

MOLECULAR AND IMMUNE LANDSCAPE OF COLORECTAL LIVER METASTASES

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ABBREVIATIONS

5-FU	5-Flurouracil
APC	<i>Adenomatous polyposis coli</i> gene
CALR	Calreticulin
CD	Cluster of differentiation
CI	Confidence interval
CIN	Chromosome instability
CLM	Colorectal liver metastases
CNA	Copy number alteration
CRC	Colorectal cancer
CTL	Cytotoxic T-cell (CD8+)
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMP	Damage-associated molecular patterns
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulating kinase
ESMO	European Society for Medical Oncology
FLV	5-FU and leucovorin
FOXP3	Forkhead box P3
HMGB-1	High-mobility group box-1 proteins
HSP	Heat-shock proteins
ICD	Immunogenic cell death
ICI	Immune checkpoint inhibition
IL-10	Interleukin -10
IM	Invasive margin
INF- γ	Interferon-gamma
IT	Intratumour
JAK	Janus kinases

<i>KRAS</i>	<i>Kristen rat sarcoma virus gene</i>
LAG-3	Lymphocyte activation gene-3
MAPK	Mitogen-activated protein kinase
mCRC	Distant metastases from CRC
MDT	Multidisciplinary team
MDSC	Myeloid-derived suppressor cells
MHC	Major Histocompatibility Complex
MMR	DNA mismatch repair genes
MSS	Microsatellite stable
MSI	Microsatellite instability
NACT	Neoadjuvant chemotherapy
N _{Cr}	Normal colon and rectum
NGS	Next generation sequencing
NK-cells	Natural killer cells
N _{Li}	Normal liver tissue
<i>NRAS</i>	<i>Neuroblastoma RAS viral oncogene homolog gene</i>
OS	Overall survival
PAMPs	Pathogen-associated molecular patterns
pCRC	Primary colorectal cancer
PD-1	Cell death protein-1
PD-L1	Programmed death-ligand 1
PFS	Progression free survival
PRR	Pattern recognition receptors
<i>PTEN</i>	<i>Phosphatase and tensin homolog deleted on chromosome ten gene</i>
RCT	Randomised control trial
RNA	Ribonucleic acid
RR	Response rate
STAT	Signal transducer and activator of transcription proteins
TCGA	The Cancer Genome Atlas project
TCR	T-cell receptor
TGF-β	Transforming growth factor-β

TMB	Tumour mutational burden
TH	Helper T-cells (CD4+)
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TME	Tumour microenvironment
TNM	Tumour-node-metastases classification
<i>TP53</i>	<i>Tumor protein p53</i> gene
Treg	Regulatory T-cells (FOXP3+)
T _{tot}	Total amount of T-cells (CD3+)
UICC	International Union Against Cancer classification
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related integration site

ARTICLES INCLUDED IN THE THESIS

- Paper I: Fretland ÅA, Dagenborg VJ, Bjørnelv GMW, Kazaryan AM, Kristiansen R, Fagerland MW, Hausken J, Tønnessen TI, Abildgaard A, Barkhatov L, Yaqub S, Røsok BI, Bjørnbeth BA, Andersen MH, Flatmark K, Aas E, Edwin B. Laparoscopic Versus Open Resection for Colorectal Liver Metastases: The OSLO-COMET Randomized Controlled Trial. *Ann Surg.* 2018 Feb;267(2):199-207. PMID: 28657937.
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INTRODUCTION

Cancer

Cancer is a heterogeneous disease, of which we still do not fully comprehend the intricate mechanisms. In 2017 cancer was the second most common cause of death worldwide according to the Global Burden of Disease database ¹⁸. Cancer has been a part of our history for thousands of years. There are 3500 year old Egyptian records of cancer, and the Romans provided detailed descriptions of cancer surgery, highlighting the importance of early treatment, details of surgical technique and preoperative and postoperative care ^{19, 20}. Cancer has been a conundrum, and many theories have sought to explain the disease. About 2000 years ago hippocratic physicians described “karkinomas” as non-healing ulcers, caused by an imbalance in body fluids. In the 17th and 18th century cancers were thought to be connected to lymphatics, inflammation, and parasites ²⁰. We now know that normal cells can transform into cancer (carcinogenesis) when cells lose the normal and well-regulated control mechanisms and gain the ability of uncontrolled growth. After the discovery of deoxyribonucleic acid (DNA) in the early 1960s, the mechanisms of cancer could be explored in more depth. The knowledge that errors in a cell’s DNA can cause cancer, has helped us better understand carcinogenesis. Initially two categories of genes harbouring errors were defined, the oncogenes and the tumour suppressor genes. Errors in oncogenes, like the *Kirsten Rat Sarcoma Virus (KRAS)* gene, were recognised for the ability to cause uncontrolled cell growth. Tumour suppressor genes on the other hand are part of a cell’s normal control mechanisms and allow the cell time to repair damaged DNA. If DNA damage is beyond repair, tumour suppressor genes will normally activate programmed cell death and the cell will perish. Damage to tumour suppressor genes, however, can lead to defects in these important control mechanisms and is a part of carcinogenesis.

Hallmarks of Cancer

In 2000, Hanahan and Weinberg published a summary of known cancer related processes, and summarised these as six biological capabilities which they called the Hallmarks of Cancer ⁶ (Figure 1). A cancerous tumour was recognised as a complex tissue that included a surrounding tumour microenvironment (TME) that actively participated in the progression of cancer. Firstly, to grow (proliferate), cancer cells acquired errors that disrupted cellular control growth mechanisms. Errors in the genes could cause a continuous production (self-sufficiency) of growth factors or increase the cell's sensitivity to growth factor stimuli. The canonical mitogen-activated protein kinase (MAPK) pathway has a key role in the response to growth factor stimuli, for example through the epidermal growth factor receptor (EGFR). In cancer cells, a constitutive activation of the MAPK pathway is caused by mutations in important genes, such as

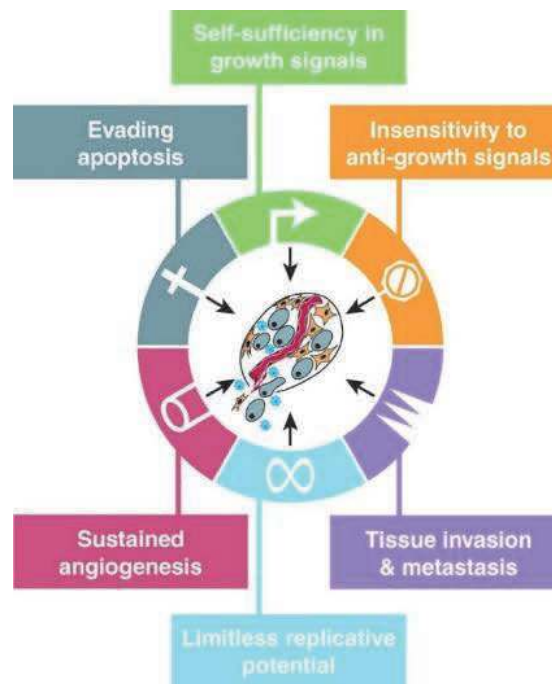


Figure 1. Hallmarks of Cancer

The six biological capabilities defining the Hallmarks of Cancer: the ability to evade apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death, ability to provide nutrients via sustained angiogenesis, limitless replicative potential and the ability to invade tissues and form metastatic tumours.

With permission. Hanahan D and Weinberg RA⁶. Copyright © 2000 Cell Press. All rights reserved.

KRAS, *neuroblastoma RAS viral oncogene homolog (NRAS)* and *B-Raf proto-oncogene (BRAF)*. These genes are all important in controlling cell proliferation and cell survival, and errors in them cause a self-sufficiency in growth signalling, a trait associated with metastatic progression^{6, 21}. For normal cells, programmed cell death (apoptosis) would be the outcome of such strong proliferative signals as gained by a continuous MAPK activation. For cancer cells, on the other hand, errors to the control mechanisms handling DNA damage enable escape from apoptosis. Active cancer cells can also create a TME lacking in oxygen and nutrients due to these cell's metabolic demands¹¹. In an attempt to overcome the lack of oxygen, cancer cells trigger signals that activate the formation of new blood vessels (angiogenesis) in the surrounding stroma by for example, secreting vascular endothelial growth factor (VEGF). VEGF can be blocked by monoclonal antibodies as a part of cancer treatment²². Cancer cells can also acquire mechanisms to generate more energy from the TME. In 2011, Hanahan and Weinberg touched upon the importance of autophagy or “self-eating” as such a mechanism. Normally, autophagy is a well-controlled physiological response where cells generate energy by degrading and recycling cellular fragments. The role of autophagy is not fully understood in cancer, and in colorectal cancer (CRC) it has been associated to both pro- and anti-tumour effects²³. Invasiveness and distant tumour cells have for a long time been associated with cancer. Several changes to a cancer cell and the TME are linked to invasive growth and metastasis. For instance, the loss of E-cadherin (an adhesion molecule) expression enables a cancer cell to detach and migrate from its origin to distant sites²². An up-regulation of proteins favouring migration and activation of a residual embryonic system called epithelial-mesenchymal transition (EMT) enable characteristics favouring invasion, survival and dissemination in cancer²⁴.

Eleven years after the initial publication, Hanahan and Weinberg extended the framework of the six hallmarks with four additional biological capabilities¹¹: deregulation of cellular energetics, genome instability and mutations, tumour promoting inflammation, and the ability to avoid immune destruction (Figure 2). Cancer cells require energy, and the ability to reprogram metabolic processes to account for this need for energy is one enabling characteristic highlighted by Hanahan and Weinberg. Genetic errors in cancer cells (genomic instability) may change the function of the cell. This enabling

characteristic will give some cancer cells an advantages through natural selection of cells in tumours that are adapted to thrive.

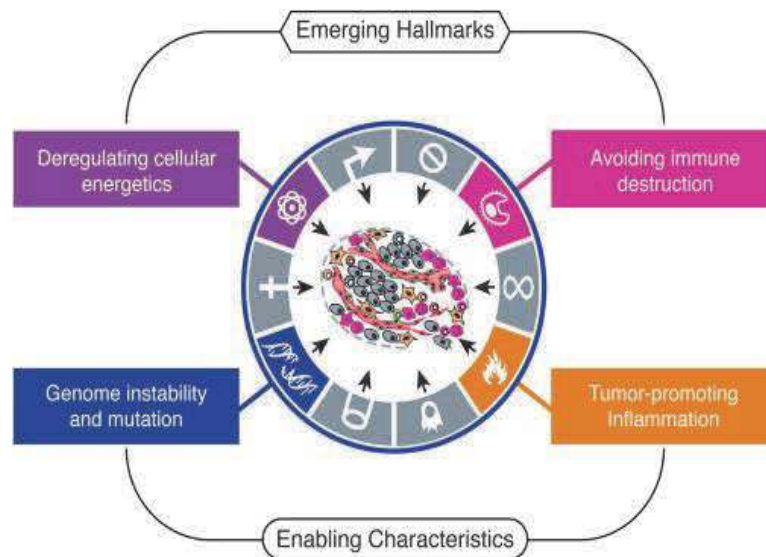


Figure 2. Enabling and emerging Hallmarks of Cancer

The characteristics of genome instability and mutations, and the role of inflammation were in 2011 introduced as enabling capabilities in cancer. Two emerging hallmarks were also introduced. These were related to a cancer cell's ability to reprogram its energy sources, and a tumour's ability to avoid destruction by the immune system.

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Colorectal Cancer

CRC is one of the most common cancers, with 1.4 million new cases diagnosed worldwide in 2012, where sixty percent of these cases were found in countries with a high human developed index ²⁵. Even when comparing countries with a high human developed index, the trends in incidence rates vary. Where the USA and several Western European countries experience stable or declining incidence rates, in Norway and Denmark the relative incidence of colon cancers is increasing ^{25, 26}. Overall the incidence of CRC is rising and is estimated to increase by another 60% by 2030 ²⁵. Worryingly, the burden of CRC is increasing in the younger population as well ²⁷. In 2018 in Norway, 4428 new CRC cases were diagnosed (3068 colon and 1360 rectum) ²⁶, making CRC the second most frequent cancer, similar to reports from other

developed countries ^{28, 29}. When comparing 5-year intervals from 2009-2013 versus 2014-2018, the increase in the number of new colon cancer diagnoses in Norway were more pronounced in women with a 5% increase versus 1% in men, while rectal cancers showed a decrease of 4% in women and 3% in men ²⁶. The age at presentation of CRC in the Norwegian population has been relative unchanged for the last 4 decades at a median age of 73 years for colon cancers and 69 years for rectal cancers.

CRC classification

Ninety percent of tumours in the colon and rectum are classified histologically as adenocarcinomas, derived from the epithelial lining of the colon and rectum ³⁰. CRC adenocarcinomas can further be classified by differentiation grade, judged by how abnormal the tumour appears compared to normal tissue, from well differentiated

Table 1. TNM-staging of CRC, from the TNM classification of malignant tumours, 7th edition

T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into pericolorectal tissues
T4a	Tumour penetrates to the surface of the visceral peritoneum
T4b	Tumour directly invades or adherent to other organs or structures
N0	No regional lymph node metastasis
N1	Metastasis in 1–3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2 - 3 regional lymph nodes
N1c	Tumour deposit(s) in the subserosa, mesentery, or nonperitonealised pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
M0	No metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ, without peritoneal metastases
M1b	Metastasis to 2 or more sites or organs, without peritoneal metastasis

Table 2. UICC stage classification of colorectal cancer

UICC-stage	T	N	M
I	T1, T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1, T2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3, T4a	N1/N1c	M0
	T2-T3	N2a	M0
	T4b	N1-N2	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
IVA	Any	Any	M1a
IVB	Any	Any	M1b

(closest to normal), to moderately differentiated and poorly differentiated (very abnormal)³⁰. In 1958 the International Union Against Cancer (UICC) published a classification system for cancer defined by the extent of Tumour, Node and Metastasis (TNM)³¹. For CRC, the TNM classification described the extent of the tumour invasion (T) into the bowel wall, the presence and extent of lymph node metastases (N), and the presence of distant metastases (M). This classification system can be applied as a preoperative staging based on clinical examination or radiological imaging like computer tomography or magnetic resonance imaging, or postoperatively by a pathologist on a surgically resected specimen. The UICC 8th edition has been in use since 2018. For this work, the UICC 7th edition of TNM classification was used (Table 1)³². Based on the TNM classification patients can be grouped into stage I to IV according to UICC (Table 2). Both TNM and UICC are used to risk stratify patients and are valuable tools for treatment planning and gaining insight into treatment effects and a robust prediction of outcomes. Equally importantly, TNM yields information essential to scientific reporting, enabling a better understanding of cancers with the worldwide applicability of the system³¹. On the other hand, the TNM classification has been criticised for its dynamic nature with frequent revisions, geographical clustering of information sources, the subjective nature of the system based on individual pathologists' examination and documented lack of inter-personal agreement, and lack of information from randomised control trials (RCT)³³.

Molecular classification of CRC in clinical use

Mutations

Testing tumours for mutations in *KRAS*, *NRAS* and *BRAF* is currently recommended when patients are diagnosed with metastatic CRC (mCRC). These mutations have predictive value for response to anti-EGFR treatment^{3, 34}, as well as prognostic value where mutations in these genes are associated with worse outcome for patients³⁵⁻³⁷.

Defective mismatch repair systems/microsatellite instability

Cells have mechanisms that detect errors that occur during DNA replication, called DNA mismatch repair systems (MMR), and cancers, including CRC can be classified based on either a functional or a deficient MMR system. In cancers, an inactivation of the MMR

genes (*MLH1*, *MSH2*, *MSH3*, *MSH6* and *PMS2*) due to mutations or epigenetic alterations can lead to an accumulation of genetic errors in the DNA. MMR deficient cancers are referred to as microsatellite instable tumours (MSI), due to multiple repeats of short DNA sequences (microsatellites) that accumulate in the genome. Cancers with a functional MMR are classified as microsatellite stable (MSS)³⁸. Lynch syndrome is an inherited error in the MMR genes that predisposes for cancer development at a young age³⁹. However, unlike the inherited Lynch Syndrome, most MSI tumours develop spontaneously due to acquired defects in the MMR genes. MSI is found in about 15% of primary CRC (pCRC) patients, who are reported to have a better prognosis⁴⁰. In the management of pCRC MSI tumours are not offered adjuvant systemic treatment due to the lack of response⁴¹.

Surgical management of primary CRC

Surgical resection with curative intent is the most optimal treatment strategy for patients with pCRC. The principles of colorectal cancer surgery comprise the resection of the tumour bearing segment of the colon or rectum with adequate proximal, distal and circumferential resection margins, along with a central division of the bowel segment's blood supply to ensure adequate harvesting of lymph nodes⁴². To achieve an optimal resection of the tumour, the surgeon endeavours to obtain margins of 10 cm in colonic cancers, 5 cm for tumours at the rectosigmoid junction and at least 1 cm in rectal resections. To further achieve better outcomes, a complete mesocolic excision is now recommended⁴³. This surgical principle was developed for rectal cancers by Heald et al in the 1980's⁴⁴. Heald described the strategy of sharp dissection in the avascular embryological planes around the mesorectum to achieve a total mesorectal excision. This technique was found to reduce the risk of local recurrence of rectal cancer from over 40%, to less than 5%. Obtaining a free circumferential resection margin (R0 resection) is important in rectal cancers. Studies showed that tumour tissue located closer than 1 mm (R1 resection) to the mesorectal fascia carried a higher risk of a local recurrence, metastasis and was thus associated with shorter survival⁴⁵. In locally-advanced rectal and colon cancers where the tumour has invaded surrounding organs, the importance of R0 resection has justified the use of multivisceral resections through

the removal of tumour-infiltrated organs along with the tumour itself. In such advanced tumours, this extensive form surgery offers the best long-term survival ⁴⁶.

Oncological management of pCRC

The use of preoperative (neoadjuvant) treatment in colonic tumours has not yet been established. Some studies report benefit when giving neoadjuvant systemic treatment (NACT) for T4b colonic tumours compared to surgery followed by adjuvant treatment ⁴⁷. These studies show that NACT increases the likelihood of free resection margins and reduces the need for multivisceral surgical resections. For rectal cancers, the use of neoadjuvant radiotherapy and chemotherapy are well-documented treatment modalities used to reduce the risk of a local recurrence. Neoadjuvant radiochemotherapy is considered when the tumour is threatening the circumferential resection margin with a risk of obtaining an R1 resection ⁴⁸. The Norwegian guidelines suggest administration of radiochemotherapy in form of a long-course regimen (50 Gy over 5 weeks) in combination with a fluoropyrimidine or a short-course regimen (25 Gy over 1 week) for any T4 rectal tumours or for T3 tumours where the distance from the tumour to the mesorectal fascia is 2 mm or less, or 1 mm or less from a suspected metastatic lymph node ⁴⁹. For postoperative colon tumours systemic (adjuvant) treatment is recommended for patients who have a high risk of CRC recurrence in form of a local recurrence or metastasis. Studies have demonstrated that adjuvant treatment for stage III colon cancer reduces the risk of death by 20% ⁵⁰. According to Norwegian guidelines, adjuvant treatment is offered for colon cancers classified as stage III, T4 tumours, if less than 12 lymph nodes were harvested during surgery, or when a perforation occurs near the tumour ⁴⁹. The recommended treatment regimen in high-risk cases comprises a 6-month course of fluoropyrimidine 5-fluorouracil (5-FU) in combination with leucovorin (FLV) and oxaliplatin ^{49, 50}. A subgroup of stage III patients with T1-3 tumours and N1 are considered at lower risk and can now be offered a 3-month course of systemic treatment ⁴⁹. In this group, reports have shown that a 3-month course combining capecitabine (a pro-drug for 5-FU) and oxaliplatin was noninferior to the standard 6-month regimen ⁵¹.

CRC outcomes for pCRC and mCRC

In the period from 2014 to 2018 in Norway, colon cancers were reported to have a 5-year overall survival (OS) of 65% in men and 68% in women, and rectosigmoid cancers were found to have an OS of 70% in men and 69% in women ²⁶. The outcome of CRC depends on the stage at diagnosis, and in 2014 to 2018, the 5-year OS for localised colon cancer (stage I and II) was reported as 96/98% in men/women, regional disease (T4 or stage III) was 82/83% in men/women, and for distant metastases (organ metastases or distant lymph nodes) the OS was 15%/18% for men and women respectively ²⁶. The outcomes in Norway are similar to international reports ^{52, 53}. In 2013, CRC was the 4th most common cause of cancer-related death worldwide, where mCRC was the main cause of mortality for CRC patients ^{25, 29}. For up to 80% of patients, systemic chemotherapy will be the considered treatment option due to an extensive burden of metastatic disease, where the median OS is approaching 30 months in this patient group ³. This accounts for a doubling in OS over the last two decades and is explained by better surveillance after pCRC resection, better biomarkers and chemotherapy regimens with more focus on down-staging prior to surgical intervention, as well as an improved overall continuum of care ³. The liver is the main site of mCRC, where about 20% of patients present with colorectal liver metastasis (CLM) at the time of CRC diagnosis ⁵⁴. Reports often quote that up to 50% of CRC patients develop CLM ⁵⁵⁻⁵⁷. However, in a study including almost 50,000 patients from Swedish registries, 30% of patients with CRC developed mCRC during follow-up. In this large dataset, 70% of mCRC was located in the liver, 32% to the thoracic cavity and 21% to the peritoneum ⁵⁸. For 20-25% of patients with CLM, surgical intervention is possible, where about 40% of patient will survive 5 years after resection; unfortunately, half of the patients experience recurrence of CRC within 18 months ⁵⁹.

Management of CLM

Key role of the multidisciplinary team

In 2016, consensus guidelines in managing mCRC were published by the European Society for Medical Oncology (ESMO) ³. The guidelines emphasise on a multidisciplinary team (MDT) approach which at a minimum requires the presence of surgeons (colorectal and hepatobiliary), oncologists, radiologists and pathologists. The

role of the MDT in managing CLM is to select patients that will be upfront resectable, potentially resectable or not resectable (Table 3), and then plan further investigations and suggest a management plan.

Surgical intervention for CLM

For technically resectable CLM with limited disease and favourable oncological signs, patients are offered either upfront surgical intervention or NACT (Table 3). There are no clearly defined criteria offered to support the decision of upfront surgery or surgery following NACT due to the lack of a convincing evidence from an RCT⁶⁰. Currently, several methods are available for eliminating CLM, ranging from radiofrequency or microwave ablations to local parenchyma sparing resections, from segmentectomies to formal hemi-hepatectomies and even liver transplantation⁶¹. Gaining access to the liver

Table 3.

Category	Contraindication
Technical (A)	
1. Absolute	Impossibility of R0 resection with $\geq 25\%$ – 30% liver remnant
2. Relative	Presence of unresectable extrahepatic disease
	R0 resection possible only with complex procedure (portal vein embolization, two-stage hepatectomy, hepatectomy combined with ablation ^a) R1 resection
Oncological (B)	
1.	Concomitant extrahepatic disease (resectable)
2.	Number of lesions ≥ 5
3.	Tumor progression

The table lists the absolute and relative technical contraindications related to resection of CLM in (A). Oncological considerations (B) are the presence of extrahepatic disease, number of lesions or tumour progression, but these are relative contraindications.

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via laparotomy has been the gold standard method for CLM resections, but minimally invasive (laparoscopic) surgery has been in use as an alternative for decades ^{62, 63}, offering a safe method for the surgical management of CLM ⁶⁴. Equipped with a solid anatomical understanding of the liver, excellent surgical skills and expert support from radiologists, anaesthesiologists and nursing staff, resection of liver metastases has low mortality and morbidity, and is the only option a patient has for a cure ^{13, 57, 65-67}. Up until 2018 when results from the randomised Oslo laparoscopic versus open liver resection for colorectal metastases (OSLO-COMET) trial were published ⁶⁸, observational studies, cohort studies, case series and reports explored the use of minimally invasive methods. High-level evidence from RCTs supporting the advantages in favour of laparoscopic CLM had not been reported ⁶⁹.

Systemic chemotherapy in potentially resectable CLM

The ESMO guidelines define a group of patients that should be offered systemic therapy if upfront resection is not possible and comprises patients where one cannot expect to achieve free resection margins without removing 70% or more of the liver parenchyma (Table 3). NACT is offered with the aim to convert patients from unresectable to resectable. In Norway, regimens involving bolus injection have been favoured and include Nordic-FLV, or oxaliplatin combined with FLV (Nordic-FLOX) or irinotecan with FLV (Nordic-FLIRI) ⁷⁰. ESMO guidelines recommend FOLFOX, which differs from the Nordic regimens by adding an intravenous infusion of 5-FU over 22 hours.

Fluoropyrimidines

5-FU, an anti-metabolite, is used for many different cancers, and has been the foundation of mCRC management since the 1990's as FLV ⁷¹. It is believed that the drug exerts its cytotoxicity by inhibiting thymidylate synthase, preventing formation of essential nucleotides required for DNA synthesis and repair, while in addition interfering with ribonucleic acid (RNA) ^{72, 73}. Response rate (RR) for IV infusion of 5-FU was reported to be 22% with a median duration of response of 46 weeks (median 12 weeks to response), but up to 30% RR has been reported when given as FLV ^{74, 75}.

Oxaliplatin

Oxaliplatin, a platinum-containing drug that has been documented to be effective in mCRC. The main cytotoxicity is exerted by disrupting DNA replication and transcription⁷⁶. Compared to FLV, oxaliplatin did not affect OS but had a better RR of 50%, which enhances resectability and gives a significant increase in progression free survival (PFS) from 6 months with FLV to 8.2 months in oxaliplatin⁷⁴. In addition to the effectiveness of oxaliplatin, the drug is currently gaining interest for a proposed additional effect, the induction of immunogenic cell death (ICD)⁷⁷.

Targeted therapies

Bevacizumab, is a monoclonal antibody that inhibits VEGF, which is overexpressed in about 50% of CRC and is associated with a more negative outcome^{11, 22, 78}. VEGF stimulates new blood vessel formation, and reports suggest that these new vessels secrete growth factors that also stimulate nearby tumor cells²². Bevacizumab is often used in combination with irinotecan and oxaliplatin containing regimens.

EGFR is overexpressed in up to 80% of CRC tumours, and anti-EGFR treatment with monoclonal antibodies (cetuximab or panitumumab) show response in mCRC patients⁴². *KRAS*, *NRAS* or *BRAF* wild type are predictive biomarkers of tumour's response to anti-EGFR treatment, but few patients experience long lasting response⁷⁹. The mechanism of failure is thought to be either development of drug resistant clones that are naturally selected for or develop during such treatment⁸⁰. There are studies showing that adding anti-EGFR treatment to NACT in resectable CLM is associated with worse outcomes⁸¹. Two more recent additions to systemic chemotherapy for refractory mCRC have not been explored in the neoadjuvant setting; the tyrosine kinase inhibitor regorafenib and the combination fluoropyrimidine trifluridine/tipiracil, both administered orally⁸².

Immunotherapies

MSI tumours have gained a great interest in the last few years the response seen after immune checkpoint inhibition (ICI) due to the immunogenic features of these tumours^{12, 83, 84}. A high number of mutations (hypermutated state) is a feature of MSI³⁸. The hypermutated state increases the chance of proteins expressed by cancer cells differ so

much from the host's proteins that they are recognised by the immune system as antigens (mutation-associated neoantigens)⁸⁵, thus recruiting a higher number of T-cells in the MSI tumours^{86,87}. Further studies have suggested that it is the high number of mutations, or tumour mutational burden (TMB), that trigger immune activation and response to ICI, regardless of MSI status^{88,89}. In mCRC, only about 5% of cases are found to be MSI⁸⁴. Since mCRC are enriched for MSS tumours, ICIs are not expected to achieve response in most of these tumours.

Genomic Alterations and Signalling Pathways in CRC

CRC carcinogenesis, the adenoma-to-carcinoma sequence

About 30 years ago, a theory was launched to explain how CRC could evolve from normal colorectal epithelium to a polyp and then to a cancer⁹⁰⁻⁹². Vogelstein and his colleagues described the adenoma-carcinoma sequence as an accumulation of genetic aberrations (Figure 3) in the epithelial cells of the colon and rectum, changing the cellular behaviour leading to pCRC⁹¹. In sporadic pCRC, 70% - 90% of cases arise from adenomatous polyps that display the molecular features of the adenoma-carcinoma sequence and following a similar pattern of events, named the classical or chromosome instability (CIN) pathway (Figure 3)^{12, 28, 93}. These events involve gain-of-function mutations in oncogenes and loss of chromosome regions (loss of heterozygosity) containing tumour suppressor genes, resulting in loss of important cell regulatory functions. The remaining 10 to 30% of CRC show molecular features that differ to CIN^{12, 28}. These tumours develop due to either epigenetic changes of CpG island methylation pathway or due to defects in MMR genes as seen in the MSI pathway (Figure 3)⁹³. The tumours of the CIN pathway are found to be MSS.

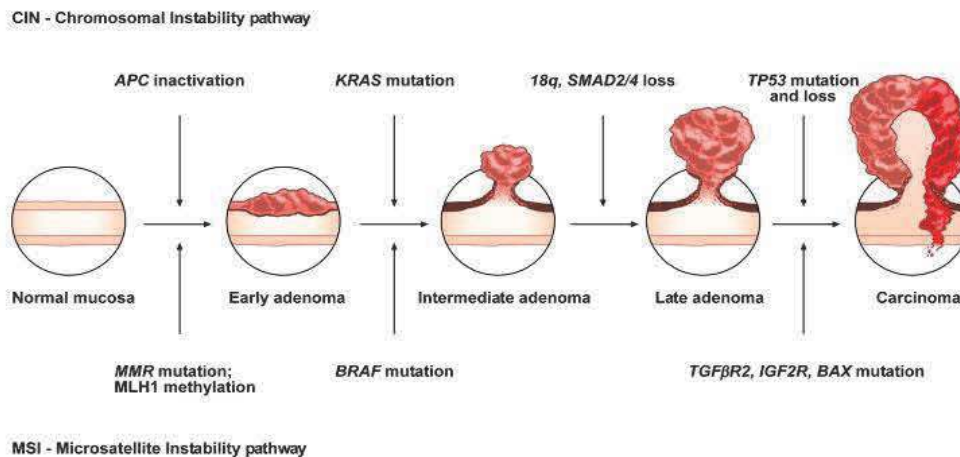


Figure 3. The adenoma-carcinoma sequence

The multi-step molecular events in the transition from normal mucosa to adenoma to carcinoma, highlighting the different molecular aberrations involved in the chromosomal instability and microsatellite instability pathways.

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Important signalling pathways deregulated in CRC

Cell signalling is activated by both external and internal cellular stimuli and control the cell's responses ⁹⁴. When aberrations in genes change the proteins they code for, the regulation of the intracellular signalling pathway is disrupted and cells carcinogenesis can occur ⁶. CIN pathway is an example from CRC where the following pathways are important: wntless-related integration site (Wnt), MAPK, transforming growth factor- β (TGF- β) and also aberrations in tumor protein p53 (TP53).⁹⁵

Wnt pathway and Adenomatous polyposis coli (APC) mutation

Aberrations in the *APC* gene caused by a loss of the long arm of chromosome 5 was described by Vogelstein et al as an early event in the development of CRC ⁹¹. *APC* is important in the regulation of the intracellular cell signalling pathway involving Wnt proteins. Wnt proteins are growth factors essential during embryonic development, which later in life are involved in maintaining tissue architecture ⁹⁶. Wnt signalling is considered fundamental in controlling cell growth, where β -catenin is a crucial transcription factor in the canonical Wnt pathway ⁹⁶. When signalling is not active, the

APC protein forms a part of a degradation complex that deactivates β -catenin (Figure 4)¹⁵. During activation of Wnt signalling on the other hand, β -catenin is released and stimulates cell proliferation and differentiation⁹⁶. In cancer, aberrations in *APC* cause an insensitivity to growth inhibitory signals through a defective degradation complex causing a continuous activation of β -catenin^{6, 91, 97}. *APC* aberrations are frequently found in CRC, where a loss of APC function is associated with the CIN pathway (Figure 3)^{98, 99}. Normally, intestinal stem cells differentiate and migrate from a crypt to a villus as an enterocyte, where it is shed after 5 days¹⁰⁰. A theory of CRC development is that perturbed Wnt/ β -catenin signalling in intestinal stem cells preserves the undifferentiated stem cell-like phenotype which results in an accumulation for these cells in the colorectal crypts instead of differentiating and migrating¹⁰¹. This theory is in accordance with the

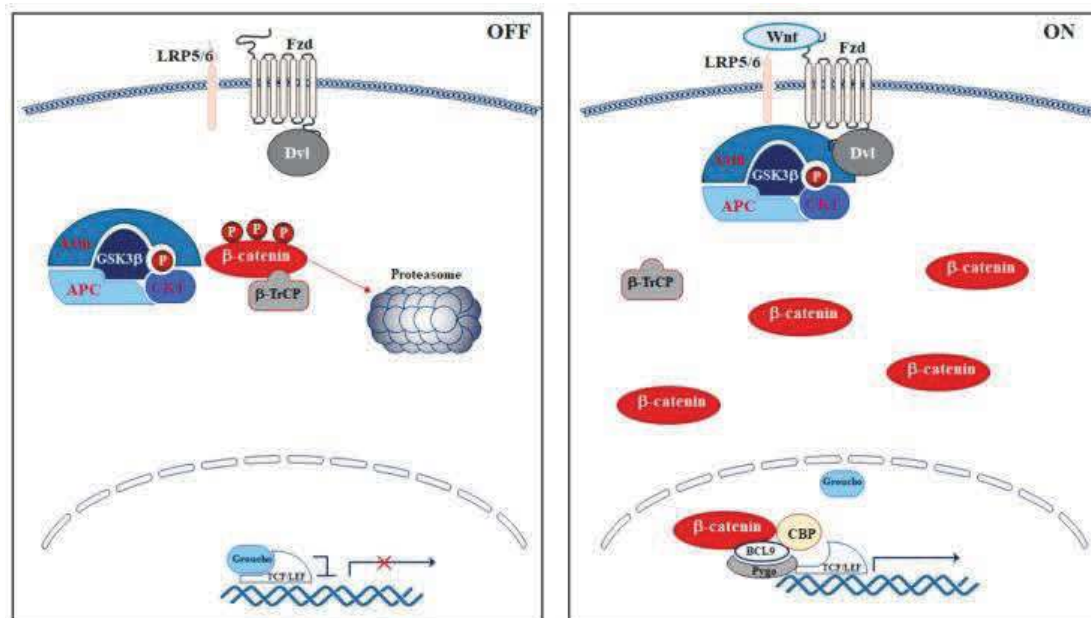


Figure 4. Proteins in Wnt signalling

The left panel shows the active degradation complex binding to and inhibiting the function of β -catenin when Wnt signalling is not active. In the right panel a Wnt signal binds to the cell surface receptors Frizzled (Fzd) and low-density lipoprotein receptor-related protein (LRP) 5/6, which activates the pathway. Stimulation of the Wnt pathway activates Dishevelled (Dvl) at the cell membrane and the degradation complex is pulled away from β -catenin. This causes an accumulation of β -catenin that then translocates to the nucleus where it activates target genes.

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adenoma-carcinoma sequence, where a perturbed Wnt signalling pathway is specifically thought to prevent migration and hence shedding, rendering the cell open for accumulation of genetic errors instead of being transported out of the gastrointestinal tract with waste ¹⁰².

MAPK signalling pathway and KRAS mutation

Deregulated proliferation is a feature in the carcinogenesis of pCRC, frequently caused by aberrations in the MAPK signalling pathway¹⁰¹ (Figure 5). MAPK signalling starts with, for example, EGFR activation, which attracts intracellular effector proteins to the plasma membrane, initiating a cascade of intracellular reactions. The end product of these cascades classically involves the activation of transcription factors in the cell nucleus via extracellular signal-regulating kinase (ERK)¹⁰³ (Figure 5). RAS proteins (such as KRAS and NRAS) are important in this cascade. These proteins are small enzymes called GTPases that function as molecular switches in MAPK signalling ¹⁰⁴. After being activated, they are rapidly switched off ^{105, 106}. Mutations in *RAS* genes eliminate the intrinsic capacity to deactivate the RAS protein causing a constitutively active MAPK pathway ^{106, 107}. *KRAS* is one of the most commonly mutated genes in all cancers, and in the Catalogue Of Somatic Mutation in Cancer (COSMIC) database, *KRAS* was mutated in 23% of all cancers, and in 13,177/38,080 (35%) of tumours of the colorectum ¹⁰⁸. In a comprehensive study of 13,336 CRC from the Foundation Medicine Incorporated, the mean frequency of *KRAS* aberrations was 49%, mostly missense mutations (<1% were amplification and frameshift mutations), mostly in codons 12, 13 and 61 of the gene. The frequency of *NRAS* aberrations was 4.5%, also mostly missense mutations ¹⁰⁹. MAPK also crosstalk with phosphatidylinositol 3-kinase (PI3K), where PI3K signalling can be activated directly via RAS proteins or by growth factors via transmembrane receptors (Figure 5). Downstream PI3K signalling activates cellular processes like proliferation and cell growth in a similar way to the MAPK pathway. PI3K is negatively regulated by the tumour suppressor gene *phosphatase and tensin homolog (PTEN)* ¹¹⁰. In CRC, a constitutive activation of PI3K signalling is caused by either an activating point mutation in the *PIK3CA* gene that encodes a catalytic subunit of PI3K, or by loss of PTEN ¹¹¹.

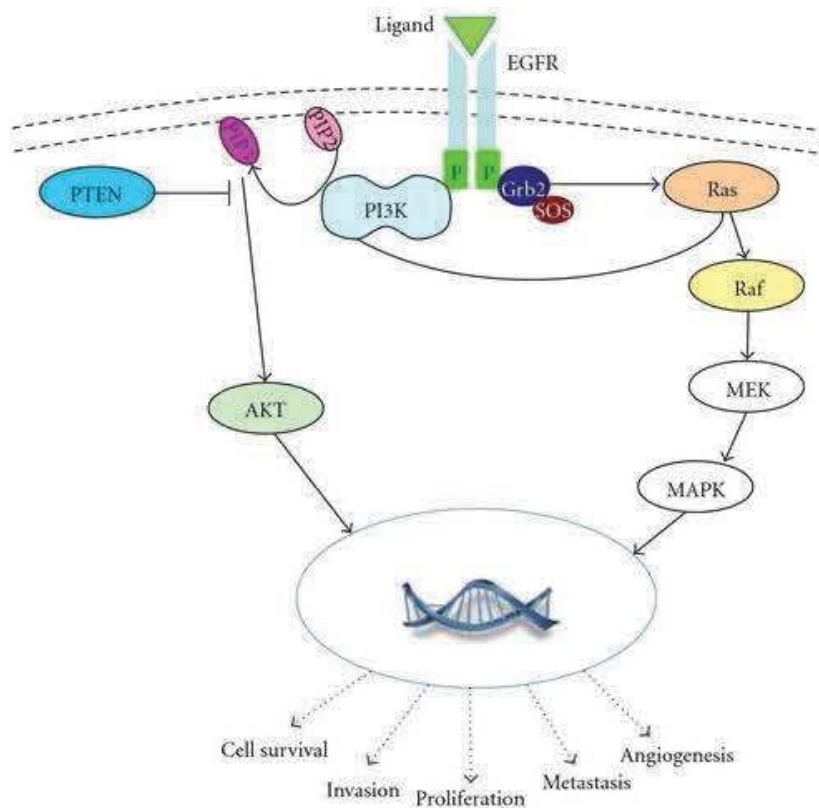


Figure 5. MAPK and phosphatidylinositol 3-kinase signalling

In the classical MAPK pathway a ligand binding to EGFR causes an activation of a signalling cascade involving Ras sarcoma oncoproteins (RAS) at the cellular membrane that attracts and activates Rapidly Accelerated Fibrosarcoma (RAF) kinase family proteins. Phosphorylation (P) of RAF kinases will further phosphorylate mitogen-activated protein kinase kinase (MEK) that eventually activates ERK. PI3K signalling is activated via RAS or EGFR receptors, that phosphorylate phosphatidylinositol (4,5)-biphosphate (PIP2) and phosphatidylinositol (3,4,5)-triphosphate (PIP3). Protein Kinase B (AKT) is then activated, a process that is inhibited by phosphatase and PTEN. In cancer a constitutive activation of MAPK and PI3K signalling gives the cells several of Hallmarks of Cancer.

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TGF- β signalling pathway

TGF- β is widely expressed in tissues and activates a multifunctional transduction pathway in cells^{112, 113}. The pathway is important in the normal function of cells, orchestrating cellular behaviour, inflammation and immune responses, as well as wound

healing and fibrosis ¹¹³. During embryogenesis TGF- β drives developmental cell programs such as EMT has a physiological role ¹¹⁴. In epithelial cells, TGF- β is important in maintaining tissue homeostasis, controlling the intracellular and the extracellular microenvironments. The main function of TGF- β is to exert a negative regulation on the cell cycle as an important protective mechanism in preventing abnormal cell growth or cell division (neoplastic growth) that can progress to cancer ^{113, 115}. In the early stages of neoplastic growth, activation of the pathway acts as a suppressor of mitogenic signals (Figure 6) ¹¹⁵. However, as a cancer develops, errors in the TGF- β pathway, especially combined with a constitutive activation of MAPK pathway, aid in cancer progression by promoting invasive and metastatic abilities, as well as promoting immune evasive mechanisms (Figure 6) ^{112, 115}. CRC with a high mitogenic activity, e.g. *KRAS* mutated tumours, will have an advantage when the regulating effect of TGF- β signalling is eliminated, and gain benefits from a TGF- β rich TME with inflammatory cytokines ¹¹³. A high TGF- β expression is correlated to worse outcome in CRC where well- to moderately-differentiated tumours are inhibited by TGF-

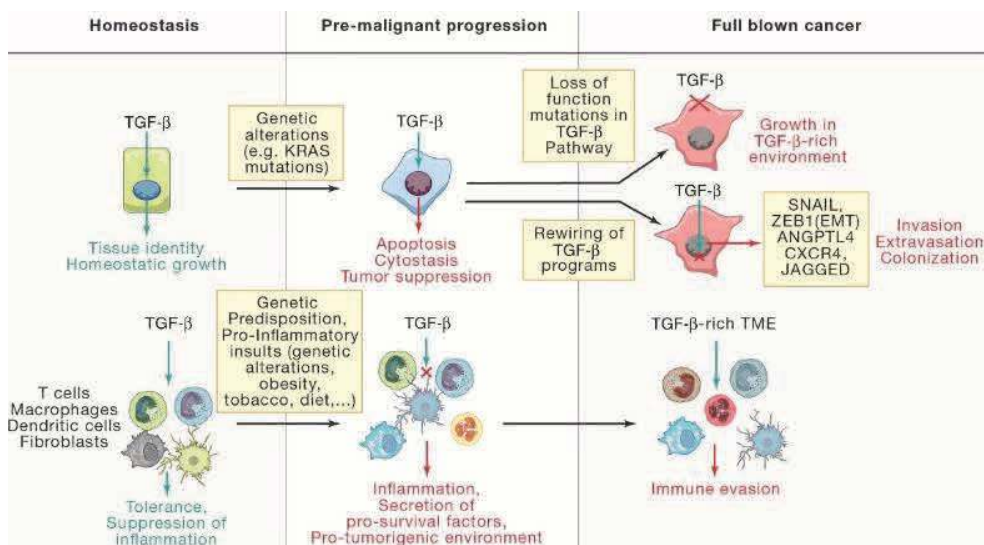


Figure 6. Transforming growth factor beta (TGF- β) signalling in cancer

TGF- β signalling is often perturbed in cancer and is involved in several of the hallmarks of cancer such as promoting EMT, angiogenesis and remodelling of the TME. TGF- β has several roles in the progression of cancer and plays a part in cancer's immune evasive mechanisms.

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β , in contrast to tumour growth being promoted in the metastatic setting ¹¹⁶. SMAD proteins are important transcription factors in TGF- β -signalling. The name SMAD is derived from the discovery of the homologous proteins coded by *Sma* genes in *Caenorhabditis elegans* and *Mad* genes in *Drosophila* ¹¹⁷. In particular, *SMAD4*, a tumour suppressor gene located on chromosome 18, codes for an important regulator of the canonical TGF- β pathway, where loss of heterozygosity of chromosome 18 is a hallmark of the adenoma-carcinoma sequence in CRC and is also found to correlate to progression to metastatic disease ¹¹⁶.

TP53 aberration

TP53, a tumour suppressor gene located on chromosome 17, was recognised as a late event in the progression from adenoma to carcinoma in CRC ⁹². This protein has the important functions of halting the cell cycle to allow for DNA repair or induction of apoptosis when the cell is faced with stress due to for example DNA damaged beyond repair. p53 is a transcription factor that has been named the “guardian of the genome” and is associated with downregulation of over 250 genes, many of which are important check-points controlling transition through the cell cycle ^{118, 119}.

CRC and Progression to Metastasis

Metastasis is a hallmark of cancer, characterised by cancerous cells moving from the primary tumor to lodge and grow in another organ ^{6, 120, 121}. For most cancers, metastatic disease is associated with poor outcome. Several theories have over the last centuries attempted to explain the mechanisms of metastasis, from metastatic spread being a random event, to tumour cells being trapped in the blood supply in the receiving organ. A theory that frequently is referred to in an attempt to explain metastasis was launched in 1889 by Stephen Paget. Paget found evidence that metastatic spread was not a random event, but rather that metastasising tumour cells, or “the seeds”, showed a preference for certain organs, or “the soil”, where tumour cells would thrive and grow ¹²². Paget’s “seed and soil” theory was supported by studies performed by Fidler and colleagues in the 1980’s, who described a multistep nature of metastatic spread ¹²⁰. It is believed that only certain tumour cells, can leave the primary tumour and grow in another organ ¹²³. In leaving the host organ, a metastasising cell must acquire certain

competencies such as increasing migratory abilities, resistance to apoptosis, and the ability to invade surrounding tissues²⁴. EMT is thought to be an important step in the initiating process of metastasis, where the reprogramming of a tumour cell enables survival without the normal polarisation and basement membrane attachment. Aberrations in both TGF- β and Wnt signalling pathways are associated with EMT, which promotes downregulation of E-cadherin, enabling metastatic cells to detach and intravasate into the blood circulation²⁴. After intravasation into the circulation, many tumour cells succumb to the mechanical forces of the blood stream, or are removed by immune cells¹²⁴. Some tumour cells acquire the ability to adhere to platelets, making them capable of surviving the shear stress of the blood circulation and avoid detection by the immune system¹²⁵. The liver drains most of the intestinal blood via the portal circulation, making the liver the first port of call for disseminated cancer cells that survive

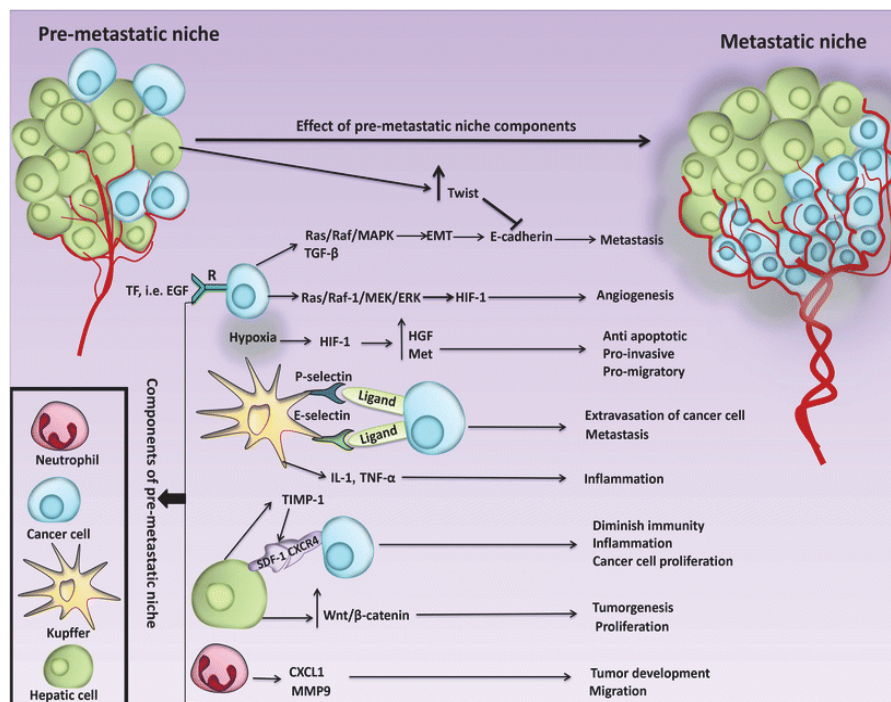


Figure 7. Metastatic niche of liver

The physiological processes in liver during liver regeneration can promote a metastatic niche for disseminated tumour cells. The environment is often hypoxic during this phase, and processes like EMT, angiogenesis and inflammation are activated as well as intracellular signalling like MAPK and Wnt, which contribute to promote a metastatic niche.

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extravasation¹²⁶. There are also theories describing the role of the pCRC in preparing a distant organ, like the liver (“the soil”), to become a recipient of disseminated tumour cells by formation of a pre-metastatic niche (Figure 7)^{8, 126}. To colonise the liver, tumour cells are thought to reverse EMT by mesenchymal to epithelial transition, where the cells again express E-cadherin¹²⁷. The process of establishing metastatic deposits is an inefficient process¹²⁶. Studies show that most of the colonising tumour cells remain dormant, with only 2% forming micrometastases, and only 0.02% going on to form macroscopic tumours¹²⁸. The liver microenvironment has physiological properties that makes it “congenial soil” for disseminated CRC cells. The rich vascular supply along with the very low flow rate and the permeable liver sinusoidal endothelial cells are some of the functional properties in favour of disseminated tumour cells reaching into the liver parenchyma¹²⁶. Unlike most other organs, liver cells have a great potential for regeneration to maintain homeostasis⁸. The innate immune system and inflammation trigger regeneration, and several molecular mechanisms are involved such as MAPK and PI3K-signalling due to growth factor activation, STAT3 activation via IL-6 signalling, tumour necrosis factor (TNF), toll-like receptors (TLR), and Wnt/ β -catenin^{8, 126, 129}. These molecular mechanisms are also known to be important players in cancer development and progression as well^{11, 130}. The liver immune cells can generate a strong immune and inflammatory response as part of homeostasis triggered by, for example, the secretion of immune suppressive cytokines such as interleukin-10 (IL-10) by Kupfer cells and myeloid-derived suppressor cells (MDSCs) that also produce TGF- β ¹³¹.

The Immune System

The immune system has developed to protect the body from harmful intruders using two functionally separate systems, the innate (natural) and the adaptive (specific) immune systems. The immune system has 4 important tasks, namely to (1) recognise intruders and then (2) activate, (3) regulate and (4) memorise immune responses¹³². The gut and the liver are two important organs in this work, and for the purpose of this thesis, the description of the normal function of the immune system will be focused on these organs.

Innate immunity

The proteins of the innate immune system are encoded by genes inherited through the germline, and function by activating a rapid, non-specific immune response^{133, 134}. The complement system is a first line of defence, and when activated involves a cascade of over 50 plasma proteins that are able to mark (opsonisation) and eliminate microorganisms by lysis¹³⁵. The cells of the innate immune system are developed from haematopoietic stem cells and include phagocytes, such as neutrophils, monocytes, macrophages, natural killer cells (NK-cells), and professional antigen-presenting cells such as dendritic cells (DC)¹³³. Monocytes, which have antigen-presenting capabilities, differentiate into macrophages and dendritic cells when entering tissues. Macrophages polarise into either an M1 subtype (secreting pro-inflammatory cytokines and eliminating pathogens and tumour cells) or an M2 subtype (anti-inflammatory properties, removing necrotic tissue and dead cells and promoting healing) dependent on the cytokine milieu¹³⁶. Antigen-presenting cells and endothelial cells express pattern recognition receptors (PRRs), which are specialised sensors with the ability to recognise specific molecular

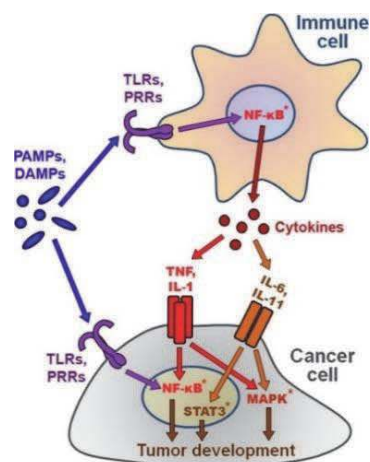


Figure 8. Toll like receptor (TLR) signalling

TLR signalling pathway activates transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and non-canonical MAPK pathway associated with cellular stress responses like p38 and Jun N-terminal kinase (JNK). IL-1 signals via NF-κB and IL-6 via STAT3. These factors are thought to aid in tumour development.

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patterns. When these patterns are specific to microorganisms they are called pathogen-associated molecular patterns (PAMPs), and when they are associated with endogenous damage to the cell, damage-associated molecular patterns (DAMPs) ¹³⁷. DCs link the innate and the adaptive immune system where they aid in generating a strong adaptive immune response ¹³⁸. DCs are a heterogenous group of immune cells that are activated when PRRs recognise and internalise PAMPs or DAMPs, which are then presented to the adaptive immune system ¹³⁸. The TLR family is comprised of PRRs. DAMPs found to activate TLR are, for example, heat-shock proteins (HSP) from necrotic tissues and high-mobility group box-1 proteins (HMGB-1) ¹³⁹. The transduction through the TLR signalling pathway activates the production of inflammatory cytokines and interferon-gamma (INF- γ) (Figure 8) ¹⁴⁰. TLRs are expressed on tumour cells including CRC and have been described as a double-edged sword in cancer. In studies of CRC cell lines, TLRs inhibit tumour cell growth, stimulate cell death and enhance anti-tumour immune responses, but TLR can also trigger pro-tumour effects by inhibiting T and NK-cells ¹³⁹.

Adaptive immunity

The adaptive immune system has, in addition to protection against recurring infections, also been shown to be a key player in cancer immunology. The adaptive immune system consists of B- and T-cells that communicate with the innate immune system and compensate for the non-specificity of its immune responses. Despite a slow initial activation process, adaptive immunity provides a dynamic ability to distinguish between pathogenic and non-pathogenic intruders. Cells of the adaptive immune system launch a swift and specific attack, and importantly develop immune memory, the hallmark of adaptive immunity ^{134, 141}.

Antigen recognition and T-cell activation

The communication between the innate and adaptive immune systems is relayed via cell surface proteins called T-cell receptors (TCR) and major histocompatibility complexes (MHCs), also known as the human leucocyte antigen complex (HLA).

TCR

Before naïve T-cells enter the circulation, they are selected and mature in the thymus¹⁴². TCRs form cell membrane-bound communication links within the immune system and have a broad ability to recognise antigens. Due to the ability to rearrange the building blocks of the receptors, each T-cell clone has a unique TCR¹⁴³. TCRs survey,

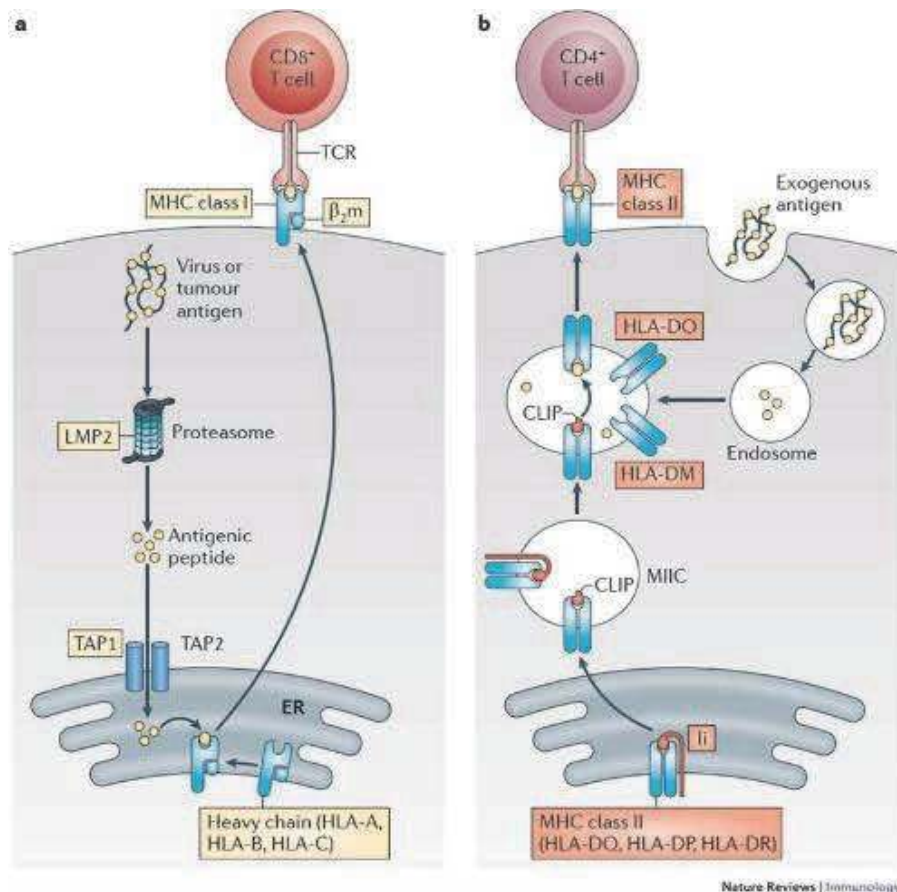


Figure 9. Antigen presentation via Major Histocompatibility Complex I and II

- (a) MHC I is expressed on all nucleated cells and presents intracellular degradation products in the form of short peptides (8-9 amino acids) in order to visualise intracellular pathology (antigen) to the immune system.
- (b) MHC II is usually expressed on antigen-presenting cells like DC, macrophages and B-cells, and present longer peptides (13-25 amino acids) consisting of exogenous proteins that have been internalised and processed.

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recognise and engage MHC presenting non-self-peptides (antigen), a prerequisite for T-cell activation ¹⁴⁴. The MHC molecules have an important role in presenting peptides (including foreign peptides or antigens) to the immune system (Figure 9) ¹⁴⁵. The genes coding for MHC classes are highly polymorphic, enabling a great diversity in the peptide presenting site of the molecule ^{146, 147}. The cluster of differentiation 3 (CD3) protein is a non-variable intracellular transduction protein that associates with the TCR forming a TCR-CD3 complex ¹⁴⁸.

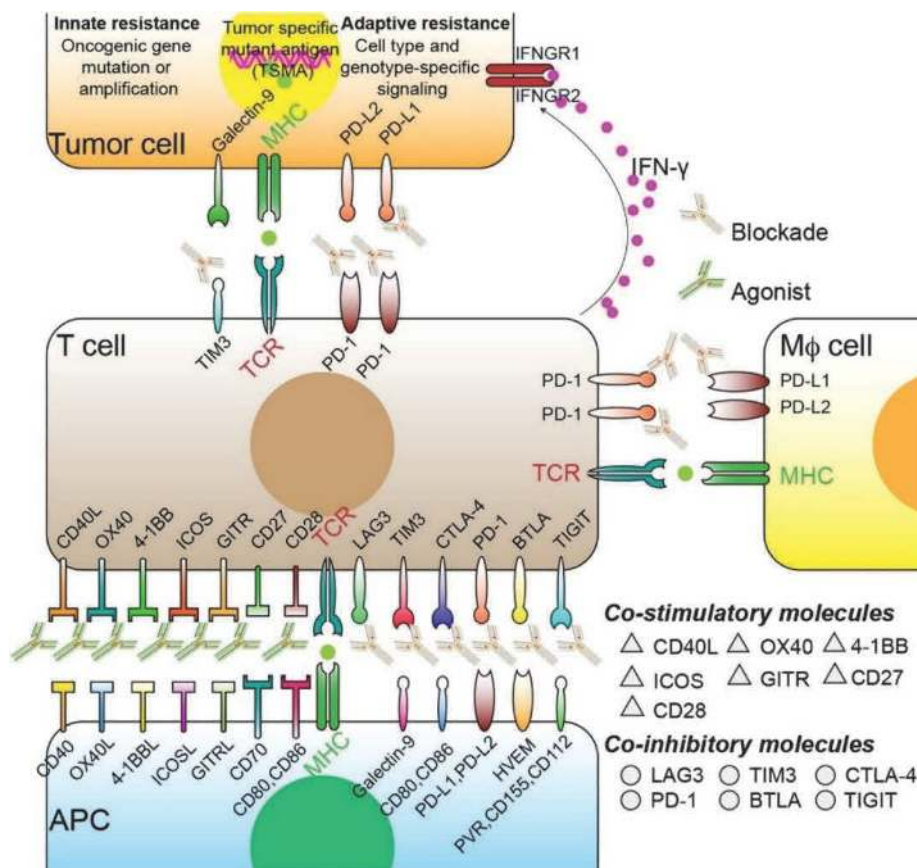


Figure 10. T-cell receptor signalling and immune checkpoints

T-cell receptor (TCR) comprises a complex of membrane bound and intracellular molecules that activate intracellular signalling cascades facilitating T-cell activation, proliferation and differentiation. TCR is engaged via the presentation of antigens on MHC by either antigen presenting cells (APC) such as DCs or tumour cells. The figure also shows the important co-stimulator CD28, and co-important inhibitory molecules such PD-1 and CTLA-4 also known as immune checkpoint .

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TCR co-signalling and immune checkpoints

Both TCR and a co-stimulatory receptor are required for a proper T-cell activation. The TCR co-stimulatory receptor CD28 is activated by ligands on antigen-presenting cells such as CD80 and CD86¹⁴⁹. Essential to peripheral tolerance are the co-inhibitors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein-1 (PD-1) (Figure 10)¹⁴⁹. CTLA-4 is a homologue for CD28 and is expressed on T-cells. PD-1, also expressed on T-cells and other immune cells, exerts a negative effect on immune cell activation when binding to programmed death ligand 1 or 2 (PD-L1 or PD-L2). PD-L1 or PD-L2 are widely expressed on many cells, including cancer cells¹⁵⁰. These co-inhibitors of T-cell activity, also known as immune checkpoints, have become important in cancer therapy in the last few years due to anti-tumour immune activation through a therapeutic blocking of for example PD-1 signalling (Figure 10)^{151, 152}. The role of other immune checkpoints is also being studied, such as T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3)¹⁵³. LAG-3 is highly expressed on CRC cells, which correlates to advanced disease, and works by inhibiting the anti-tumour function of effector T-cells. LAG-3 is also required for full regulatory T-cells (Treg) anti-immune functions¹⁵⁴. Both TIM-3 and LAG-3 are associated with PD-1 expression and also considered promising targets for therapy^{154, 155}.

Lymphocytes – cells of the adaptive immune system

Lymphocytes, comprising both B-cells and T-cells, are also derived from hematopoietic stem cells. These cells are described as the heart of immune recognition and are major players in the adaptive immune response¹⁵⁶. T-cells can be classified into two subgroups based on the expression of co-receptors, where cytotoxic T-cells (CTLs) are CD8+ and helper T-cells (THs) are CD4+.

CTL

On CTLs the CD8 is a co-receptor to the TCR-CD3 complex that specifically monitors MHC I (Figure 9), which is expressed on all nucleated cells. The function is to detect intracellular antigens from for example viruses and tumours. When the MHC I receptor recognises its cognate antigen, an immune synapse forms between the TCR-CD3

complex and MHC I. This causes expression of Fas ligand on CTLs leading to release of granzymes and perforin, triggering apoptosis in the target cell via cell-cell contact ¹⁵⁷. Activated CTLs release TNF and INF- γ , cytokines that aid in shaping the immune response. Further, activated CTLs differentiate and acquire different phenotypes, where some become effector CTLs (strong cytolytic activity and little cytokine production), others differentiate into memory CTLs (proliferate and produce cytokines, but no cytolytic activity), and some become an intermediate CTLs with weak cytolytic activity, but produce high levels of cytokines ¹⁵⁷. The pool of effector CTLs consists of two subsets. The majority (95%) are terminal effectors that are destined to die following activation, and the minority, termed memory precursors, survive to give rise to the pool of long-lived memory T-cells ¹⁵⁸. During an immune activation, effector CTLs will, after exerting their cytolytic activity, succumb to apoptosis, a control mechanism to restore immune homeostasis while retaining memory CTL ¹⁵⁹.

Helper T-cells (TH)

T-cells expressing the CD4 co-receptor are given the name “helper” due to their ability to assist B-cells in antibody production. THs are key orchestrators of immune responses and comprise multiple phenotypes (subsets) responsible for maintaining immune homeostasis by both activating and inhibiting immune responses ¹⁶⁰. Unlike CTLs, THs are activated when MHC II, expressed primarily on antigen-presenting cells like DCs, presents the TCR-CD3 complex with its cognate antigen and forms an immune synapse (Figure 11) ¹⁴⁵. TH function is shaped by the cytokine milieu (Figure 11) and these immune cells are classified according to their role in the immune system. Th1, Th2 and Th17 subsets are all pro-inflammatory T-cells. Th1 subset differentiation is stimulated in the presence of antigens from intracellular pathogens like bacteria and viruses. Th2 subsets are crucial during adaptive immune responses fighting infections of extracellular parasites, and also a part of the pathogenesis of asthmatic and allergic inflammatory diseases ¹⁶⁰. Th17 cells are characterized by the IL-17 cytokine which induces a strong inflammatory response. These cells are correlated with the development of Crohn’s disease ¹⁶⁰.

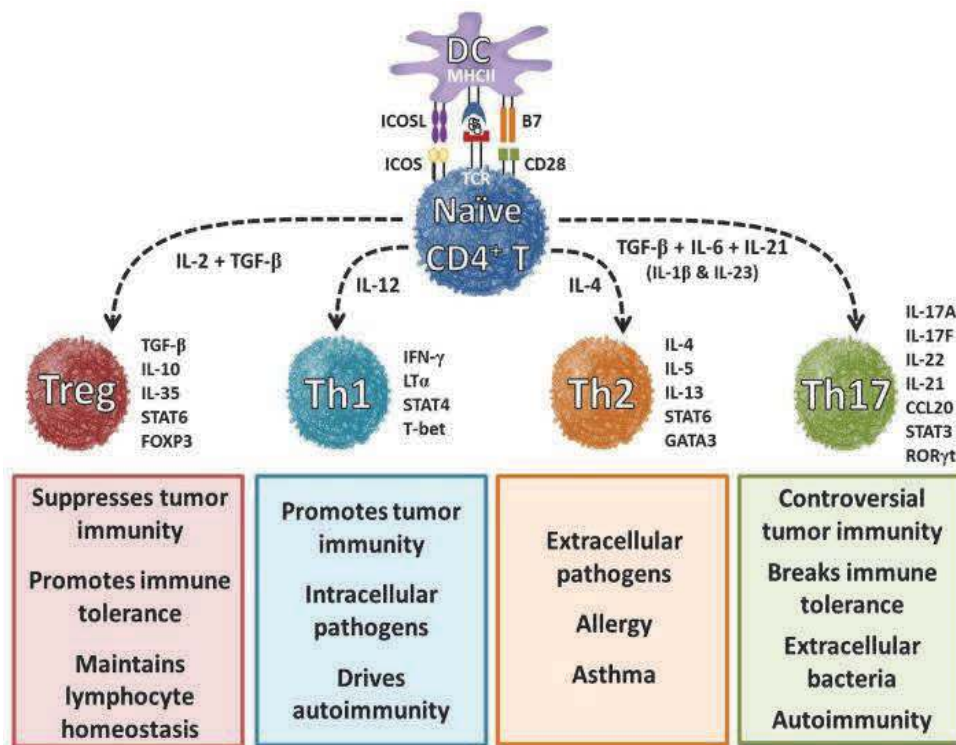


Figure 11. T-cell differentiation and important cytokines

Figure shows cytokines involved in differentiation of naïve TH into TH subsets as well as associated pathological processes. Reused by Creative Commons Attribution License (CC BY). Courtesy of Bailey SR et al ².

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The Janus kinases (JAK) and signal transducer and activator of transcription proteins signalling (STAT) also known as JAK-STAT signalling is important in the regulation of growth factors and cytokines related to, for example, the immune system where it aids in deciding the differentiation of T-cells. The effect of the balance between STATs is complicated, but important in the development of TH subsets, where for instance, INF-γ signalling and a development of Th1 cells is regulated by STAT1 and STAT4, while Th17 cells are dependent on STAT3 signalling ¹⁶¹. The JAK-STAT signalling pathway has been found important in tumour growth and metastasis in CRC ¹⁶².

Regulatory T-cells

The regulatory function of THs is essential, where Tregs can downregulate immune responses and maintain immune homeostasis. These TH subsets are recognised by the

transcription factor forkhead box P3 (FOXP3)¹⁶³, which is important in maintaining the immune suppressive function of the Tregs (Figure 11) ¹⁶⁰. Tregs express CD25 and CD4, and will differentiate in the presence of IL-10 and TGF- β . Their role is to dampen immune responses by reducing the action of effector T-cells. Depletion of Tregs plays an important role in the pathogenesis of autoimmune diseases, including inflammatory bowel disease ¹⁶⁴. Similar to CTL and TH, Tregs require the presentation of a cognate antigen on the TCRs and CD28 co-stimulation. Of the co-inhibitory mechanisms, the immune checkpoint CTLA-4 has been found important in the function of Tregs. CTLA-4 exerts an immune suppressive effect through its stronger affinity for the ligands CD80 and CD86 than CD28, and will capture these ligands from antigen-presenting cells and internalise them, in addition to increasing Treg motility enabling an increase in range of reaching more antigen-presenting cells ^{150, 163}.

The immune system in the colon and rectum

The lining of the colon and rectum is colonised by an abundance of commensal bacteria, the microbiome, living in symbiosis with the host without triggering an overwhelming immune response. To protect the host, the mucosa has been found to harbour immune suppressive capabilities ¹⁶⁵. The microbiome of the gut is thought to be closely connected to the normal function of the whole organism. Emerging evidence highlights the innate immune system's role as orchestrator of epithelial cells, immune cells and microorganisms, where PRRs play a role in maintaining immune homeostasis, and where a dysfunctional symbiosis is linked to diseases such as colorectal cancer ¹⁶⁶. Pathogen infection and inflammation will, after a failure of the protective function of the gut microbiota, cause toxic damage to DNA and carcinogenesis, especially in the presence of *APC* mutations ^{167, 168}. The gut mucosa and more so the lamina propria contain antigen-experienced T-cell populations including memory T-cells. Many T-cells, especially THs, will remain in the lamina propria. CTLs on the other hand tend to migrate to the mucosa ¹⁶⁵. In the presence of the microbiota in the gut, Tregs are important in maintaining the tolerogenic environment by dampening the activation of effector T-cells ^{165, 169, 170}.

The immune system in the liver

The liver has important immunological functions in the body^{131, 171}. Liver-resident macrophages (Kupffer cells, NK-cells and NK T-cells) are important players in the innate immune system¹⁷². Eighty to ninety percent of complement proteins and secreted PRRs are produced in the liver, mostly by hepatocytes and not by the immune cells^{171, 172}. The liver has a unique connection to the gut and functions as filtering system where 80% of the intestinal blood supply drains via the portal venous system. Gut microorganisms thus challenge the liver immune system with debris and food residues that are all potential activators of immune responses¹⁷².

The Immune System in Cancer

The immune system and inflammation have an intricate role in cancers, where products of inflammation can promote carcinogenesis as well as anti-tumour immune responses¹¹. Chronic infections or inflammatory conditions can predispose to several cancers, including CRC¹⁷³. The association of immune response to prognosis has been established, where a high T-cell density has been linked to a good prognosis¹⁷⁴. When the immune system is medically suppressed (e.g. after solid organ transplantation) an increased cancer risk has been reported¹⁷⁵. Interestingly, there are studies documenting very good outcomes in patients after liver transplantation for CLM where patients were treated with long-term immune suppressants⁶¹. Research into these immune related processes has given important insight into the mechanisms of cancer progression and has recently become important in treating cancer. The acquired understanding of inflammation and immune responses is now being implemented in cancer therapy, and the questions relating to the dual mechanisms of the immune system have culminated in a great research interest worldwide.

Immunoediting in cancer

In 2004, Dunn et al summarised several studies demonstrating the immune system's ability to remove cancers, and at the same time hypothesised why cancers still grow in the presence of a functional immune system¹⁷⁵. This hypothesis was named the three E's of cancer immunoediting describing 3 phases, namely elimination, equilibrium and escape (Figure 12)¹⁷⁵. The elimination phase is recognised by the normal functions of

both the innate and adaptive immune responses in eliminating cancers, similar to how bacteria and viruses are removed. A growing cancer disrupts the surrounding stroma, producing inflammatory proteins that attract immune cells. These inflammatory proteins act as danger signals that can alert the innate immune cells to the presence of cancer cells. In addition, changes in the cancer cell's genome will produce and present the immune system with altered proteins, recognised as antigens. When the innate immune system senses and presents tumour proteins to the adaptive immune system, DCs can activate Th1, which further facilitates presentation of tumour specific antigens to CTLs via MHC I. The removal of tumours is induced via INF- γ -dependent mechanisms. If the

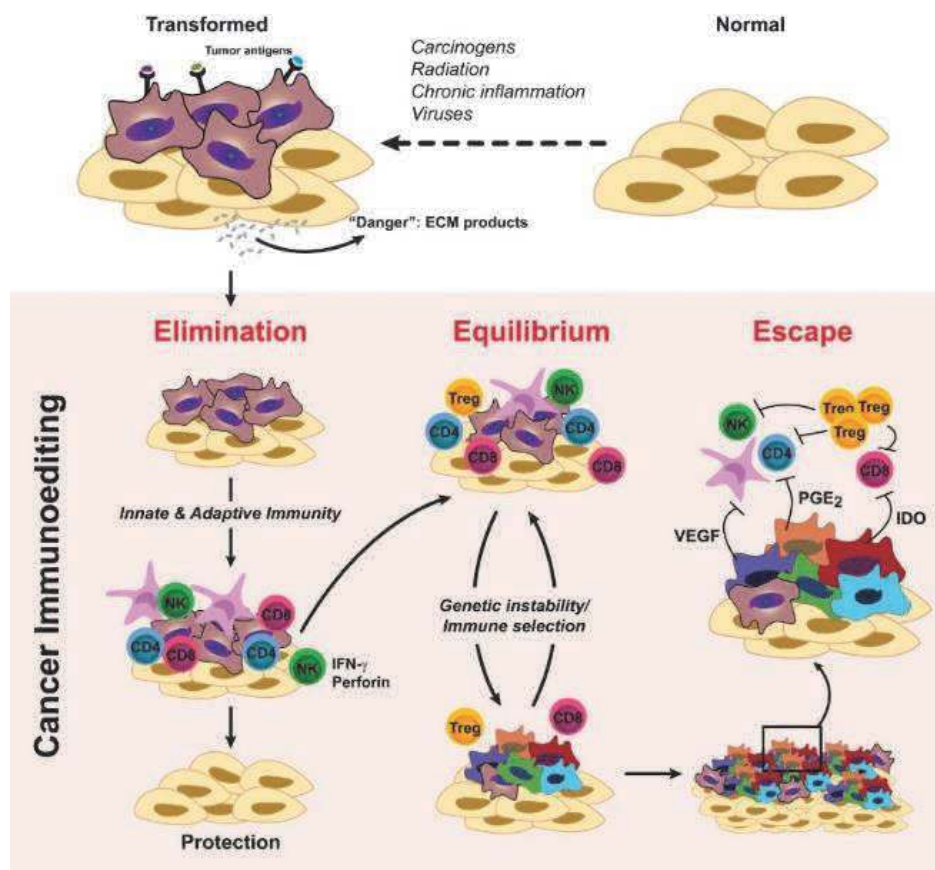


Figure 12. Cancer immunoediting

Tumour cells are initially eliminated by the immune system, but the development of aberrations in tumour cells that are not recognised by the immune system allow for a long-lasting equilibrium phase, where tumours are usually not detected clinically. Eventually tumours develop mechanisms to avoid a fully functional immune system and become clinically overt cancers.

Figure courtesy of Sheraz Yaqub. Used with permission

cancer is not eliminated by the immune response, the equilibrium phase starts. In this phase the immune system actively attacks the tumour cells without eliminating the tumour. Dunn et al explained this using Darwinian selection, where tumours with less immunogenic properties could avoid the immune system and survive due to favourable changes caused by genomic errors, such as for example aberrations in CIN¹⁷⁵. Immune escape then occur if the less immunogenic tumours also develop hallmarks of cancers that enable growth¹¹. Several mechanisms have been proposed to explain why cancers such as CRC are less immunogenic. Antigen presentation is frequently hampered by either loss or reduced expression of MHC I, or by acquired errors in its antigen presenting machinery. Cancers can downregulate the expression of ligands that normally trigger an immune response, such as natural killer group 2D (NKG2D) ligands or increase the secretion of immune suppressive cytokines like TGF- β and indolamine-2,3-dioxygenase (IDO). These mechanisms both prevent activation of the immune system or work by dampening the immune responses^{175, 176}. Further, the infiltration of immune suppressive Tregs and MDSCs exert an inhibitory effect on TH and CTL¹⁷⁶.

Non-immunogenic and immunogenic cell death

Both cell regeneration and cell death are prerequisites for an organism to survive. A great many cells die each day without activating immune responses. Only the cells with errors or cells containing infections will trigger immune activation associated with an immune memory¹⁷⁷. As part of evolution, our immune system has evolved methods to recognise different types of cell death¹⁷⁸. In the physiological state, cells dying by apoptosis usually avoid immune activation, while the elimination of unhealthy cells elicits an immune response causing the removal of cellular debris by phagocytosis. Unhealthy cells emit danger signals like DAMPs that trigger immune responses via PRR, and dying tumour cells thus represent a potential risk to the cancer of being seen by the immune system. Cancers can avoid eliciting an immune response thereby grow and metastasise undetected by the immune system¹⁷⁵. However, certain cancer treatments have been found to elicit an immunogenic response where less immunogenic tumours become immunogenic. ICD is a multi-step theory that explains how radiotherapy and certain cytotoxic drugs, such as oxaliplatin, induce stress responses in cancer cells leading to both innate and adaptive immune responses. Studies hypothesise that ICD inducing

therapies cause an insult to cancer cells that leads to a release of neoantigens from dying cancer cells, an activation of autophagy and the release of intracellular nucleotides like adenosine triphosphate of which all contribute to recruit and activate DCs. These therapies also cause stress to the endoplasmic reticulum (ER), which increases the expression of DAMPs such as calreticulin (CALR) and HMGB1, proteins that are involved in the maturation of DCs. CALR for example, activates PRRs, attracts DCs and facilitates antigen uptake. These innate immune responses activate the adaptive immune system by recruiting, priming and causing clonal expansion of T-cells with the ability to attack remaining tumour cells as well as generating memory ¹⁷⁷. The details of the ICD theory have been summarised by Kroemer and Galluzzi in several publications over the last decade ¹⁷⁷⁻¹⁷⁹. Observations leading up to the theory of ICD were that

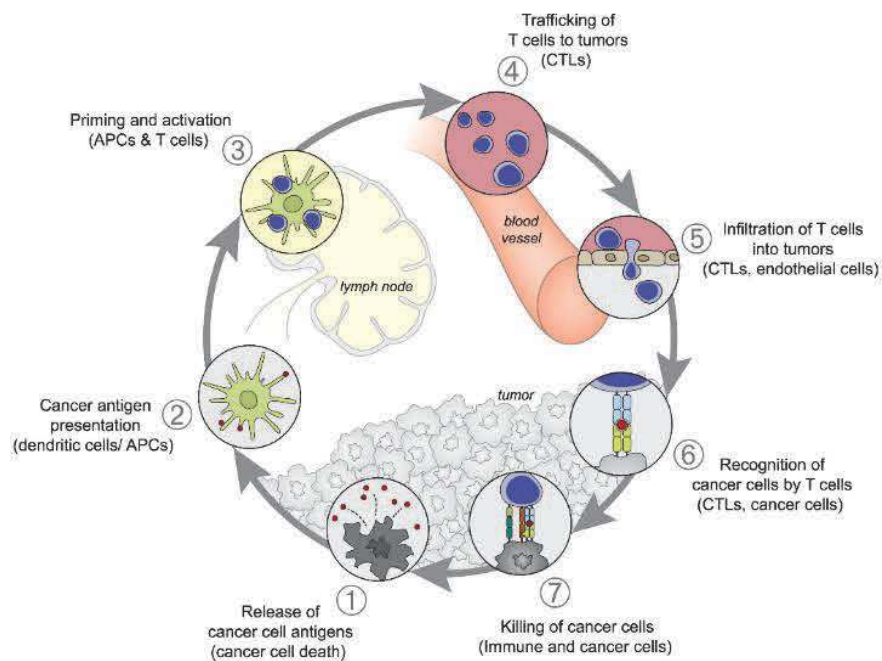


Figure 13. Cancer-immunity cycle

The figure shows how tumours can activate the immune system, broken down into 7 steps. After treatment tumours release antigens that are (1) presented to innate immune cells (2) causing priming and activation of antigen presenting cells (APCs) (3), which in turn causes trafficking of CTLs to (4) and infiltration into tumours (5). T-cells then recognise (6) and kill tumour cells (7). There are anti-immune responses related to each of these steps.

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syngeneic immunocompetent mice treated with ICD inducers (anthracyclines and oxaliplatin) had a much better response to treatment compared to immunodeficient mice. Similarly, mice treated with non-ICD chemotherapeutics and a cardiac glycoside (strong inducer of ICD) responded better than non-ICD chemotherapeutics alone. Lastly, clinical data from breast cancer and CRC showed a superior outcome in patients with non-ICD chemotherapy taking a cardio glycoside versus non-ICD only ¹⁷⁹. Two criteria were suggested to define an agent as causing ICD, which were only testable in animal models. Firstly, cells killed in vitro by assumed ICD agents prior to administration must elicit an immune response and activate memory, comparable to immune effects of vaccinations. Then, administration of these in vivo ICD agents must generate a local immune response in the tumour, where both innate and adaptive immune cells are activated resulting, in part, to a treatment response ¹⁷⁹. The concept of ICD entails treatment strategies that expose intracellular proteins otherwise concealed from the immune system, so that they can elicit an immune response, which includes memory towards cancer cells (Figure 13).

Immunoscore an emerging classification of CRC

The cancer's ability to evade the immune system has been acknowledged as a hallmark of cancer, and immune responses have in the last 15 years been tied in as a predictive biomarker (like MSI) as well as a prognostic marker ¹¹. In the early 2000s, work on CRC found that T-cell densities were associated with the prognosis of patients, where a high density of T-cells in and around tumours was reported to be correlated with a superior outcome ¹⁷⁴. This created the basis for a scoring system defined by T-cell densities in the centre of tumour and in the surrounding invasive margin (IM) by quantifying the total amount of T-cells (CD3+ T-cells) and CTLs (CD8+ T-cells) (Figure 14) ¹⁷⁴. Patients with CRC could thus be stratified into high and low risk for CRC recurrence and death. This system, created by Galon and his colleagues, was later refined and named the Immunoscore. The scoring system was suggested as a better predictor of prognosis compared to the universally used TNM classification, giving a better account of the heterogenous outcomes seen in patients within the same TNM tumour stage ¹⁸⁰. In the last few years, work on the Immunoscore has been supported by an international worldwide task force ⁴. A validation and refinement of the score has been performed in

2681 patients, stratifying Immunoscore into high, intermediate and low scores ¹⁸¹. The method has been validated as a tool to predict CRC outcomes, where it has been proposed as a useful method for selecting high risk stage II CRC that may benefit from adjuvant chemotherapy ¹⁸¹.

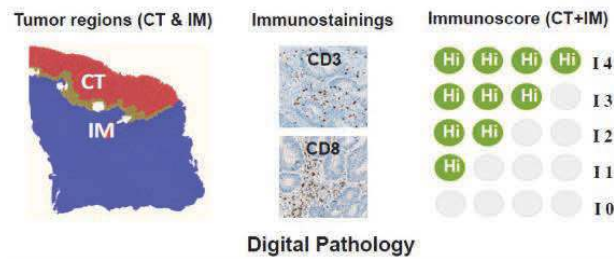


Figure 14. Immunoscoring by digital pathology

The total number of T-cells (CD3+) and CTL (CD8+) is quantified and analysed by digital pathology. The score is either 0 for low T-cell density of 1 for high, and is quantified in both CT and IM for both CD3+ and CD8+ cells giving a possible score from 0-4. The Immunoscore is further stratified into low (0-2) and high (3-4).

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AIMS OF THE STUDY

CRC is one of the most common cancers in the Western world, and metastatic progression is the main cause of mortality. The liver is a frequent site of CRC metastasis, and surgery is the main treatment for patients with resectable CLM. The possibility of implementing minimally invasive surgical procedures in the management of CLM has until now lacked high-level evidence, forming the rationale for the OSLO-COMET trial. The extensive trial biobank provided a unique opportunity to investigate molecular and immune features of resectable CLM, focusing on multilevel molecular characterisation and analysis of infiltrating tumour T-cells.

Specific aims of the study:

1. To compare short-term outcomes and costs after laparoscopic and open liver resection of CLM by executing the OSLO-COMET trial
2. Analyse mutational and transcriptional profiles in tumor and normal tissue biopsies of resectable CLM
3. Quantify and compare total T-cell and T-cell subtype densities in pCRC and matched CLM
4. Compare T-cells densities in CLM in patients with or without previous administration of NACT

SUMMARY OF ARTICLES

Paper I: Laparoscopic Versus Open Resection for Colorectal Liver Metastases: The OSLO-COMET Randomized Controlled Trial

Laparoscopic liver resections have been used for decades in the management of CLM. However, no high-level evidence existed to show that laparoscopic resections were superior to the gold standard open technique. The aim of the study was to perform a randomised controlled trial comparing laparoscopic and open resections in the management of CLM. The trial was designed as an assessor-blinded, randomised superiority trial, where patients were recruited from a single centre, Oslo University Hospital, Oslo, Norway. All patients available for parenchyma sparing resections were eligible. The accrual of patients started in February 2012 and ended in January 2016 with a total of 280 patients with resectable CLM included and randomised, n=133 in the laparoscopic group and n = 147 in the open group. The primary outcome was to compare postoperative complications within 30 days (Accordion severity grade 2 or higher) in the two groups. Secondary outcomes looked to explore any differences between laparoscopic and open resections with regards to postoperative hospital stay, resection margins, blood loss, operation time, cost-effectiveness and quality-adjusted life years. The results showed that the laparoscopic group had a significantly lower complication rate at 19% compared to 31% in the open group, $P = 0.021$. The laparoscopic group also had a significantly shorter admission time in hospital at 53 hours for the laparoscopic group and 96 hours for the open group, $P < 0.001$. Further, there were no significant differences in 90-day mortality, (no patients died in the laparoscopic group and 1 died in the open group), nor any significant differences in R0 resections rates or frequency of missed lesions between the two groups. After 4 months, the laparoscopic group had gained a significant number of quality-adjusted life years, with a similar cost to open surgery. As the first study to publish data from a randomised control trial the OSLO-COMET trial concluded that laparoscopic surgery had a lower complication rate, was cost effective, obtained similar frequency of R0 resection with shorter stay in hospital and a significant gain in quality-adjusted life years. The results

advocated the implementation of laparoscopic surgery in the management of patients with CLM eligible for parenchyma sparing resections.

Paper II: Molecular Signatures Reflecting Microenvironmental Metabolism and Chemotherapy-induced Immunogenic Cell Death in Colorectal Liver Metastases.

mCRC is a major cause of death in CRC patients and is associated with highly variable clinical outcome and response to therapy. Multi-level analysis of CLM is increasingly used to identify molecular characteristics of metastatic disease for both prognostic parameters and predicting response to treatment with for example monoclonal antibodies. In 2015, the identified consensus molecular subtypes (CMS1-4) were found to be associated with prognostic and therapeutic implications in pCRC, but the validity in CLM was not explored. CLM and tumor-adjacent liver tissue from 46 patients (10 patients had two metastases sampled) included in the OSLO-COMET trial were analysed by targeted sequencing and genome-wide copy number alterations (CNAs) to explore the type and frequency of genetic aberrations of 50 cancer related genes. Gene expression analyses were performed to explore any differentially expressed genes related to clinicopathological parameters. The most common mutations found were *TP53* in 76% of patients, *APC* in 61% and *KRAS* in 59%, followed by *PIK3CA* in 20%, *SMAD4* in 15% and *NRAS* in 11%. The 10 patients with 2 metastases (metastatic pairs) had the same mutations in both tumours, but 8 of the 10 metastatic pairs suggested intra-patient differences in CNAs between tumours sampled at the same operation. A CMS classifier tool applied to gene expression data, revealed the cohort to be highly enriched for CMS2. Hierarchical clustering of genes with highly variable expression identified two subgroups separated by high or low expression of 55 genes with immune-related and metabolic functions. Importantly, the induction of genes and pathways associated with ICD was identified in metastases exposed to neoadjuvant chemotherapy (NACT). The uniform classification of CLM by CMS subtyping may indicate that novel class discovery approaches need to be explored to uncover clinically useful stratification of CLM. Detected gene expression signatures support the role of metabolism and chemotherapy in shaping the immune microenvironment of CLM. Furthermore, the

results point to the need for rational exploration of immune modulating strategies in CLM, particularly by exploiting NACT-induced ICD.

Paper III: Low Concordance Between T-cell Densities in Matched Primary Tumours and Liver Metastases in Colorectal Cancer

Exploring the immune microenvironment in cancer has gained great interest in the last decades. Both the aspects of cancer immune evasion, and how to reactivate anti-tumour immune responses in the therapeutic setting are being explored. The location, type and densities of T-cells are associated with the prognosis of patients with pCRC and CLM, but few studies have explored the immune landscape in pCRC and matched CLM. This OSLO-COMET trial substudy included 58 patients with pCRC and matched CLM. Immunohistochemistry was used to define hotspots and quantify T-cell densities (cells/mm²) of the total amount of T-cells (T_{tot}), helper T-cells (TH), cytotoxic T-cells (CTL) and regulatory T-cells (Treg) in the invasive margin (IM), intratumor (IT), and normal tissue from the colorectum (N_{Cr}) and from normal liver (N_{Li}). IM had the highest T-cell density of all regions where T_{tot} in the IM of CLM (2838 cells/mm² (2292 - 3841) versus 1244 cells/mm² (933 - 1749)) in pCRC. In comparison IT had a lower T-cell density than IM, at 485 cells/mm² (284 - 706) in IT pCRC versus 340 (184 - 569) in IT CLM. Our data showed a very low correlation between T-cell densities in pCRC and matched CLM for T_{tot} and for T-cell subtypes, exemplified by a correlation coefficient (R²) = 0.07 between IM pCRC and CLM for T_{tot}. IT pCRC had the highest ratios of TH:CTL at 2.94 (1.70 - 4.35) and Treg:TH at 0.44 (0.27 - 0.59) and IT CLM had ratios of 1.84 (1.07 - 3.04) and 0.24 (0.12 - 0.41), respectively.

The accumulation of T-cells in the IM of these tumours with low penetration into IT combined with high TH:CTL and Treg:TH ratios in IT was suggestive of an immune suppressed TME. T-cell densities of pCRC differed markedly from the matched CLM, warranting development of more efficient methods for evaluation of this potential biomarker in CLM, particularly for analysis of unresectable cases.

Paper IV: Neoadjuvant Chemotherapy is Associated with a Transient Increase of Intratumoral T-cell Density in Microsatellite Stable Colorectal Liver Metastases

Patients with CLM commonly receive NACT prior to surgical resection to increase the chance of R0 resection. In the past, the use of ICI in the metastatic setting of several cancers, has seen a good response in immunogenic tumours, such as MSI CRC. However, most mCRC are found to be of the non-immunogenic MSS type. There are reports suggesting that NACT induces immunogenic cell death recruiting T-cells into the tumor microenvironment. T-cell infiltration is recognised as a biomarker for response to ICI, and in theory NACT could support the use of ICI in obtaining responses in MSS CRC as well. However, evidence to suggest optimal treatment schedules are lacking. Using immunohistochemistry (IHC), the densities of total-, TH, CTL and Tregs were quantified. The aim of this study was to explore if NACT had any effect on T-cell densities or T-cell composition in the IM and IT in resected CLM. Ninety-two patients included in the OSLO-COMET trial were examined, where all but one patient had MSS tumours (91/92). Associations between T-cell densities and clinicopathological parameters were analysed. Fluoropyrimidine-based NACT (in most cases with addition of oxaliplatin or irinotecan) was administered to 45 patients and completed at a median of 8 weeks prior to surgical resection. No overall association was found between NACT administration and IT T-cell densities. However, within the NACT group, a short time interval (< 9.5 weeks) between NACT completion and CLM resection was strongly associated with high IT T-cell densities compared to the long-interval and no NACT groups (medians 491, 236, and 292 cells/mm², respectively; $P < 0.0001$). Only one patient displayed expression of PD-L1. The results from this study suggest that the observed increase in intratumoral T-cells after NACT administration may be transient. The significance of this finding should be further explored to ensure that optimal treatment schedules are chosen for studies combining cytotoxic chemotherapy and ICI.

METHODOLOGICAL CONSIDERATIONS

The Oslo Laparoscopic versus Open Liver Resection for Colorectal Metastases (OSLO-COMET) Trial

The patients in this work were all included in the OSLO-COMET trial (NCT01516710) (Figure 15). The trial's primary endpoint was the difference in 30-day perioperative complication rate when comparing minimally invasive (laparoscopic) surgery to open liver resection in the management of CLM ¹⁸². Secondary endpoints were intraoperative

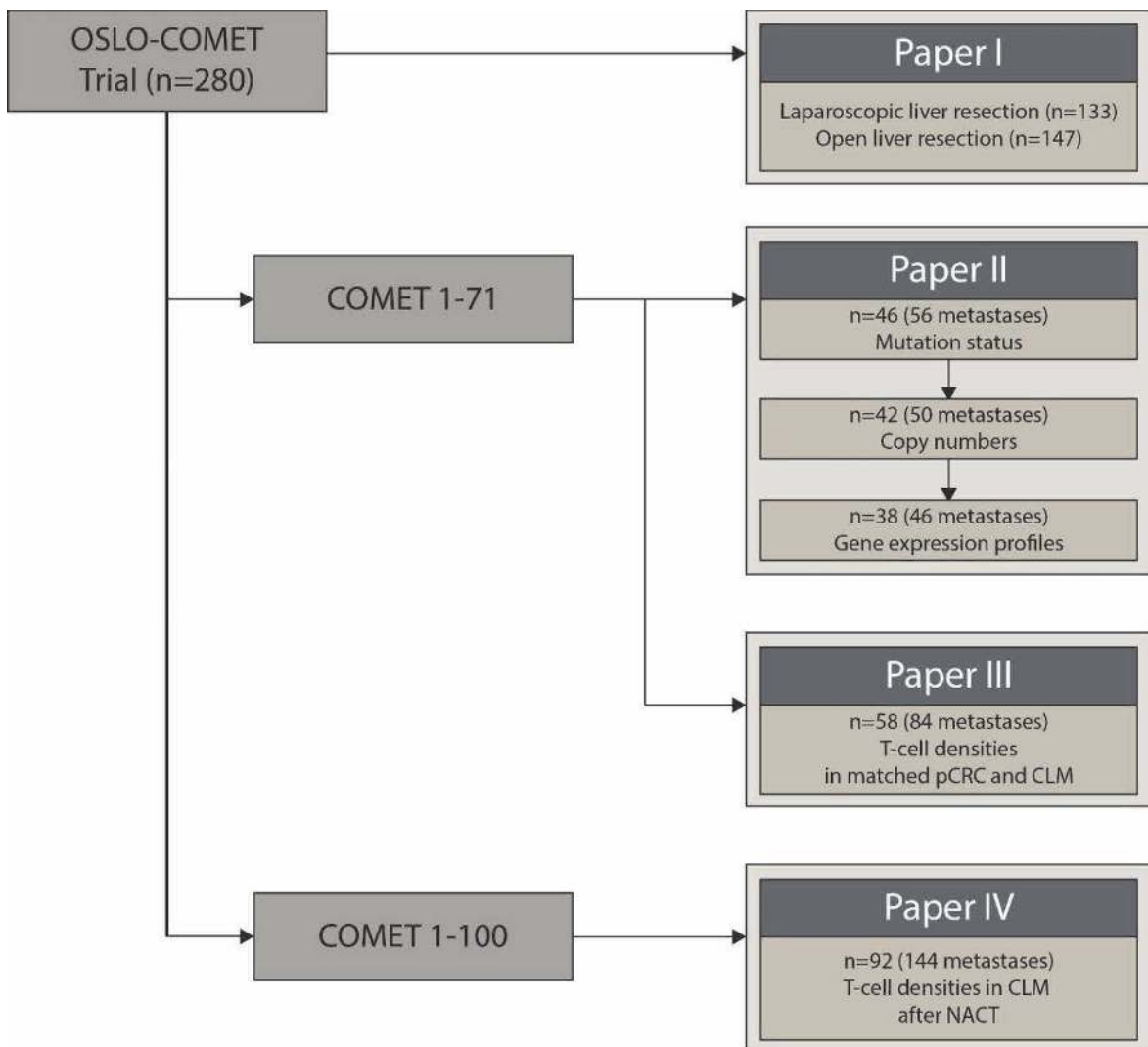


Figure 15. Overview of patient cohorts in paper I to IV

Consort diagram showing the patient cohorts used in the 4 papers in this work. Details on excluded patients are specified in each of the papers.

incidences like blood loss, perioperative pain management, and then length of hospital stay, health-related quality of life, mapping the molecular and immunological environment in tumours, R0-resection rates and the long-term outcomes. The trial was designed as a single centre, open-label and assessor blinded trial. The primary endpoint was important to explore, but if laparoscopic surgery were to be considered as an alternative to open surgery in the management of CLM, R0 resection rates and long-term outcomes would need to be similar or better in the laparoscopic group. For CLM resections, the potential benefits of laparoscopic surgery such as less pain, shorter hospital stays and better quality of life were hypothesised, but these comparisons to open surgery had not previously been explored in an RTC. Internationally there was a strong wish for such a trial to be completed⁶⁹. Patient recruitment began in February 2012 and ended in January 2016 after inclusion of the planned 280 patients. The trial was approved by the Regional Ethical Committee of South Eastern Norway (REK Sør-Øst B 2011/1285) and the Data Protection Officer of Oslo University Hospital.

Patient recruitment

The department of hepatopancreatic and biliary surgery at Rikshospitalet, Oslo University Hospital, Norway is the only referral centre for patients with resectable or potentially resectable CLM in the health region of South-Eastern Norway. Patients potentially eligible for CLM resection were referred and discussed at the liver MDT meeting, where a treatment plan was formed. During the accrual period, 294 patients were assessed for eligibility for inclusion. Four did not meet the criteria and 10 declined to participate, leaving 280 patients for randomisation. At accrual, patients were registered in the OSLO-COMET trial database after consenting to participate in the study, after which the randomisation was performed. Clinical parameters such as TNM classification, histological grade, time of primary operation, previous liver resections and, importantly, information regarding previous or planned chemotherapy, type of chemotherapy and number of cycles were registered. The prospective registration of clinical data would thus be an asset in further substudies branching from the OSLO-COMET trial. Clinical data were entered into a study database at the Oslo University Hospital's information technology department.

NACT

A detailed account of chemotherapy received prior to the study CLM resection became important. For each patient who had chemotherapy and in particular NACT, the timing of chemotherapy in relation to the study CLM resection, the number of cycles and the number of weeks between the last chemotherapy cycle and CLM resection was determined for each case. In papers II and IV (Figure 15) the potential effects of chemotherapy on the immune and molecular landscapes were investigated by analysis of the resected CLM tissues. CLMs exposed to NACT were defined as metastases that were visible on radiological imaging at the time of chemotherapy administration. For the 45 patients that were defined as exposed to NACT, the median time from NACT completion to CLM resection was 8 weeks but ranged from 3-38 weeks. Thirty-nine of 45 patients had a resection within 11 weeks of completing NACT. Six patients followed a complex treatment schedule, resulting in a longer interval between NACT completion and CLM resection, as detailed in Table 4 (unpublished data).

Table 4

Weeks from NACT completion to CLM resection

Weeks	Reason for delay
16	Recurrence of vanished lesion
16	Delay in referral for surgery
18	Prolonged observation due to extra-hepatic metastases
19	CLM present during adjuvant treatment, but acknowledged on follow-up scan
37	Prolonged observation due to extra-hepatic metastases
38	2 stage procedure, NACT was given prior to first stage

The OSLO-COMET Molecular and Immunological Substudies

Patient cohorts

The first 71 consecutive patients included up until April 2013 were available for molecular characterisation and thus created the basis for the patient cohort in paper II and III when the work for this thesis started in 2014 (Figure 15). An extension of the

patient cohort was warranted to further examine results related to NACT in paper II and the 100 patients included up until September 2013 comprised the patient cohort for paper IV.

Patient representativeness

The patients included in the OSLO-COMET trial were eligible for local liver resections, and the protocol did not allow for inclusion of patients in need of formal hemihepatectomies. As most CLM resections at Oslo University Hospital are performed as parenchyma sparing local procedures, the protocol in fact excluded very few of resectable patients. Using tumour tissue from resectable CLM to gain insights into the mechanisms of cancer progression, and to identify new biomarkers and treatment targets are important steps toward improving treatment for this group of patients. Since only 20-25% of CLM patients will be eligible for surgical intervention, the OSLO-COMET cohort represents a minority of patients with CLM, which is a possible limitation of the study and of other similar studies^{56, 59}. Acquiring adequate tumour tissue specimens from unresectable patients is a challenge, as it raises important ethical questions when an invasive biopsy procedure does not provide direct benefits to the patients. When biopsies are available in this setting, the amount of tissue is generally low, suggesting that alternative methods to IHC for immune cell quantification are necessary. Finding good circulating biomarkers could also contribute to solving this challenge. Still, with these limitations, the results and hypotheses generated from studies like the OSLO-COMET trial may form an important body of knowledge that may be relevant also to patients with unresectable CLM.

Tissue samples for analyses

The protocol for the OSLO-COMET trial contained plans for substudies examining molecular characteristics of the resected CLM. Patient consent had been obtained for collecting fresh tumour and tumour adjacent tissue, as well as the collection of routine formalin-fixed paraffin-embedded tissue blocks. To ensure optimal quality of the fresh samples, a pipeline was set up to minimise time between sample collection and delivery to the Department of Pathology at Rikshospitalet, Oslo University Hospital. For the fresh tissue, a designated person collected the surgical specimen for transfer immediately

after the CLM resection. At the pathology department, the surgical specimen was immediately examined and representative tissue samples from the tumour and tumour adjacent tissue were collected and snap frozen. Samples were transported and stored at -80°C in the OSLO-COMET trial biobank located at Institute for Cancer Research at the Norwegian Radium Hospital, Oslo University Hospital. Several patients had multiple metastases sampled, ranging from 2-6. Having patients with multiple metastases gave us the opportunity to study intra-hepatic similarities and differences which was a strength in our study. However, multiple metastases also posed a challenge when analysing both fresh frozen tissue and routinely processed histological sections. To investigate per metastasis response to NACT, it was particularly important to map the anatomical location of each metastasis as seen on radiological imaging to the data in the OSLO-COMET database. For most patients this was a straightforward procedure, but it became a challenge when mapping the anatomy for patients with a high number of metastases, especially where they were located within the same liver segment. All these pre-planned measures ensured that the quality of the tissues and quality of the data was optimal for analysis and interpretations.

Molecular analyses

Tumour content of each sample was assessed by a pathologist, and DNA and RNA were then extracted and processed from the fresh tissue samples using standard methods. Next generation sequencing (NGS) of DNA was performed using the Ion AmpliSeq Cancer Hotspot Panel (v2) from Thermo Fischer using the Ion Torrent (Life Technologies) covering mutations in 50 cancer-related genes (Table 5). Using NGS gave us the advantage of analysing multiple genes with as little as 10 nanograms of DNA with good accuracy^{183, 184}. Reports show that NGS methods are neither more time consuming nor more expensive compared to standard single gene tests¹⁸⁴. The MetAction study (NCT02142036) has demonstrated the feasibility of using NGS in the routine clinical practice, as well as showing that treatment options increase when changing from 50 gene panel to a larger panel¹⁸⁵.

Table 5 Ion AmpliSeq Hotspot Panel V2 genes

<i>ABL1</i>	<i>EZH2</i>	<i>JAK3</i>	<i>PTEN</i>
<i>AKT1</i>	<i>FBXW7</i>	<i>IDH2</i>	<i>PTPN11</i>
<i>ALK</i>	<i>FGFR1</i>	<i>KDR</i>	<i>RB1</i>
<i>APC</i>	<i>FGFR2</i>	<i>KIT</i>	<i>RET</i>
<i>ATM</i>	<i>FGFR3</i>	<i>KRAS</i>	<i>SMAD4</i>
<i>BRAF</i>	<i>FLT3</i>	<i>MET</i>	<i>SMARCB1</i>
<i>CDH1</i>	<i>GNA11</i>	<i>MLH1</i>	<i>SMO</i>
<i>CDKN2A</i>	<i>GNAS</i>	<i>MPL</i>	<i>SRC</i>
<i>CSF1R</i>	<i>GNAQ</i>	<i>NOTCH1</i>	<i>STK11</i>
<i>CTNNB1</i>	<i>HNF1A</i>	<i>NPM1</i>	<i>TP53</i>
<i>EGFR</i>	<i>HRAS</i>	<i>NRAS</i>	<i>VHL</i>
<i>ERBB2</i>	<i>IDH1</i>	<i>PDGFRA</i>	
<i>ERBB4</i>	<i>JAK2</i>	<i>PIK3CA</i>	

Limitations to molecular analyses

All fresh tissues sampled were evaluated by a pathologist macroscopically to identify potential tumour-containing regions prior to biobanking. Later, the tumour content of each sample was evaluated microscopically prior to analyses. In our work, samples with less than 10% tumour were not used and 10 patients had to be excluded from NGS due to no tumour (N=6) or low tumour content (N=4) (Figure 15). Interestingly, 5 of the 6 patients with no tumour found in the biobank sample had a tumour score of 10% or more when assessing the whole section for paper III and IV, indicating that refining the initial sampling protocol could improve the quality of the samples in a biobank. Interestingly, NACT was administered to 9 of the 10 patients that were excluded because of low tumour content; response to treatment is therefore a possible explanation for the low tumour content (unpublished data). Ensuring representative tissue sampling is a challenge in routine practice as well as in research, with small biopsies or poor quality of fixed tissue potentially giving rise to sampling bias with subgroups of patients not being analysed.

Analysing bulk tissues

The same freshly sampled bulk tissue was used for analyses of RNA. Using bulk tissue implies that the samples will contain tumour cells and non-tumour cells found in the surrounding tumour microenvironment. This means that DNA/RNA from other cells, such

as fibroblasts, immune cells and normal endothelial cells would be included in the analyses with the tumour cells. It is likely that this may influence the results obtained in this work. For instance, a high proportion of non-cancerous cells could prevent the detection of mutations present with very low frequency and only found in small subclones within the tumour. For RNA expression it is difficult to assess the relative contribution of tumour cells versus other cells in the TME. On the other hand, the importance of TME in addition to tumour cells is being increasingly emphasised, and results from this work also gave a representation of the entire tumor section that was sampled.

T-cell densities

For quantifying T-cell densities, formalin-fixed and paraffin-embedded tissue blocks from pCRC and corresponding CLM were used. Representative blocks containing both tumour and adjacent normal tissue were selected if possible. The presence of tumour with IM and intratumoural (IT) regions on the same slide was a requirement. Serial sections were made to assess T-cells and always stained in a set order to detect total amount of T-cells (T_{tot}), TH, CTL and Tregs. The stained sections were digitised and all stained sections were scrutinised to capture the most relevant areas with the highest T-cell density, the hotspots, using a method well documented by Galon et al ¹⁷⁴. Multiple hotspots were then selected from 3 regions: tumour adjacent normal tissue (2 hotspots), invasive margin (up to 4 hotspots) and IT (up to 3 hotspots) aiming to keep the total size of the sampled areas similar between regions. The T-cell density (cells/mm²) was determined by manual counting of the number of cells in a hotspot and dividing the number by hotspot area, and an average T-cell density was calculated in each of the 3 regions for each tumour. In this work median T-cell densities (interquartile range) were reported. At present, T-cell density as a prognostic score, known as the Immunoscore, is also performed on digitally scanned, whole slide sections. In the process of validating whole sections, Galon and his colleagues validated the use of hotspots versus whole slides where results suggested that both methods were valid ¹⁸¹. For our work the inter-investigator correlation was validated and found to be extremely good ($R^2 = 0.99$). An obvious limitation to this method is the amount of time required when performing manual

counting. Unfortunately, no appropriate automatic software was available to us, and still would have required manual validation.

RESULTS AND DISCUSSION

The OSLO-COMET Trial – Short-Term Outcomes

Over a period of 4 years (2012 to 2016), 308 patients were eligible for the trial, and of these 294 were screened for inclusion. From the 294 patients screened, 280 patients were included and randomised in the OSLO-COMET trial and the results are presented in paper I. Of the included patients, 133 were randomised to laparoscopy. Four of these did not receive the allocated treatment due to unresectable disease (n=3) and 1 was found to have benign disease when re-evaluated preoperatively. One-hundred-and-twenty-nine patients from the laparoscopic group and 147 in the open liver resection group were included in the analyses (Figure 15). Only 1 of 280 (0.04%) patients died within 90 days postoperatively. The primary endpoint was 30-day perioperative morbidity. In the laparoscopic resection group, 24 of 119 (19%) experienced Accordion grade 2 complications or higher compared to 44 of 147 (31%) in the open resection group, $P = 0.02$. The hospital stay was significantly shorter in the laparoscopic group at 53 hours versus 93 hours in the open group, $P < 0.001$. With regards to oncological outcome, the R0 resection rate was equal in both groups. The overall costs of laparoscopic resection were equal to open resection when accounting for equipment used, hospital stay and postoperative follow-up, even if the cost of laparoscopic procedure in itself was higher. The laparoscopic group had a gain in quality-adjusted life years compared to open resections.

With the results from the OSLO-COMET trial in paper I, as the first RCT to publish data comparing laparoscopic and open resections of CLM, the conclusion was that laparoscopic resection was noninferior to open resection with regards to oncological principles and costs, but with less morbidity and a shorter hospital stay. The overall mortality rate was very low. Subsequent results from the OSLO-COMET trial, showed that the quality of life was better for patients who had laparoscopic surgery⁶⁶. The results from the OSLO-COMET trial has paved the way for a wider use of laparoscopy in the management of CLM, and the technique is now offered as a routine operation for patients in need of parenchyma sparing CLM resection at Oslo University Hospital. In

addition, the study standardised principles for enhanced recovery after surgery important for laparoscopic as well as open surgery.

Clinicopathological Parameters of the Substudy Cohorts

For the purpose of this summary we chose to present clinicopathological parameters for only the 92-patient cohort presented in paper IV (Table 6) as these are representative of

Table 6. Clinicopathological parameters (n=92)

Variable		Number (%)
All cases		92
Gender	Male	55 (60)
	Female	37 (40)
Data pertaining to pCRC		
TNM-classification	T1-2	6 (7)
	T3	71 (77)
	T4	15 (16)
	N0	40 (43)
	N1	28 (30)
	N2	24 (26)
	M1	36 (39)
pCRC Location	Right colon	24 (26)
	Left colon + rectum	68 (74)
Data pertaining CLM		
Age	Median (IQR)	68 (61 - 73)
Performance status	ECOG 0	66 (72)
	ECOG 1 and 2	22 (24)
	NA	4 (4)
Clinical risk score	0-2	68 (74)
	3-4	23 (25)
	NA	1 (1)
Microsatellite instability		1 (1)
	NACT	
	No NACT	47 (51)
	NACT	45 (49)
Resection interval (weeks), median (range)		8 (3 - 38)
Number of NACT cycles, median (range)		4 (3 - 12)
NACT regimens	Fluoropyrimidine + oxaliplatin	31 (69)
	Fluoropyrimidine + irinotecan	6 (13)
	Fluoropyrimidine + other	7 (16)
	Oxaliplatin monotherapy	1 (2)
	Partial response	19 (43)
	Stable disease	19 (43)
RECIST response (n=44)	Progressive disease	6 (14)

The table shows the most relevant parameters for the patients in paper IV. The distribution of patients for the different variables are similar in paper II and III. Clinical risk score¹³ was calculated by giving 1 point for each of the following parameters: lymph node metastases in pCRC, <12 months from pCRC to diagnosis of CLM, multiple metastases, largest metastasis > 5 cm, CEA > 200 µg/L. A patient with CRS ≤ 2 was considered to have a low risk of colorectal cancer recurrence.

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the results found in paper II and III. Of the 92 patients, 86 patients (93%) had T3/T4 pCRC, and 52 patients (57%) had lymph node metastases. Thirty-six (39%) patients had mCRC when diagnosed with pCRC. Sixty-eight (74%) had pCRC located in the left colon or rectum. The median age at study CLM resection was 68 (interquartile range 61 - 73), where slightly more patients were male 55 (60%). Sixty-six (72%) patients had an Eastern Cooperative Oncology Group (ECOG) status equal to 0¹⁸⁶. At CLM resection 68 (74%) patients had a low clinical risk score of 0-2¹³. NACT was administered to 45 (49%) patients, where 44 of 45 had fluoropyrimidine containing regimens, and 35 of the 45 patients were exposed to oxaliplatin. Response to NACT was assessed using the Response Evaluation Criteria in Solid Tumours (RECIST 1.1) in 44 patients, where 38 (86%) showed either stable disease or partial response to treatment¹⁸⁷. Only 1 patient was classified as MSI.

Molecular Landscape of CLM from OSLO-COMET Trial

Exploring the molecular aberrations has been important in understanding the cellular behaviour of cancers and importantly, for finding therapeutic targets. Exploring tumour DNA has become more efficient with the development of high-throughput DNA sequencing, also known as NGS¹⁸⁸. This technology has increased the possibilities of exploring multiple genomic aberrations to better understand features of carcinogenesis and metastasis⁹⁴. Up until 2016, the focus when employing NGS methods had been on exploring pCRC^{1, 189, 190}, but even for pCRC, NGS studies with high-quality, prospectively registered clinical data were lacking¹⁹⁰. For studies including CLM, the main focus areas were on establishing NGS as a feasible method for detection of mutations and analysing concordance with pCRC, and studies were mostly performed with a limited number of cases¹⁹¹⁻¹⁹⁶. Studies involving multilevel characterisation of CLM, including NGS and gene expression profiling, especially with good clinical data were virtually non-existent.

Mutations and CNAs in CLM

Exploring the molecular characteristics of CLM was an important substudy in the OSLO-COMET trial. Fresh frozen tissue was used for NGS analyses of 46 patients (Figure 15) using a 50 cancer-gene hotspot panel (Table 5). For comparison, 2 pCRC and 1 mCRC

cohorts with publicly available datasets were obtained via cbioportal.gov (Figure 16). In 2012, the Cancer Genome Atlas (TCGA) network reported on multidimensional molecular data from 224 pCRC patients aiming to explore pCRC biology and therapeutic targets¹⁸⁹. In 2016 Giannakis et al performed large scale sequencing of 619 cases of pCRC mapping the frequencies of mutations and exploring the correlations to immune cell infiltration in tumours¹⁹⁰. The only publicly available dataset pertaining to mCRC was reported by Yaeger et al in 2018¹⁹⁷, where 979 patients with mCRC were analysed. For the purpose of this work, analyses of 353 patients with CLM was extracted from the dataset.

TP53 was the most frequently mutated gene found in 76% of patients in the OSLO-COMET cohort. Reports from pCRC showed a lower frequency of *TP53* mutations at 54%¹⁸⁹ (TCGA) and 56%¹⁹⁰ (Giannakis) compared to our data. In the cohort of CLM (Yaeger)¹⁹⁷ the frequency of *TP53* mutations of 82% was comparable to findings in the OSLO-COMET trial cases. This signifies the importance of *TP53* aberrations in the development of CLM, which are also associated with poor outcome and poor response to treatment^{91, 198}. *APC* was the second most commonly mutated gene found in 61% of patients in the OSLO-COMET trial cohort. In the publicly available datasets, the frequency of *APC* mutations was variable from 63 to 84% (Figure 16). *APC* is considered a molecular gatekeeper in CRCs¹⁹⁹, and its aberrations have been associated with loss of β -catenin regulation. Aberrations in *APC* cause insensitivity to anti-growth signals and are recognised as important events in the CIN pathway^{6, 92}. *KRAS* was the third most frequent mutation found in 59% of cases, where 70% of the mutations were in codon 12 and 19% in codon 13. The frequency of *KRAS* mutation ranged from 30 – 42% in the other cohorts (Figure 16), meaning that the OSLO-COMET trial cases had a up to 2-fold higher mutation frequency compared to the two cohorts of pCRC patients and the one of CLM. Further, our analyses found that *NRAS* was mutated in 11% and *BRAF* in 7%. One patient in our study had both *NRAS* and *BRAF* mutations in the same tumour. *KRAS*, *NRAS* and *BRAF* are important genes in the MAPK pathway suggesting that a large proportion of the tumours had perturbed MAPK signalling, providing self-sufficiency in growth signalling⁶. When analysing CNAs, *APC* was deleted in 36% of OSLO-COMET trial samples, *SMAD4* was deleted in 88%

(mutated in 15%) and *TP53* was deleted in 69%. All the aberrations found in OSLO-COMET trial CLM cases are previously well described in CRC tumours and in line with the study patients harbouring well known features of the CIN pathway^{91, 93}

Two metastases were sampled and analysed from ten of the 46 patients (metastatic pairs). The mutation profiles were pairwise identical in the 10 metastatic pairs. We did not perform NGS analysis of the corresponding pCRC, but several studies have previously shown a very high concordance between pCRC and the corresponding metastatic tumours for the most common mutations found in CRC^{192, 200, 201}. The finding of a similar mutation profile in the metastatic pairs can be explained by the use of the focused 50-cancer gene panel, which limits the possibilities of discovering rarer mutations private to either pCRC or CLM. In contrast, when analysing CNAs, there were differences between the CLM derived from the same patient in 8 of the 10 cases. An explanation for this could be that the metastases may originate from different cancer cell clones in the primary tumour, or that they have developed different characteristics upon reaching the liver microenvironment.

Seventy-four percent of the OSLO-COMET trial patients had mutations in *KRAS*, *NRAS* and *BRAF*. *RAS* mutations are generally associated with a worse outcome after CLM resection³⁵, exemplified in a study of 421 patients where the 5-year OS for *RAS* mutated patients were 42% compared to 65% in *RAS* wild-type tumours²⁰². At the time of accrual into the study, anti-EGFR treatment emerged as an option for mCRC, where *KRAS*, *NRAS* and *BRAF* wild-type tumours were predicted to respond, meaning that only 26% of OSLO-COMET patients would have been eligible for such treatment. With *KRAS* being a frequent and important driver mutation in cancer, the mutated gene theoretically poses as an interesting target for oncological treatment. However, due to the conformation of the mutated *KRAS* protein, this has proven very difficult. Hence, *KRAS* has been considered an “undruggable target”^{203, 204}. There are currently phase I and phase II trials where targeting the *KRAS* variant G12C is being studied in solid cancers (NCT04006301, NCT04111458)²⁰⁵. In a case report where treatment targeted the mutated *KRAS* G12D (NCT01174121), which is the most frequently mutated variant in CRC, adaptive immune cells, and more specifically CTLs recognised the *KRAS*

variant G12D and induced a partial radiological response in a lung metastasis from CRC, which lasted 9 months after treatment ²⁰⁶.

The molecular landscape of CLM in the OSLO-COMET trial reveals a pattern of mutations and CNAs consistent with tumours following the CIN pathway, where the metastatic pairs harbour the same mutations, but show differences in CNAs. The high frequency of MAPK pathway perturbation, as evidenced by the high frequency of *KRAS*, *BRAF* and *NRAS* mutations, excludes the majority of these patients from receiving anti-EGFR treatment and these mutations are associated with a worse outcome compared to wild-type tumours.

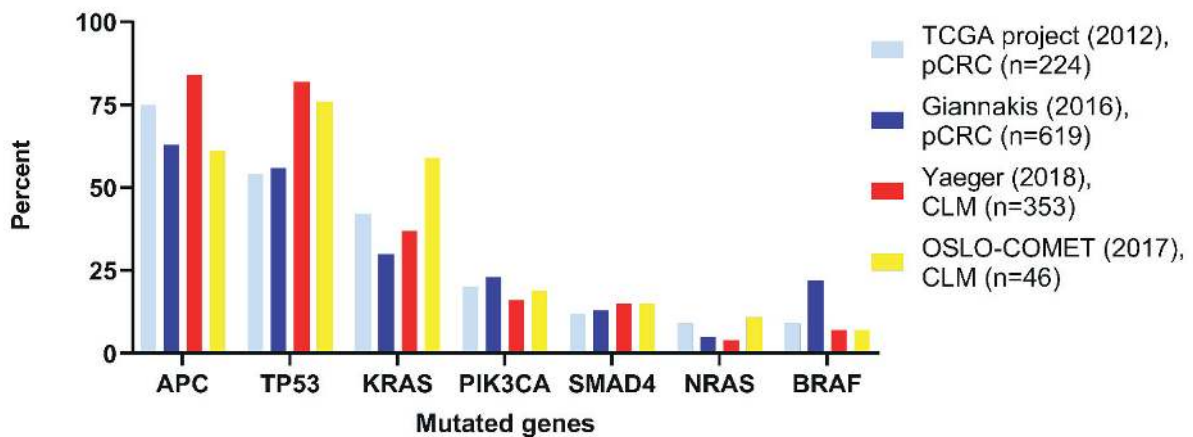


Figure 16

Figure visualising the frequency of common mutations from 4 studies including the OSLO-COMET trial. The TCGA project in light blue and Giannakis et al in blue represent analyses from pCRC. Yaeger et al in red show CLM selected from analyses of mCRC cases. The yellow bars represent the frequency of mutations found in the OSLO-COMET patients.

Consensus molecular subtypes (CMS) in CLM

Microarray analyses provided gene expression profiles for 38 patients included in the OSLO-COMET trial (Figure 15), which were used to classify the study patients according to the CMS classification. Our study was the first to report such analyses performed on CLM. In the seminal work by Guinney et al, a consensus was reached on how to capture the diverse phenotypes of pCRC in 4 molecular subtypes, classified based on an aggregate of multi-platform gene expression data from 18 datasets comprising 4151

pCRC samples¹. The algorithm developed categorised the samples into 4 clusters representing CMS 1-4 (Figure 17). Clinical and molecular data were connected into each of the subsets to further characterise them with clear associations to the transcriptomic analyses. CRC is a heterogeneous disease, and the use of transcriptomic data (gene expression analysis) is considered better for capturing the additive effects of molecular aberrations in CRC²⁰⁷. The CMS classification has been shown to have prognostic value, where the CMS2 patients have the best outcome after treatment of pCRC and after developing a disease relapse. The CMS2 subtype also seems to respond well to oxaliplatin containing chemotherapy and anti-EGFR treatment^{1, 208, 209}.

When the CMS classifier script was applied to the CLM cases in the OSLO-COMET trial, 84% (37/44) of the tumours were classified as CMS2, which is much higher than what was found in pCRC patients in the original report, where 37% were classified as CMS2¹. CMS2 is described as the “canonical” subtype, comprising the MSS pCRC, located in the left colon or rectum and with activation of MYC and Wnt signalling, which is in agreement with the OSLO-COMET cases mostly being distal cancers, displaying features of the CIN pathway with a high number of aberrations in *APC*, *TP53* and *SMAD4*^{1, 210}. The frequency of *KRAS* mutations (59%) was, however, higher than other reports on CMS2, where *KRAS* was reported mutated in 28% of CMS2 cases^{1, 209}. In contrast to the pCRC setting, the CMS classification has been less extensively used for analysis of metastatic tissues. In three identified studies, where CLM was analysed, the frequency of CMS2 was intermediate compared to our study and the results from pCRC (51-56% based on analysis of 51-62 cases)²¹¹⁻²¹³.

In the report by Guinney et al, CMS2 cases had a 73% 5-year relapse free survival, while all our cases had CLM, of which 77% developed within the first year of primary surgery, suggesting that CMS2 in CLM may reflect a different biology. The observed differences might be related to differences in the TME and might have clinical implications. Therefore, although several of the molecular and clinicopathological features of the OSLO-COMET trial are consistent with the description of the CMS2 pCRC in the original report, it remains unclear whether the CMS classification will be useful in the metastatic setting.

CMS1 MSI Immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
<i>BRAF</i> mutations		<i>KRAS</i> mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGFβ activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 17

Figure shows the 4 consensus molecular subtypes (CMS), frequencies of different CMS in CRC, with associated phenotypical traits and outcomes.

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T-cell Densities in Matched pCRC and CLM

The immune system and immune escape mechanisms are recognised as hallmarks of cancer and considered important in progression to metastasis ^{11, 175}. The immune system has gained particular interest with the emerging use of ICI in the treatment of immunogenic tumours, where MSI and high TMB are suggested to be predictive of response to ICI ^{85, 214-216}. High densities of effector T-cells such as CTL are associated with MSI and high TMB and may serve as additional biomarkers for response to ICI ^{85, 87, 215, 217, 218}. Results from the analysis of T-cell densities have been established as prognostic markers in both CRC and CLM ^{174, 181, 219, 220}. As most mCRC are of the

immune ignorant MSS subtype that do not exhibit response to ICI ²¹⁷, understanding the immune TME in these tumours is of importance. Where many studies have compared genomic aberrations in pCRC and matched CLM ^{200, 201}, few studies have explored correlations between T-cell densities in matched cases.

T-cell concordance in matched pCRC and CLM

In paper III the aim was to explore the densities of T_{tot} and subtypes (CTL, TH and Tregs) in 58 patients in pCRC and matched CLM (Figure 15). Interestingly, there was a poor correlation between pCRC and matched CLM with regard to the density of T_{tot} and T-cell subtypes where R² ranged from less than 0.01 to 0.18 in IM and IT. In the clinical setting, our data suggest that the immune cells in the metastatic tumours will have to be examined and that one cannot rely on analysis of T-cell densities in pCRC, which is in contrast to the high concordance seen for mutation status and MSI ^{192, 200, 201}.

Type and localisation of T-cells in pCRC and CLM

Further in paper III, we compared T-cell densities in IM and IT regions of pCRC and CLM. IM CLM was the region harbouring the highest density of T-cells with a median of 2838 cells/mm² (2292-3841), over double that found in IM pCRC with a median of 1244 cells/mm² (933 - 1749), *P*-value <0.0001. Similar observations in IM have been made in other studies in both pCRC and CLM ^{174, 219, 220}. It is an interesting observation that the peri-tumoral region localised between normal tissue and tumour tissue hosts such a high density of adaptive immune cells. The colon and rectum, as well as the liver, are both immunologically potent organs, but with different functions, where the bowel mucosa represents a barrier to microorganisms, harbouring cells educated to trigger an adaptive immune response, whereas the liver relies more on an innate immune response ^{131, 153, 165, 166}. In IT pCRC the T_{tot} density of 485 cells/mm² (284 - 706) was less than half of IM CRC, but similar to tumour adjacent colorectal tissue (N_{Cr}). The T_{tot} in IT CLM had a median density of 340 cells/mm² (184 - 569), only 1/10th of IM CLM, but significantly higher than N_{Li}. This striking difference between IM and IT found in both pCRC and CLM was similar to several other reports ^{174, 220}, and showed that both organs are capable of recruiting T-cells to the tumor-normal tissue interface, but that the T-cells are less able to penetrate into the tumour. The large difference in T-cell densities between IM and IT can

be explained by a lack of T-cell migration into tumours, and reports recognise this as an immune escape mechanism in pCRC ²²¹.

In our data, TH was the dominating T-cell subtype for both IM and IT of pCRC and CLM. THs are important in organising the immune responses and differentiate into different subsets with different roles ¹⁶⁰. The TH:CTL ratio ranged from 1.71 (1.09 - 2.39) in IM CRC to 2.94 (1.70 - 4.35) in IT CRC, and IM/IT CLM was in-between at 1.83 (1.36 - 2.50) and 1.84 (1.07 - 3.04) respectively. Normal adjacent tissue had a ratio closer to 1.0, where N_{Cr} had a ratio of 1.16 (0.89 - 2.03) and N_{Li} 0.72 (0.44 - 1.14). A higher ratio of TH to CTL, as seen in our data, was previously associated with an invasive and metastatic phenotype in pCRC ²²². Our data also show a high Treg:TH ratio in tumour regions, in particular in IT pCRC at (0.44 (0.27 - 0.59) versus 0.24 (0.12 - 0.41) in IT CLM, P-value < 0.0001). Tregs are important in dampening the adaptive immune responses ²²³, and the results suggest the presence of an immune suppressed TME in both pCRC and CLM.

In this work, the correlation between T-cell densities in pCRC and matched CLM was poor, implying that the T-cell density in pCRC did not accurately reflect T-cell densities in matched CLM. The striking accumulation of T-cells in the IM, with a T-cell composition favouring TH over CTL, and with a high Treg:TH ratio, suggests an immune suppressive TME, especially in IT pCRC.

NACT Changes the Immune Landscape in CLM

Due to the enrichment of MSS in pCRC, methods that could overcome resistance to ICI treatment in these non-immunogenic cancers would be of high interest. A possible strategy could be to find ways of attracting and activating effector T-cells in MSS tumours ²²⁴. Cytotoxic drugs, such as oxaliplatin, are of interest in this context, because of their hypothesized ability to induce ICD ¹⁷⁹. However, since reliable assays are not available to capture the occurrence of ICD in the clinical setting, surrogate parameters might be identified on the transcriptional level or by analysing the presence of infiltrating immune cells ²²⁵. Several studies report an increase in T-cell density within a window of 2-10 weeks after NACT administration ²²⁶⁻²²⁸. However, no studies had patient samples

that could explore and compare associations between NACT, T-cell densities especially over a wider time span between NACT completion and CLM resection.

NACT exposure and differentially expressed immune related genes

In paper II (Figure 15), 12 (n=15 metastases) of the 38 patients (44 metastases) with gene expression analyses had received NACT. A striking result was that 208 differentially expressed genes were found when comparing expression profiles of patients who had NACT to the ones that had not (no-NACT). When exploring the functions of the 208 genes, processes related to both innate and adaptive immune responses were prominent. Nine out of 12 (73%) patients received oxaliplatin containing chemotherapy with a median of 8 weeks (3-19) from NACT completion to CLM resection. Oxaliplatin is interesting as it has been recognised as an inducer of ICD¹⁷⁹. In our study the NACT group displayed up-regulated genes related to innate immunity such as expression of PRR, and maturation of DCs. In vitro studies have demonstrated that oxaliplatin can activate T-cells via DCs, by binding to intracellular proteins, that triggers an immune response, unlike the damage the drug causes to the DNA, which is thought to cause apoptosis²²⁹. Oxaliplatin is therefore thought to be an inducer of ICD, unlike other platinum-containing anti-cancer drugs¹⁷⁷. Oxaliplatin treatment releases intracellular CALR from cancer cells, a protein that serves as an “eat me” signal to DCs²²⁹. Our results also show an up-regulation of genes related to T-cell activity, where the cytokine INF- γ was the predicted top activated upstream regulator. INF- γ is secreted by activated T-cells (TH and CTL) and by antigen-presenting cells (DC and macrophages), and has been linked to anti-tumour activity of tumour infiltrating lymphocytes through the promotion of inflammation and attraction of immune cells²³⁰. This fits with our data, where the recruitment and activity of other immune cells was suggested in the NACT group, where genes related to “cellular movement”, “immune cell trafficking”, “natural killer cell signalling”, “phagosome formation” and “production of nitric oxide and reactive oxygen species in macrophages” were enriched compared to the no-NACT group. Some of the up-regulated genes related to macrophages in the NACT samples point to a polarisation towards the pro-inflammatory M1 subtype²³¹, while simultaneously, up-regulation of *CD163* is associated with the anti-inflammatory M2 subtype. Further

balancing the immune activating signals, up-regulation was also observed of immune suppressive genes related to IL-10 and *STAT3* signalling in the NACT group.

Our results suggest that at the transcriptional level, NACT administration may have triggered up-regulation of genes related to both innate and adaptive immune responses in CLM as well as immune suppressive genes. ICD induction by oxaliplatin containing chemotherapy may explain some of the findings, but since there are no validated transcriptional markers of ICD, we could not confirm this. The results suggest the need for further studies to dissect the transcriptional changes after NACT administration in CLM.

NACT exposure and T-cell densities in MSS CLM

The immune responses seen in CLM after NACT exposure on the transcriptional level described in paper II warranted further exploration, and a study to analyse the effect of NACT on T-cells densities CLM was initiated. In paper IV, we extended the cohort to include 92 patients bearing 144 metastases (Figure 15), where 91 of the 92 patients had MSS tumours. T-cell densities were quantified by IHC. Forty-five (49%) of the 92 patients had NACT. The number of NACT cycles was not associated with differences in T_{tot} density. A short time interval between NACT completion and CLM resection, however, was associated with significantly higher IT T_{tot} density in multivariable analyses compared to a long interval. Exploring the time interval from NACT completion to CLM resection further via receiver operating characteristics defined 9.5 weeks as the most optimal cut-off value to distinguish between high and low T_{tot} density in IT. We could thus explore 3 groups, comprised of no-NACT patients, and two NACT subgroups consisting of 30 patients in a short-interval group with less than 9.5 weeks from NACT completion to CLM resection and 15 patients in the long-interval group. The short-interval group had a significantly higher T_{tot} density IT at 491 cells/mm² (271 - 926) versus 236 cells/mm² (102 - 323) in the long-interval group and 292 cells/mm² (187 - 491) in the no-NACT group, $P < 0.0001$. The densities of THs and CTLs mirrored T_{tot} . An association between NACT and T-cell densities has previously been demonstrated for pCRC^{232, 233} and CLM^{226, 228, 234} as well as other cancers²²⁷. A plausible explanation for the higher T-cell densities in NACT exposed CLM was the activation of immune

responses due to ICD, since most of our study patients were treated with oxaliplatin containing NACT¹⁷⁷. The lack of response to ICI in MSS tumours is a challenge in cancer treatment and one suggested explanation is the low number of T-cells in the tumours (cold tumours)^{85, 214, 224}. ICIs work by eliminating the co-inhibitory signals enforced by checkpoint inhibition on T-cells, and not by recruiting T-cells into tumours¹⁵¹. To achieve responses to ICI, a high number of T-cells in the tumour would be favorable²¹⁵, and the T-cell density may thus serve as an additional marker to predict responses to ICI²¹⁷.

Changes in IT composition of T_{tot}, CTL and Tregs were also found in the different NACT groups after NACT. Treg densities in IT displayed only marginal differences between the NACT subgroups at 50 cells/mm² (28-88) in the no-NACT group, to 61 (30-104) in the short-interval group and 37 (20-68) in the long-interval group. While the short-interval group had the highest absolute density of Tregs, the long-interval group had the highest Treg to CTL ratio at 0.60 (0.21-1.30) versus 0.29 (0.20-0.53) in the short-interval group and 0.49 (0.29-0.75) in the no-NACT group, where both comparisons had *P*-value < 0.05. Tregs are important in regulating immune responses, and are also important players in tumour immune escape mechanisms¹⁶⁰. The higher Treg to CTL ratio in the no-NACT group can be interpreted as the TME favouring immune escape¹⁷⁵.

NACT did not up-regulate the expression of PD-L1 as only one of the CLM in our study expressed PD-L1 (>5%). PD-L1 expression is used as a predictive biomarker for response to ICI in several other cancers^{235, 236}. Cold tumour such as MSS CRC are recognised by a low expression of PD-L1 and are sometimes referred to as “immunologically ignorant”^{217, 236}. The use of PD-L1 as a biomarker for response to ICI is debated, as cancers that respond to ICI show high variability in PD-L1 expression ranging from 14% up to 100%²³⁶. Even in CRC, response to PD-1 inhibition was seen even in tumours lacking a high PD-L1 expression²¹⁴.

Based on our results we hypothesise that the increase in T-cell densities found in the short-interval group after NACT could pose as a window of opportunity for response to ICI also in MSS tumours. Several strategies are currently being explored to achieve this where one challenge is to overcome the lack of neoantigens, absence of activated T-

cells and the impaired trafficking and infiltration of T-cells into MSS tumours. Results from the OSLO-COMET substudy are currently being explored in the RCT “Colorectal Cancer METastasis - Shaping Anti-tumor IMMunity by OXaliplatin” (METIMMOX trial - NCT03388190) where responses are compared in patients receiving oxaliplatin containing chemotherapy with or without sequential PD-1 inhibition.

Analysing patients with multiple metastases

Thirty-three of the 92 patients in our cohort had multiple metastases (Paper IV). Our data showed that overall, the intra-hepatic variation (range) in T_{tot} for patients with multiple liver metastases was low at 686 cells/mm² compared to the much larger variation of 4035 cells/mm² between individual patients. There is currently no consensus on how to approach the challenge of analysing data from patients with multiple metastatic tumours and outcomes in patients with mCRC. For patients with multiple metastases in paper III and IV it was therefore necessary to decide on using either the values from one of the metastases or an average of all when exploring T-cell densities and associations with outcomes. The low variation in patients with multiple metastases allowed the use of a mean T-cell density value for these patients.

Associations with Long-term Outcomes After CLM Resection

Paper IV comprises all patients in the OSLO-COMET molecular substudy (Figure 15 and Table 6). After the study CLM resection, the median follow-up time was 63 months (95% confidence interval (CI), 61 - 64), with a median OS of 61 months and an estimated 5-year OS of 51%. Overall, mCRC is associated with a poor OS^{3, 26, 52, 53}, but patients who can be offered surgical intervention for CLM have a better OS⁵⁵. Patients in the OSLO-COMET trial were all eligible for parenchyma sparing resections and our results are similar to other reports⁶⁷, although resectable CLM also displays heterogenous outcomes where reported 5-year OS varies between 40% to over 70%^{13, 60, 65, 237}. For the 92 patients in paper IV, three parameters were associated with a worse OS: N2 status of pCRC with a HR of 2.7 (95% CI 1.40 - 5.22), P -value < 0.0001) compared to N0, ECOG 1-2 with a HR of 2.12 (95% CI 1.13 - 3.96), P -value = 0.02 compared to ECOG 0 and increasing age with a HR of 1.05 (95% CI 1.01 - 1.08), P -value = 0.01. From paper II, the 7 patients with *SMAD4* mutations had inferior OS compared to wild-

type, HR 3.3 (95% CI 1.20 – 9.50), *P*-value = 0.02. None of these are novel biomarkers for outcomes in CRC/mCRC^{1, 58, 238, 239}. Sixty-seven percent of the patients had an immune score of 3 or 4 (paper III) which is associated with a favourable prognosis¹⁷⁴¹⁸¹. Furthermore, 74% of patients had a low clinical risk score¹³ (Table 6), and 84% (analysis of CLM) had CMS2 tumours (paper II), both parameters associated with favourable outcome^{1, 212}. Even though our data shows that half of the patients were alive after 5 years, the majority of patients in this study experienced a recurrence of CRC after CLM resection. In a follow-up study of the OSLO-COMET trial exploring long-term outcome, there were no differences in 5-year OS comparing the 147 patients having open surgery to the 133 having laparoscopic surgery for CLM, at 55% and 54%, respectively. However, the study had insufficient power to exclude differences up to 10% in either direction. (Aghayan DL et al, *Ann Intern Med*, *in press*).

For PFS after CLM resection, patients were followed up for a median of 57 months (95% CI: 51 - 62), and 75% of the 92 patients experienced an event after CLM resection. Thirty-nine percent of the first registered events were in the liver, 10% were in the lungs, 16% developed a recurrence outside of the liver and lungs, 22% had metastases at multiple sites and 9% developed other cancers. Four percent died without a known recurrence of CRC. The median PFS was 19 months (95% CI 11 - 27), which is in accordance with other reports^{3, 57}. Clinical risk score and immune score were not associated with OS or PFS, nor was NACT. The influence of NACT on the OS of resectable CLM is not fully established^{3, 60}. Due to the recurrence rate, and as many of the recurrences were in the liver, the prospect of re-resection must be considered in such a patient group. As resectable recurrent CLM has a prognosis mirroring the good outcomes seen after the first resection²⁴⁰⁻²⁴², the use of parenchyma sparing resections is important as the technique was found to increase the possibility of a re-resection from less than 10% after open surgery to 22% after laparoscopic surgery²⁴³.

The OSLO-COMET trial patients represent a cohort of patients with a long OS after CLM resection but where the majority of patients experience a recurrence of mCRC. Few variables were associated with long-term outcome, including NACT, which suggests that a relatively homogeneous group of patients were selected based on resectability.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As the first RCT to publish data comparing laparoscopic and open resections of CLM, the OSLO-COMET trial concluded that laparoscopic resection was noninferior to open resection, with the advantages of fewer complications and shorter hospital stays. The laparoscopic technique was associated with better quality of life at a similar cost. This unique trial has been important in validating the use of laparoscopy and facilitating further implementation of the technique in the management of CLM. It is now a standard surgical method at Oslo University Hospital.

Our data show that the OSLO-COMET trial cohort was, probably because resectability was the main inclusion criterion, a selected group of patients with a favourable prognosis. The high immune score and enrichment for CMS2 also suggested a homogeneous group of patients. The cohort was almost exclusively composed of MSS cases with a high frequency of *KRAS* mutations, which excludes this patient subgroup from receiving ICI treatment and EGFR-targeted therapies. Based on the NACT induced transcriptomic changes pointing to an adaptive immune response, and the transient increase in CTL densities in CLM, this work has led us to hypothesise that NACT induced a window of opportunity that could be exploited by ICI. As most CLM are of the MSS subtype, enabling the use of ICI in such tumours may improve patient outcome. To answer this question, we designed the ongoing METIMMOX trial where MSS mCRC patients are randomised to oxaliplatin-based chemotherapy followed by ICI (experimental study arm) or the oxaliplatin-based standard-of-care (control arm).

Identifying biomarkers to predict and monitor responses to ICI is important, and since immune cell infiltration is believed to be essential for response to ICI, quantification of immune cells poses as a possible option. As we have emphasised, quantification by IHC is labour intensive, and not a very efficient method, and is essentially only applicable to resectable patients where a surgical specimen is available. Based on our results, the T-cell density of the pCRC does not reflect the situation in the CLM. Therefore, less invasive methods are needed, such as methods utilising core biopsies, imaging techniques, or circulating biomarkers. Well-described CLM cohorts, such as ours, could be suitable for validation purposes in future studies.

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