancer, and there are several studies on their prognostic significance in CRC (Birgisson et al., 2012, Gaber et al., 2010, Louhimo et al., 2002, Lundin et al., 2000, Lundin et al., 2001, Koskensalo et al., 2012a). We found that preoperatively elevated TATI in plasma (>13 μg/l) associated with poor 3-year DFS after liver resection, and postoperatively elevated hCGβ (>1.0 pmol/l) with poor 3-year OS. Otherwise no statistically significant associations were found, when all patients were included in the analyses.

Earlier, elevated hCGβ (>2.0 pmol/l) measured before the operation on the primary colorectal tumor was shown to predict poor OS in a material including 204 patients with Dukes' A–D disease (Louhimo et al., 2002). In a cohort of 334 CRC patients undergoing operation on the primary tumor, preoperatively elevated serum TATI (>15.59 μg/l) predicted impaired DFS and OS (Gaber et al., 2010), and preoperatively elevated hCGβ (>1.55 pmol/l) associated with shorter survival in a cohort of 324 patients, especially among women (Birgisson et al., 2012).
The previously unpublished results presented in this thesis showed that both hCGβ and TATI were prognostic in specified subgroups of patients. First, elevated hCGβ after liver resection indicated worse survival among males and in primary rectal cancer. The association among men is contradictory to the results of Birgisson et al. (Birgisson et al., 2012), who found an

association between elevated preoperative serum hCGβ levels and shorter survival among women. There are several possible explanations to this difference: the patients included in the study of Birgisson et al. had primary colorectal tumors instead of liver-metastatic disease only; the hCGβ measurements were performed using serum samples instead of plasma; the samples were drawn before the operation on the primary tumor and not in conjunction with liver resection; and the cut-off level used was 1.55 pmol/l instead of 1.0 pmol/l. However, it seems clear that the prognostic value of hCGβ is partly dependent on the patient's gender and, based on the novel findings in this thesis, possibly also on the location of the primary tumor.

Our results concerning TATI were not as promising as the previously published ones, but preoperatively elevated values were found to associate with shorter DFS, especially in synchronous disease. A substantial difference between the earlier studies and ours is that our material included only patients with stage IV (Dukes' D) disease, and 47.0% (79/168) of the patients had metachronous liver metastases, possibly referring to lower tumoral volume than in primary Dukes' D disease. Thus, it seems possible that TATI is a potential biomarker in metastatic CRC with a larger tumor volume.

Further analyses in a larger patient cohort and more specific subgroup analyses may give additional information on the prognostic significance of both hCGβ and TATI. As TATI can inhibit the activation of latent MMPs that contribute to cancer invasion (Moilanen et al., 2003), their relationship is also of interest. However, in the light of the present findings, the prognostic value of hCGβ and TATI seems to be inferior to that of CEA and CA19-9 in CRC with liver metastases.

3. YKL-40, IL-6, CRP, CEA, and CA19-9 combined (II)

In study II, elevated serum concentrations of the inflammatory biomarkers YKL-40 and IL-6 associated with shorter RFS and OS, when measured either before or after liver resection, and postoperatively elevated CRP indicated poor survival. However, only the association of preoperative YKL-40 with OS was significant in the multivariate analyses.
The prognostic value of CEA and CA19-9 individually is discussed in the previous **section 1**.

Interestingly, when all the five biomarkers – YKL-40, IL-6, CRP, CEA, and CA19-9 – were combined, 2–5 elevated biomarkers either pre- or postoperatively indicated clearly worse RFS and OS after liver resection. The number of patients with 2–5 elevated biomarkers was 43.9% preoperatively and 41.4% postoperatively, accounting for a significant proportion of the patients.

In an explorative analysis, it was noted that CEA and CA19-9 seemed to be the main drivers of the associations between the elevated biomarkers and survival, but the risk of relapse increased with every additional elevated biomarker.

Earlier, elevated plasma levels of YKL-40 have been shown to predict poor survival in patients with metastatic CRC (N=566) treated with first-line oxaliplatin-based chemotherapy alone or combined with cetuximab (Tarpgaard et al., 2014). The results of our study are similar, showing that YKL-40 is an independent predictor of poor OS in patients with colorectal liver metastases. Concerning CRP, elevated values (>10 mg/l) before liver resection were earlier found to predict poor survival in a cohort of 492 CRC patients undergoing liver resection (Kostner et al., 2016). In our study, preoperative CRP did not have prognostic significance, but the postoperative values indicated poor OS. Noteworthy, we used a cut-off level of 5 mg/l instead of 10 mg/l, and it is possible that also the preoperative values would have been prognostic with a higher cut point.
YKL-40 has been found to promote angiogenesis in malignant tumors via the regulation of VEGF (Yeo et al., 2019), and it also contributes to cancer metastasis by increasing the migration and invasion of cancer cells (Jefri et al., 2015). IL-6 stimulates the production of YKL-40 and CRP in the liver (Nishikawa et al., 2008, Nielsen et al., 2011, Tanaka and Kishimoto, 2012). In study II, we decided to investigate YKL-40, IL-6, and CRP in combination, because YKL-40 is also induced by proinflammatory cytokines and other biological processes (Yeo et al., 2019). We also estimated that the production of CRP might be affected by the liver metastases.

Inflammation is an established characteristic of cancer (Nasr et al., 2018, Hanahan and Weinberg, 2011), and our results confirm the link between systemic inflammation and impaired prognosis in patients with colorectal liver metastases. A biomarker panel including the inflammatory biomarkers YKL-40, IL-6, and CRP and the cancer biomarkers CEA and CA19-9 could be used to identify the patients at a high risk of relapse already before liver resection. However, the role of IL-6 and CRP in this panel seems to be inferior to that of the other biomarkers.

4. MMP-8, MMP-9, and MPO in serum (III)

MMP-8 and MMP-9 are promising new prognostic biomarkers in gastrointestinal malignancies, and their expressions in tumor tissue specimens and concentrations in serum have been actively investigated in the last years. Earlier, elevated serum levels of MMP-8 have been associated with impaired prognosis in CRC (Böckelman et al., 2018, Sirniö et al., 2018, Väyrynen et al., 2012). However, no studies concentrating on the prognostic significance of MMP-8 or MMP-9 in a purely metastatic setting have been published previously.
Serum MPO, on the other hand, has not been studied in metastatic CRC earlier, and in Study III, novel data on its prognostic significance was obtained.

In our patient cohort, both preoperatively low and high serum concentrations of MMP-8 indicated shorter OS, which corresponds to the results of a study by Laitinen et al. on MMP-8 in gastric cancer (Laitinen et al., 2018). It is worth mentioning that the pre- and postoperatively elevated serum values of MMP-8 associated with shorter survival especially among over 65-year-old patients and among those with primary colon tumors, and postoperatively intermediate and high values of MMP-8 in the same subgroups as well as in synchronous disease. Postoperatively elevated serum MMP-9 associated with poor survival only among over 65-year-old patients.
MMP-8 seems to have more prognostic value in primary colon cancer rather than rectal cancer, and the significance of both MMP-8 and MMP-9 appears to be dependent on the patients' clinical characteristics, especially age. MMPs can promote immune responses (Egeblad and Werb, 2002), and elevated levels of MMP-8 have been observed in several inflammatory conditions (Lauhio et al., 2016). Thus, the stronger prognostic value observed among elderly patients may be related to immune responses that become weaker with age. It is possible that the negative prognostic value of MMP-8 and MMP-9 is compensated by stronger immune responses among younger patients, which would explain why no association with prognosis was found among them.

One factor to be taken into consideration is that the cut-off levels used in the studies on serum MMP-8 in CRC have varied. In Study III of this thesis, the ROC curve analyses did not provide any statistically significant results, and thus, we used the tertiles of the pre- and postoperative serum concentrations as cut points. In previous studies, at least the cut-off levels of 60.0, 63.4, and 100.0 ng/ml have been used (Böckelman et al., 2018, Väyrynen et al., 2012, Sirniö et al., 2018). In addition, the methods used in the serum determinations may affect the results, but the measurements in the above-mentioned studies were performed congruently (Mauramo et al., 2018). Thus, an optimal cut-off level for prognostic significance remains to be established.

MPO is an interesting biomarker, and no previous studies have been published concerning the prognostic value of its serum concentrations in CRC. However, elevated serum values have been associated with absence of lymphovascular invasion in breast cancer (Coelho et al., 2014), and high infiltration of MPO-positive inflammatory cells have signified improved prognosis in both breast cancer (Zeindler et al., 2019) and CRC (Droeser et al., 2013).
In our patient cohort, elevated preoperative serum concentrations associated with significantly improved prognosis after liver resection and, correspondingly, low concentrations indicated poor prognosis. The positive prognostic value was most significant among women, under 65-year-old patients, and in patients with synchronous liver metastases. The role of the postoperative concentrations remained controversial, as high values associated with impaired prognosis and only in over 65-year-old patients. On the other hand, this association among elderly patients was similar to that found concerning serum MMP-8 and MMP-9.

These results refer to a protective role of MPO in metastatic CRC. What makes this finding interesting is that MPO is known to catalyze the production of the pathogenic acid HOCl (Gomez-Mejiba et al., 2010) and to oxidatively activate proMMP-8 and -9 (Saari et al., 1990), which contribute to tumor invasion. On the other hand, MPO is needed for the proper functioning of the immune responses (Parry et al., 1981) that have importance in suppressing cancer metastasis. Thus, the prognostic value of serum MPO may reflect the immune defense mechanisms against cancer.

Another aspect to be speculated on is the strong association observed in women, which could refer to hormonal regulation, possibly via estrogen. Low levels of estrogen have been linked to increased activity of MPO in experimental menopause (Pósa et al., 2015) and, in theory, the positive prognostic value of MPO in women might signify lower estrogen levels in those patients, and thus, less aggressive disease. In men, this difference was probably not observed because of generally lower levels of estrogen.
It is also worth mentioning that preoperatively low MPO indicated poor survival among patients who received neoadjuvant chemotherapy before liver resection, while no difference in survival was observed among those who did not receive neoadjuvant treatment. Thus, MPO may also reflect response to treatment, possibly via immunological mechanisms.

Earlier, Linkov et al. observed that the serum levels of MMP-8, MMP-9, and MPO are individual (Linkov et al., 2009), suggesting that knowing the baseline for each patient might be required in order to use these biomarkers as a prognostic tool in CRC. It is probable that the normal levels differ with age in healthy individuals the same way as those of several tumor markers (Lopez et al., 1996), even though that was not confirmed in the study by Linkov et al. Our results strongly support this hypothesis.

In conclusion, MMP-8, MMP-9, and MPO are potential prognostic biomarkers in CRC with liver metastases. However, their prognostic significance seems to depend on the patients' clinical characteristics, such as gender and age, and further knowledge of their biological characteristics is needed before they can be adapted to clinical use.
Especially MPO is a biomarker of interest, since its elevated serum levels were shown to associate with improved prognosis. Thus, high levels may refer to stronger anticancer immune responses. In addition, a possible link with hormonal regulation mechanisms was noted, as the prognostic value of MPO was significant especially in women. Among patients receiving neoadjuvant treatment before liver resection, low preoperative MPO indicated early postoperative recurrence, suggesting that those patients might benefit from more efficient perioperative chemotherapy.

5. MMP-2, MMP-8, and MMP-9 in tumor tissue (III)

In study III, we found that high expression of MMP-9 in the primary colorectal tumors associated with a significantly improved prognosis after liver resection compared to low expression. The association was stronger in patients with metachronous liver metastases than in those with synchronous metastases, that is, in patients with no existing liver metastases at the time of the operation on the primary tumor. This finding is similar to that concerning the prognostic value of TKTL1 in study IV.

In addition, the expression of MMP-9 in the primary tumors was lower in synchronous than in metachronous disease. Previously, Takeha et al. found that the number of MMP-9-positive cells along colorectal tumors' invasive margin was significantly smaller in patients with liver metastases compared to those without metastases (Takeha et al., 1997). These findings suggest that the expression pattern changes depending on the stage of the disease. It can be speculated that low expression of MMP-9 in the primary colorectal tumors reflects either already existing (synchronous) or shortly developing (metachronous) liver metastases, and thus, worse prognosis.

The expression of MMP-9 in the primary tumors was also a stronger prognostic factor among women than among men, and among patients with primary colon rather than rectal cancer. The variance in the prognostic significance according to the exact location of the primary tumor is presumable, as CRCs originating from different parts of the colon or rectum are currently classified as different types of disease with divergent prognoses (Loree et al., 2018, Shida et al., 2020). The association in women, on the other hand, has not been observed before, and it raises speculation about a possible link with hormonal regulation, similarly as with serum MPO. Estrogen is known to promote tumorigenesis (Heijmans et al., 2014), and stimulation of estrogen receptor α (ERα) has been shown to induce the upregulation of MMP-9 (Ahmad et al., 2018). It can be hypothesized that estrogen levels are

higher in women than in men, leading to the release of MMP-9 from inside the tumor cells into the ECM via stimulation of the estrogen receptors – observed as a low expression in the tumor cells – and thus, impaired prognosis.

Somewhat surprisingly, the expressions of MMP-2 and MMP-8 in the primary colorectal tumors did not associate with prognosis. In addition, the expressions of all three biomarkers – MMP-2, -8, and -9 – in the liver metastases were not prognostic, except for an association between low MMP-2 expression and shorter DFS in women, and that between high MMP-8 expression and shorter OS among patients with primary colon cancer.
Altogether 59.5% of the patients included in Study III received neoadjuvant chemotherapy before liver resection, and it seems possible that it affects the expression and prognostic significance of the investigated MMPs in the liver metastases. Indeed, when only the patients who did not receive neoadjuvant chemotherapy were included in the analyses, it was found that low expression of both MMP-8 and MMP-9 in the primary tumors associated with worse DFS after liver resection, while no difference was observed between the low and high expression groups among patients who did receive neoadjuvant therapy. In the liver metastases, some differences were also observed, but they remained non-significant.
It is also worth mentioning that there was no correlation between the expressions of MMP-2, MMP-8, and MMP-9 in the primary tumors and the liver metastases, and only the expression of MMP-8 in the liver metastases correlated with the serum concentrations of the corresponding biomarker. Thus, it seems that there is variation in the tissue expression of these biomarkers between the primary tumors and the liver metastases, and at least serum MMP-9 may be mainly produced by extratumoral cells or tissues.

As a conclusion, high expression of MMP-9 in the primary colorectal tumors indicated improved prognosis after liver resection, especially among women and in patients with primary colon cancer. High expression in the primary tumors was also prognostic of better DFS and OS in patients with metachronous liver metastases. Hence, liver resection seems to be especially effective among patients with high MMP-9 expression in the primary tumors, but among those with low expression, efficient perioperative chemotherapy and more intensive follow-up after resection may be beneficial. The possible connection with hormonal regulation should be examined further.
MMP-2 and MMP-8 in the primary colorectal tumors were not prognostic in the whole patient cohort, but it seems that the patients with low MMP-8 expression may also benefit from neoadjuvant chemotherapy before liver resection. The expression in the liver metastases was neither indicative of prognosis, which may be due to the neoadjuvant chemotherapy that the majority of the patients received.

6. TKTL1 (IV)

In patients with synchronous liver metastases, high TKTL1 expression in both the primary colorectal tumors and the liver metastases associated with poor prognosis. By contrast, in patients with metachronous metastases, high expression in the primary tumors and the metastases indicated better prognosis.
In previous studies, high TKTL1 expression has been shown to predict poor prognosis in lung and cervix cancer (Kayser et al., 2011, Chen et al., 2009), oral squamous cancer (Grimm et al., 2014), ocular adnexal tumors (Lange et al., 2012), and also in locally advanced rectal cancer (Schwaab et al., 2011). In a material of 733 CRC patients, of whom 22.5% (165/733) had a stage IV disease, high TKTL1 expression in primary colorectal tumors associated with poor disease-specific survival (Ahopelto et al., 2016). Our results in a cohort comprising only patients with resectable colorectal liver metastases offer a new dimension to this biomarker, as high expression in the primary tumors and the liver metastases indicated improved prognosis in patients with metachronous metastases.

A possible explanation for our findings could be that synchronous and metachronous CRC are two different types of disease. TKTL1 is suggested to catalyze the non-oxidative part of the PPP, enabling the tumor cells to consume glucose in the absence of oxygen and to produce lactate, consequently enhancing the invasiveness of cancer (Langbein et al., 2006). In a study by Diaz-Moralli et al., the expression of TKTL1 in colorectal tumors was shown to increase progressively in disease stages I to III, but in stage IV, a strong decrease in the expression was observed (Diaz-Moralli et al., 2011). A similar pattern was found concerning the incidence of *KRAS* mutations, and it was speculated that the change in TKTL1 expression could be linked to the mutational status, and that *RAS* mutations might activate TKTL1.

Based on the results of our study, we argued that high expression of TKTL1 increases the invasiveness and metastatic ability of CRC, but in the patients with high expression in the primary tumors and no existing liver metastases (metachronous setting), there may be some other factors that strongly diminish the cancer's metastatic potential. As such, it remains unclear whether a high expression in metachronous CRC truly indicates a better prognosis or if it reflects other, yet unknown characteristics that reduce the invasiveness of the disease. *KRAS* mutational status might provide important further information concerning this. However, among patients with synchronous liver metastases and high TKTL1 expression in the primary colorectal tumors, more aggressive chemotherapy could be considered in addition to or instead of liver resection because of a very poor prognosis.

7. STRENGTHS AND LIMITATIONS

The strengths of the four studies presented here are the long follow-up period of up to 20 years as well as comprehensive and reliable follow-up data. The patient material was uniform, consisting solely of patients who had liver metastases of CRC operated on at a single institute. In study II, the number of patients was exceptionally large. In studies III and IV, on the other hand, the study material was unique, consisting of tumor tissue samples of both the primary colorectal tumors and the liver metastases in addition to serum samples drawn before and after liver resection.

A limitation of the studies is the missing information concerning the patients' *RAS* and *BRAF* mutation status as well as the MSI/MMR status. These were not routinely analyzed at the Helsinki University Hospital prior to years 2013 and 2018, respectively. In study II, a validation cohort would have further increased the value of the findings. In addition, the diurnal variation of IL-6 was not considered, which may confound the results. In studies III and IV, the major limitation was the quite small size of the patient cohort, which restricted the subgroup analyses and decreased the strength of the results. However, the statistically significant findings most probably are reliable, since they were obvious despite of the size of the cohort.

In addition, it can be speculated that the advances in the surgical techniques and the oncological treatments over the past couple of decades may partly confound the results presented here. Disease-free and overall survival of the patients with metastatic CRC have improved significantly, and it can be assumed that the patients who underwent liver resection later during the study period of this thesis had a better prognosis to start with than those who underwent resection in the early years. The long study inclusion period can also confound the results concerning the tissue biomarker expressions, as the age of the tissue specimens may affect the quality of the immunohistochemical stainings. However, no obvious changes in the stainings of the older versus newer tissue specimens were observed.

CONCLUSIONS

Based on the results of the four studies and the previously unpublished results presented in this thesis, the following is concluded:

1. CEA is a useful prognostic biomarker for approximately half of the patients following liver resection for colorectal metastases. However, a notable proportion of patients do not have elevated serum levels despite of metastatic disease, and some of those patients may benefit from measuring CA19-9, especially preoperatively. For patients who have normal CEA and/or CA19-9 levels, other biomarkers might be tested and considered instead in the follow-up after liver resection.

2. The prognostic value of hCGβ and TATI was found to be inferior to that of CEA and CA19-9, but they may identify some patients at a high risk of recurrence or death after liver resection.

3. A biomarker panel comprising YKL-40, IL-6, CRP, CEA, and CA19-9 could be used to identify the patients with a poor prognosis after liver resection. Two or more elevated biomarkers before or after resection indicate future recurrence and shorter survival. With this biomarker panel, the prognosis might be assessed already before liver resection, and the surgical and oncological treatments adjusted accordingly.

4. Elevated preoperative serum levels of MPO associate with improved prognosis, especially among women, under 65-year-old patients, and in patients with synchronous liver metastases. High expression of MMP-9 in the primary colorectal tumors indicates improved prognosis after liver resection, especially among women, in colon cancer, and in patients with metachronous liver metastases. Neoadjuvant chemotherapy seems to be especially beneficial for patients with low expression of MMP-8 and/or MMP-9 in the primary tumors, and low preoperative serum levels of MPO despite of neoadjuvant therapy indicate poor prognosis. The prognostic value of MMP-2, MMP-8, and MMP-9 in tumor tissue as well as MMP-8, MMP-9, and MPO in serum seems to be strongly related to the clinical characteristics of the affected patients, possibly via immunological and hormonal mechanisms.

5. High expression of TKTL1 in the primary colorectal tumors and the liver metastases is a sign of poor prognosis in colorectal cancer with synchronous liver metastases, and more efficient treatment should be considered for those patients. On the contrary, high expression in the primary tumors and the liver metastases indicates better disease-free survival in metachronous disease. This difference may be due to stage-dependent metabolic changes in colorectal cancer that need to be investigated further.

FUTURE PROSPECTS

Over the past years, it has become obvious that colorectal cancer is a disease with individually varying characteristics. The factors affecting the patients' prognosis are diverse, and they include molecular, metabolic, inflammatory, and hormonal regulation mechanisms. The search for reliable, easily accessed, and universally valid biomarkers for the follow-up of both local and metastatic CRC has been intense, but by far, it has been challenging to find biomarkers that would be applicable to all patients.

All research is based on reliable technical methods, and the results should be repeatable and comparable worldwide. At present, there is variation in the determination methods of several new serum/plasma biomarkers as well as in the interpretation of immunohistochemical stainings of tumor tissue samples. This causes challenges in the comparability of the results. The optimal methods for determining the concentrations or expressions of all biomarkers of interest should be established in order to produce generally comparable scientific data.

Different inflammatory biomarkers have great prognostic potential in various malignancies, as inflammation contributes to cancer invasion and progression. However, there is fluctuation in the normal physiological serum/plasma concentrations, and it would be valuable to define the reference levels according to age, gender, and other potentially confounding factors. Subsequently, the levels could be compared to those in CRC patients with local and metastatic disease. In addition, consecutive samplings and measurements of serum/plasma biomarkers after surgical interventions or during and after oncological treatment might give prognostic information and help estimate the response to treatment.
Biomarker panels including several different biomarkers, measured at the time of diagnosis of CRC or before liver resection, could be applied to clinical use, as they seem to have more prognostic value when used in combination. YKL-40, IL-6, CRP (or hsCRP), MMP-8, MMP-9, and MPO are promising biomarkers in this respect.
The biomarkers' tissue expressions in the liver metastases should be evaluated in larger patient cohorts with precise information about the possible neoadjuvant treatments. Biomarker expression in normal liver tissue outside the tumor cells might possibly provide novel information on the mechanisms of invasion.
The advances in genetic testing and the adoption of liquid biopsies to clinical use are expected to enable more extensive research shortly. After all, understanding the regulatory mechanisms behind the invasion and metastasis of CRC creates the basis for all treatment.

Estimating the prognosis of CRC with liver metastases already prior to liver resection is important, because it enables making necessary changes in the treatment plan. Hopefully, the biomarkers studied in this thesis will be useful in this respect. They increase our understanding of this complex disease and the possible ways towards cure.

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Reetta Pelts

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