MALIGNANCY, METASTASIS AND IMMUNE MODULATION

Experimental tumor immune regulation and observational clinical studies in ovarian and colorectal cancer

Simer Bains, Oslo 2015

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Acknowledgments

This Thesis summarizes work carried out at the Biotechnology Centre of Oslo, University of Oslo, and Centre for Molecular Medicine, University of Oslo, in the period august 2007 to march 2015. The research study was part of the Medical Student Research Program and the PhD program at the Faculty of Medicine, University of Oslo. After completing my M.D. in 2012 and internship in 2014, I received a 1 year PhD-fellowship from Centre for Molecular Medicine, University of Oslo, to complete my work.

1 Abbrevations

AA – arachidonic acid

Ab – antibody

AC – adenyl cyclase

A.D. – anno domini

Ag – antigen

Akt – protein kinase B

AMC – ascites mononuclear cells

AP-1 – activator protein 1

APC (cell) – antigen presenting cell

APC (dye) – allophycocyanin

APC (gene/protein) – adenomatous polyposis coli

ASA - acetylsalicylic acid

ATC – anatomical therapeutic chemical classification

Bcl-2 – B cell lymphoma 2

bGFG – basic fibroblast growth factor

BRCA1 – breast cancer type 1

BRCA2 – breast cancer type 2

cAMP – cyclic adenosine monophosphate

CA125 – cancer antigen 125

CD – cluster of differentiation

CD25 - alpha chain of the IL-2 receptor

CD45RO - Protein tyrosine phosphatase, receptor type, C

CD71 - transferrin receptor protein 1

CD95 - tumor necrosis factor receptor

CD127 – interleukin-7 receptor

CD147 - basigin/emmprin

CD148 - receptor-type tyrosine-protein phosphates-eta superfamily member 6

CEA – carcinoembryonic antigen

CFSE – carboxyfluorescein diacetate succinimidyl ester

CI – confidence interval

CIMP – CpG island methylator phenotype

CIN – chromosomal instability

CNR – Cancer Registry of Norway

CO_2 – carbon dioxide

COX – cyclooxygenase

CRC – colorectal cancer

CRLM – colorectal cancer liver metastasis

CRP – C reactive protein

Csk – C-terminal Src kinase

CT – computed tomography

CTL – cytotoxic T lymphocyte

CTLA-4 – cytotoxic T lymphocyte antigen-4

DC – dendritic cell

DDD – defined daily dose

DNA – deoxyribonucleic acid

EDTA - ethylenediaminetetraacetic acid

EGFR – epidermal growth factor receptor

EP – E Prostanoid receptor

ERK – extracellular signal-regulated kinases

FACS - fluorescent activated cell sorting

FAP – familial adenomatous polyposis

FasL – Fas ligand

Fcm - flow cytometry

FCS – fetal calf serum

FIGO – International Federation of Gynecology and Obstetrics

FITC - fluorescein isothiocyanate

FKH – forkhead

FoxP3 – forkhead box protein 3

GITR – glucocorticoid-induced TNF receptor

GM-CSF – granulocyte-macrophage colony-stimulating factor 3

GPCR – G protein coupled receptor

Her-2 – human epidermal growth factor receptor 2

HE4 – human epididymis 4

HLA – human leukocyte antigen

HNPCC – hereditary non-polyposis colon cancer

HR – hazard ratio

ICD-O-03 - International Classification of Diseases for Oncology, 3rd edition

ICD-10 – International Classification of Diseases, 10th edition

IDO - indoleamine 2,3-dioxygenase

IFN – interferon

IL – interleukin

IL-8 – neutrophil chemotactic factor

IL-10 - human cytokine synthesis inhibitory factor

IPEX - immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

ITAM – immunoreceptor tyrosine-based activation motifs

JAK – janus kinase

K-ras – Kirsten rat sarcoma viral oncogene homolog

LAG-3 – lymphocyte activation gene 3

LC – liquid chromatography

Lck - lymphocyte-specific protein tyrosine kinase

LPS - lipopolysaccharide

MAPK – mitogen-activated protein kinase

MHC – major histocompatibility complex

miRNA – micro ribo nucleic acid

MMR – mismatch repair gene

MRI – magnetic resonance imaging

mRNA – messenger ribo nucleic acid

MS – mass spectrometry

MSI – microsatellite instability

NFAT – nuclear factor of activated cells

NFκB – nuclear factor kappa B

NK – natural killer

NorPD – Norwegian Prescription Database

NY-ESO-1 - New York esophageal squamous cell carcinoma 1

OC – ovarian cancer

PAMP – pathogen-associated molecular patterns

PBMC – peripheral blood mononuclear cells

PBS - phosphate-buffered saline

PD-1 – programmed death 1

PE - phycoerythrin

PerCP - peridinin chlorophyll protein

PET – positron emission tomography

PFA – paraformaldehyde

PG – prostaglandin

PI - phosphoinositide

PI3K – phosphoinositide 3-kinase

PLD – pegylated liposomal doxorubicin

PMA – phorbol myristate acetate

PTEN - phosphatase and tensin homolog

PTK – protein tyrosine kinase

p53 – protein 53

RAS – rat sarcoma

RCMRE - Regional Committee for Medical Research Ethics

RAR – retinoid acid receptor

RFA – radiofrequency ablation

RCT – randomized controlled trial

ROME – risk of ovarian malignancy algorithm

RORC - RAR-related orphan receptor C

Src – Proto-oncogene tyrosine-protein kinase sarcoma

STAT - Signal Transducer and Activator of Transcription

TAA – tumor associated antigen

TAMs – tumor associated macrophages

TCR – T cell receptor

TGF – tumor growth factor

Th – T helper

TNF – tumor necrosis factor

TNM – tumor, node, metastasis

Tp53 (gene) – tumor protein 53

Treg – regulatory T cell

TXA2 – thromboxane A2VEGF – vascular endothelial growth factor

WHO – world health organization

Wnt – wingless intergration 1

XIAP – X-linked inhibitor of apoptosis protein

Zap70 – ζ-chain-associated protein kinase

2 List of Publications Included

I. Solstad T, *Bains SJ*, Landskron J, Aandahl EM, Thiede B, Taskén K, Torgersen KM.(2011): *CD147 (Basigin/Emmprin) identifies FoxP3$^+$CD45RO$^+$CTLA4$^+$-activated human regulatory T cells.*
Blood, Nov 2011 10;118 (19): 5141-51

II. *Bains SJ*, Yaqub S, Landskron J, Bjørge L, Rokkones E, Taskén K*: Characterization of immunosuppressive properties of malignant ascites in ovarian carcinoma.*
Manuscript.

III. *Bains SJ*, Mahic M, Myklebust TÅ, Cvancarova MS, Yaqub S, Dørum LM, Bjørnbeth BA, Møller B, Brudvik KW, Taskén K**:** *Impact of Aspirin as Secondary Prevention in an Unselected Cohort of 25,644 Patients with Colorectal Cancer – A Population-Based Study.*
Submitted

IV. Brudvik KW, *Bains SJ*, Seeberg LT, Labori KJ, Waage A, Taskén K, Aandahl EM, Bjørnbeth BA. (2013): *Aggressive treatment of patients with metastatic colorectal cancer increases survival: a scandinavian single-center experience.*
HPB Surg, June 2013;2013:727095

3   Introduction

Cancer is a leading cause of deaths world-wide, and current research is on-going to develop new means of early diagnosis and treatment. Early detection of disease is an important means to prolong survival, and several screening programs have been developed with this purpose. Traditionally, cancer management has been centered around surgery, radiotherapy and chemotherapy, alone or in combination. In recent years, the potential of using the immune system to combat tumor development has been launched as a 4th pillar in treatment of cancer patients, and cancer immune therapies are now focus of increasing attention.

Cancer immunology is a growing field that explains some of the complex cellular and molecular interplay between tumors and host immunity. The basis for tumor immunology is the biological pattern of tumor cells and a functional immune system. The term immunogenicity encompasses tumor antigen recognition and actions of cytotoxic killer cells. Immunotherapy focuses on essential immunogenic elements to evoke tumor-specific humoral and cell-mediated immune response [1], and how to circumvent tumor-induced immune suppressive mechanisms.

In contrast to the anti-tumor immune response, tumors employ several mechanisms to escape attack by the immune system, including recruitment of regulatory T cells (Tregs) to foster tolerance. In this Thesis we have focused on several aspects of anti-tumor immunity and tumor development. Firstly, we have conducted a study to distinguish the characteristics and function of Tregs in healthy humans. Regulatory T cells are an important mediator in tumor immune evasion, but due to the lack of a distinct surface-marker, they have proven difficult to target for immune-regulatory purposes. Furthermore, we have looked into the interplay between immune cells and tumor microenvironment in a disease model, specifically in human ovarian cancer patients. Ovarian cancer (OC) was chosen as a model due to presence of malignant ascites in advanced stages. Malignant ascites fluid from OC patients contains cytokines, extracellular matrix components and free-floating immune and tumor cells, and represents the tumor microenvironment in a soluble fashion, making it an easily accessible model to study anti-tumor immunity. A parallel study was conducted in our lab where the cellular compartment of the malignant ascites was assessed, specifically with regards to Tregs and tumor-infiltrating and tumor associated lymphocytes [2]. My main focus has been

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characterization of the cell-free ascites, in order to unravel the effect of soluble immune mediators in a malignant environment.

Furthermore, we conducted two observational studies in colorectal patients. One of the studies looked into the use of aspirin as a potential treatment/secondary preventive remedy in colorectal cancer patients. Aspirin influences both the tumor directly and also the immune cells, through inhibition of cyclooxygenase (COX) isoenzymes. COX-2 facilitates the accumulation of prostaglandin E2 (PGE_2), a known facilitator in tumor development and tumor immune evasion. Lastly, we carried out a cohort study regarding novel treatment options for colorectal cancer patients with liver metastases.

3.1 The immune system

The immune system is the human body's defense mechanism against invading pathogens (virus, bacteria and fungi). Other functions include monitoring tissue homeostasis and to preserve the host tissue intact. An immune reaction is the result of interplay between numerous defense mechanisms that work in concert and augment individual responses. A number of innate properties (specificity, diversity, discrimination, memory and self-limitation) are of vital importance for the normal function of the immune systems, and all its components are meticulously controlled to secure an equilibrium[3]. However, autoimmune diseases develop when there is immunological overshoot leading to self-attack. Regulatory immune mechanisms are thus needed to ensure that appropriate responses are elicited when the host is under attack, and to separate self from non-self.

3.1.1 Innate and adaptive immunity

When faced with an invading pathogen or tissue damage, the immune system reacts promptly. Our innate immune system is the first line of defense, after the outer barrier (skin and mucosa) and attacks invading pathogens based on pathogen-associated molecular pattern (PAMP) recognition, in an unspecific and "simple" mode. Central effectors include monocytes/macrophages, dendritic cells (DCs), granulocytes, natural killer (NK) cells, mast cells and soluble parts like complement factors, cytokines and acute phase proteins [4].

Our innate immunity collaborates with the adaptive immune system in a coordinated manner [5], as the adaptive system mounts a later, more specific response. The adaptive immunity also confers an immunological memory, that enables a much more proficient reaction upon a re-infection with the same pathogen [6].

The main effectors of the adaptive immune system are the B- and T cells. Naïve B cells develop in the bone marrow, further advance into isotype-switched memory B cells, and may differentiate into immunoglobulin producing plasma cells and produce antibodies that target specific antigens. T cells develop in the bone marrow and mature in the thymus, and are responsible for cell-mediated immune responses [5]. T cell immunological memory is related to an increase in the antigen specific cell population with the presence of surface molecules with homing properties, thus ensuring a faster and more extensive secondary immune response.

A prerequisite for an adaptive immune response is presentation of foreign antigens by innate immune cells, such as macrophages and dendritic cells. These antigen presenting cells (APCs) bind antigens on their surface receptor, next they engulf and process the antigens, before presenting them on major histocompatibility complex (MHC) molecules to T cell receptors in germinal centers of lymph nodes. MHC molecules are found in all vertebrates, originally identified as an antigen system of the leukocytes, therefore called Human Leukocyte Antigen (HLA) in humans. The purpose of MHC antigens is to serve as an identity marker on the surface of cells and present foreign antigen peptides (MHC-antigen complex) to the T cell receptor (TCR). MHC-class I molecules are expressed on the surface of all nucleated cells and present antigens to $CD8^+$ cytotoxic T cells. MHC-class II molecules on the other hand, are exclusively expressed on the surface of APC's, and thus present antigens to $CD4^+$ T helper cells [4].
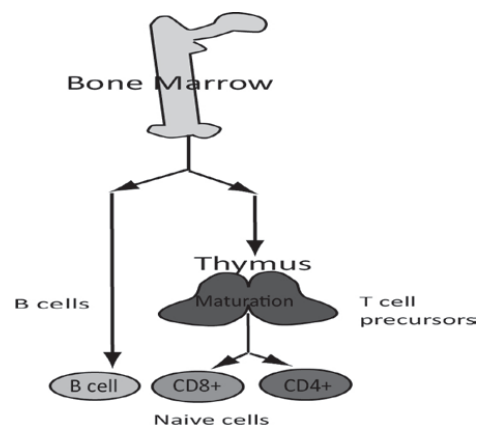
FIGURE 1: B and T cells develop in the bone marrow and thymus

3.1.2   T cell-mediated immunity

Immunity involves a panoply of different cell types (as mentioned above), but this Thesis focuses mainly on $CD3^+$ T lymphocytes which serve to orchestrate most immune responses. Depending on the cytokine environment in the lymph node and the specific APC, the T cells develop into cytotoxic $CD8^+$ T cells that combat foreign pathogens by killing them directly, or $CD4^+$ T helper cells which achieve the same through secretion of chemokines and cytokines to recruit other effector immune cells.

The $CD4^+$ T helper (Th) cells are further divided into subpopulations, including the traditional Th1 and Th2 cells, and the more recently discovered Th17, Th9, follicular T helper cells (Tfh), Th3, Tr1 and regulatory T cells (Tregs).

Th1 cells secrete IFN-γ, IL-2 and lymphotoxin (LT) to stimulate macrophages and cytotoxic T cells, and trigger subsequent killing of intracellular pathogens or viruses. Th2 cells produce IL-4, IL-5 and IL-13, and are important in the activation of B cells and antibody production, providing the host with an extracellular immunity.

Recently, additional T helper subsets have been identified: Th17 cells are IL-17 producing $CD4^+$ Th cells, and differentiate under the influence of IL-1 and IL-23 [7]. Besides IL-17, these cells also produce IL-21 and IL-22, protecting surfaces against extracellular bacteria [8]. In 2009 the idea of a Th 9 cells was launched. Veldhoen et al. proposed that the new addition to the Th cell-family was in fact "reprogrammed" Th2 cells that lose their characteristic profile under TGF-β influence, switching to IL-9 production [9]. Follicular T helper (Tfh) cells are an antigen experienced $CD4^+$ T cells found in abundance in the B follicles of secondary lymphoid organs, such as spleen and lymph nodes. The Tfh cells mediate transition of B cells to antibody-producing plasma cells. Th3- and Tr1 cells are subsets of regulatory T cells (Tregs) that can suppress immune responses.

3.1.3   T cell activation

The T cell receptor (TCR) complex consists of two functional parts – the heterodimeric glycoprotein of one α- and β-chain, and the CD3 and ζ-chain homodimer. The TCR is subject to random gene rearrangement in individual developing T cells, thus providing the huge diversity in T cell repertoire. T cells require two signals to reach a state of full activation. Initially, the TCR needs to engage a MHC/peptide complex as presented by antigen presenting cells (APC), to determine the antigen (Ag) specificity of the response. Following this first step of activation, the T cells require an additional co-stimulatory signal, to avoid

anergy or death by apoptosis. Co-stimulation materializes through assignation of T cell surface receptors with their associated ligands on APCs. Among the positive co-stimulatory molecules, is CD28 that is constitutively expressed on T cells, and that binds to either B7-1 (CD80) or B7-2 (CD86) on APCs. Succeeding co-stimulation, T cells achieve complete activation, which encompasses cytokine production, clonal expansion and T cell survival [10]. On the other hand, interaction of cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed on T cells with APC-ligands B7-1 or B7-2, leads to a negative co-stimulatory signal, thus preserving tolerance and preventing immunological overshoot.
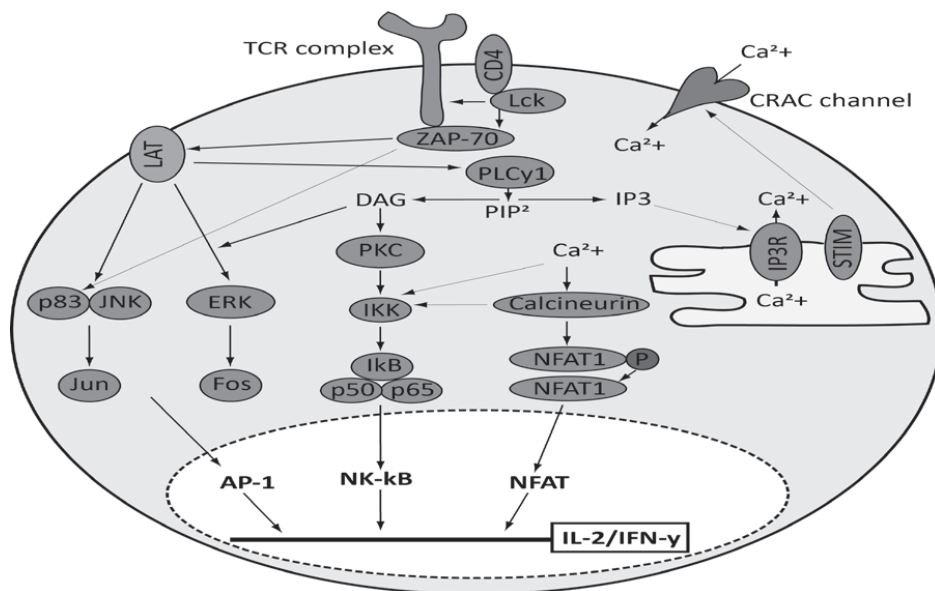
FIGURE 2: TCR signaling involves several intracellular signaling pathways that activate transcription factors AP-1, NFkB and NFAT (Adapted from Schmidt A, Frontiers of Immunology, 2012).

Inside the T cell, the cytoplasmic domains of CD3 proteins contain structures called immuno-receptor tyrosine-based activation motifs (ITAMs). The TCR-complex is additionally associated with either CD4 or CD8 molecules (depending on the T cell subtype). These molecules have a cytoplasmic domain which upon activation will associate with cytoplasmic protein tyrosine kinases (PTKs) of the Src-family, such as Fyn and Lck, which further phosphorylates tyrosine residues on the ITAMs. This kick-starts the signaling cascade, and leads to the binding of signaling molecules to the phosphorylated ITAM domains. The next step is facilitated by the binding of ζ–chain-associated protein kinase 70 (Zap70) to the phosphorylated ITAM tyrosine domains, that further activates three important downstream

intracellular signaling pathways. The end-product of this process is the induction of transcription factors such as NFAT (nuclear factor of activated cells), NFkB and AP-1. The combined action of these three transcription factor turn on the expression of genes required for T cell proliferation and differentiation, such as IL-2 [11].

3.1.4 Regulatory T cells

The functional definition of a regulatory T cell (Treg) is a T cell that inhibits immunity by manipulating the activity of another cell type [1]. Regulatory T cells play a central role in modulation of immune reactions to self-antigens, allergens and transplants, as well as immune responses to tumors and infectious microbes [12;13]. Both humans and mice deficient in or with dysfunctional Tregs, develop severe allergy, autoimmune and immune pathological diseases [14]. Regulatory T cells are also crucial in the maintenance of allograft tolerance and fetal-maternal tolerance during pregnancy [15]. On the other hand, Tregs can also suppress antitumor immune responses and favor tumor progression.

Regulatory T cells express the transcription factor forkhead box P3 (FoxP3) and are naturally present in the immune system. Most of FoxP3$^+$ Treg cells are CD4$^+$ T cells that express CD25 (the IL-2 receptor a-chain), and can suppress the activation, proliferation and effector functions of a wide range of immune cells, such as CD4$^+$ and CD8$^+$ T cells, NK cells, B cells and antigen presenting cells.

However, recent findings propose that Tregs are functionally and phenotypically diverse, with assorted suppressive mechanisms, identity and stability [16]. Many cell subsets with regulatory activity have been described, including TGF-β producing Th3, IL-10 producing Tr1, CD4$^-$CD8$^-$ T cells and CD8$^+$CD28$^-$ T cells and NKT cells [17;18]. These cells are peripherally induced Tregs (adaptive, aTregs), derived from naïve CD4$^+$ T cells in the periphery [19-21], which means that they acquire regulatory functions following specific antigen stimulation in particular cytokine environments. The aTregs develop during chronic antigen stimulation, and some but not all express Foxp3 [22]. PGE$_2$ exposure can induce Tregs by up-regulating FoxP3$^+$ expression in CD4$^+$ CD25$^+$ T cells and consequently enhancing their suppressive capacity [23]. In addition, there are the TGF-β producing Th3 cells that are crucial in inducing and maintaining peripheral tolerance by driving the differentiation of Ag-specific Foxp3$^+$ Tregs in the periphery [17]. Tr1 is a CD4$^+$ T regulatory type 1 cell that down-modulates immune responses through the production of the immunosuppressive cytokines IL-10 and TGF-β [18]. Through IL-10 and TGF-β secretion Tr1 also facilitate CD4$^+$CD25$^-$ T cell

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conversion into CD4^{+}CD25^{+}FoxP3^{+} Tregs, while they require IL-2 for their peripheral maintenance [24].

This is in contrast to the naturally occurring CD4^{+}CD25^{+}Foxp3^{+} regulatory T cell (nTreg), that our studies have focused on. These nTregs comprise a distinct T cell subpopulation that is developmentally determined in the thymus and is specialized for suppressive functions, rendering it critical in the maintenance of immunological self-tolerance and immune homeostasis [25]. These nTregs account for up to 5-10% of peripheral CD4^{+} T cells [21], and are crucial to the fine balance between sustaining peripheral tolerance by suppressing potential autoimmune responses, while also controlling satisfactory responses to infections [20].
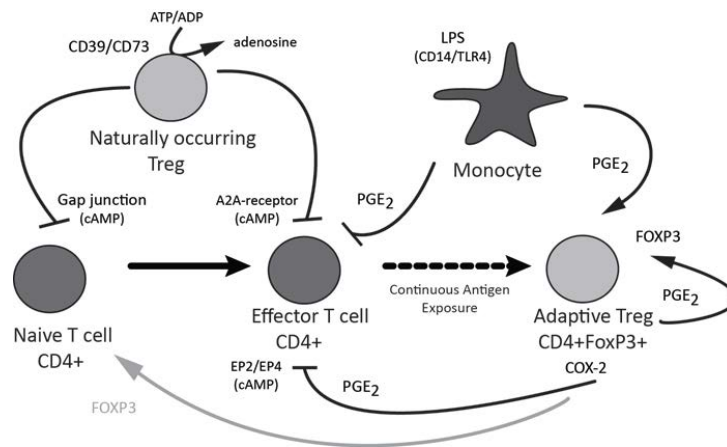
FIGURE 3: Naturally occurring regulatory T cells suppress naïve and effector T lymphocytes. CD4^{+} T lymphocytes develop into adaptive Tregs during chronic antigen stimulation (adapted from Yaqub S, 2008).

In **Paper I** of this Thesis we studied nTregs in peripheral blood from healthy donors to identify novel surface molecules to identify functional subsets.

3.1.4.1 Treg characterization

Regulatory T cells are CD4^{+} T cells characterized by their expression of the trans-membrane IL-2 receptor α chain (CD25) and the transcription factor forkhead/winged-helix family transcriptional repressor/activator p3 (Foxp3). Foxp3 controls the expression of many genes, and its forkhead (FKH) domain is critical for DNA binding and nuclear localization [26;27]. FoxP3 is a transcriptional repressor for promoters of genes for the key cytokines IL-2 and granulocyte-macrophage colony-stimulating factor 3 (GM-CSF). Furthermore, FoxP3 interacts with transcription factors that take important part in the expression of many cytokine genes, including nuclear factor of activated T cells (NFAT). Furthermore, ectopic FoxP3

expression induces suppressive activity in conventional T cells. And it is known that stop or frameshift mutations in the FoxP3 gene lead to Treg deficiency and a severe multi-organ autoimmune and inflammatory disorder, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) [28]. These findings suggest that FoxP3 is a master regulator of Treg cell differentiation and function. CD127 is another Treg "defining" molecule. Being the α-chain of the interleukin-7 receptor, CD127 expression correlates inversely with the FoxP3 expression and suppressive function of Tregs [24]. However, conventional CD4^{+} T cells are known to down-regulate CD127 expression after activation, making CD127 negative status less useful to discriminate Tregs from activated T cells.

In addition, several other molecules are expressed by Tregs, including the glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), CD28/CTLA-4, CD95 (Fas), chemokine receptors, Toll-like receptors (TLRs), membrane bound TGF-β, CD45RO/CD45RA and other molecules, such as neuropilin-1, lymphocyte activation gene-3 (LAG-3) and granzyme [19]. None of these molecules are however uniquely expressed by Tregs alone, and there is still a race to find the best marker to characterize Tregs and differentiate them from activated T cells.

The surface marker CD25 is an activation marker, and is expressed by all activated lymphocytes. Peripheral human blood contains up to 30% CD4^{+}CD25^{+}T cells, but only 1-2% of cells containing the highest CD25 expression have shown to be functionally suppressive. The transcription factor Foxp3 remains the most specific functional and phenotypic marker for Tregs, but being located in the nucleus it is difficult to access when trying to isolate or target viable Tregs for interventional purposes. Furthermore, Foxp3 is known to be induced in naïve CD4^{+}FoxP3^{+} T cells upon stimulation, without conferring suppressive activity [29]. These finding indicate that not all FoxP3^{+} T cells are inhibitory.

Due to the heterogeneous nature of the function and phenotype of FoxP3^{+} T cells, Tregs have been divided into suppressive and non-suppressive subsets based on CD45RA and CD45RO expression. Naïve/resting Tregs that express CD25^{+}CD45RA^{+}FoxP3^{+} (rTregs) are in a quiescent state and have not experienced TCR stimulation-mediated maturation. These recent thymic emigrants are highly resistant to apoptosis, and they will proliferate, upregulate Foxp3 expression and convert to CD45RA^{-}FoxP3^{++} Tregs (aTregs) upon activation. The effector aTregs are an activated and functionally differentiated subset, known as CD45RA^{-}FoxP3^{++}. These effector aTregs are mainly derived from naïve rTregs and have potent suppressive capability. Following activation and suppression, these aTregs are highly susceptible to

apoptosis. The effector Tregs can be further subdivided based on their expression of ICOS and HLA-DR. While CD45RA$^-$CD25$^+$FoxP3^{++} ICOS$^+$ effector Tregs produce the suppressive cytokine IL-10, the ICOS$^-$ cells actively secretes TGF-β. Furthermore, expression of HLA-DR identifies an effector Treg subset that has a more profound ability to suppress T cell proliferation and to secrete cytokines when compared to HLA-DR$^-$effector Tregs. HLA-DR$^+$ Tregs are believed to represent a terminally differentiated subset in the effector Treg pool. Lastly, there is the non-Treg, CD45RA$^-$FoxP3$^+$ population that is non-suppressive and cytokine-secreting [30]. The difference between non-regulatory and functional FoxP3 Tregs may be linked to the methylation status of the Foxp3 gene, which is incompletely demethylated in CD45RA$^-$FoxP3$^+$ non-regulatory T cells but is completely demethylated in FoxP3$^+$ Tregs with suppressive activity. This is partly helpful when assessing Treg function and when isolating cells.

TABLE 1: Regulatory T cell subsets

CD 4+ Treg subset	Origin	Phenotype	Function	Methylation status of FoxP3 gene
Naïve/resting Treg (*rTreg*)	Thymus	CD45RA+FoxP3+	Suppressive	Complete demethylation
Activated Treg (*aTreg*)	Periphery	CD45RA-FOXP3++	Highly suppressive	Complete demethylation
FoxP3+ non-regulatory T cell	?	CD45RA-FoxP3+	Non-suppressive,cytokine-secreting	Incomplete demethylation

Previous attempts to find a unique Treg maker have been to no avail. Earlier studies have looked at the differences of DNA- and mRNA expression between CD4$^+$CD25$^+$Foxp3$^+$ and CD4$^+$CD25$^-$Foxp3$^-$ T cells [31]. However, many proteins undergo posttranslational modifications and not all mRNA is in fact further processed to proteins.

No one has yet found a specific functional and phenotypic marker that truly sets the Treg population apart from the other CD4$^+$ T cells in the human immune system, and Foxp3 remains the most valid marker for Tregs. A possible way to discover a specific Treg biomarker might be with a proteomic approach, trying to find differences in molecules expressed on a protein level. That way one can target proteins specifically, in the nucleus, cytosol and membrane fraction, and this strategy was pursued in **Paper I** of this Thesis.

3.1.4.2 Suppressive mechanisms

Regulatory T cell suppressive activity requires prior activation of the Tregs through their αβ T cell receptor (TCR), but once activated they suppress in an antigen-nonspecific manner [22].

The suppressive capacity of Tregs was for a long time considered to be dependent on a cell-to-cell contact mechanism, leaving the concept of a soluble factor mediating Treg suppression controversial. However, although the precise molecular mechanisms of

suppression by human Tregs remains somewhat intangible, several studies report that Tregs employ several mechanisms to suppress and regulate an immune response, either by directly targeting T cells or antigen presenting cells. These mechanisms include inhibitory cytokines, cytolysis, metabolic disruption of target cells, and modulation of antigen presenting cell (APC) function (through decreased co-stimulation or decreased antigen presentation) [32;33]. Numerous in vivo studies describe the importance of Treg-derived IL-10, PGE_2, TGF-β, LAP, galectin-1 and IL-35 in the suppression of various immune responses [20;22;32;34]. It has also been proposed that Tregs can directly suppress responder T cells through granzyme-A-mediated cytolysis in a perforin-dependent, Fas-FasL-independent manner [35]. Moreover, Tregs express CTLA-4 that binds tightly to the B7 molecules on APCs. This binding decreases the APC co-stimulation and antigen presentation, thereby limiting the activation of effector T cells (Th1, Th2, and CTL) that is needed to mount an efficient immune response. Regulatory T cells can also suppress immunity by FoxP3 directly repressing the IL-2 gene transcription and consequently down-regulating T cell activation [29], or through IL-2 consumption and cytokine deprivation. $CD8^+$ Tregs suppress antigen-specific $CD4^+$ T cells through a MHC I-independent way, through direct contact between Tregs and effector T cells.
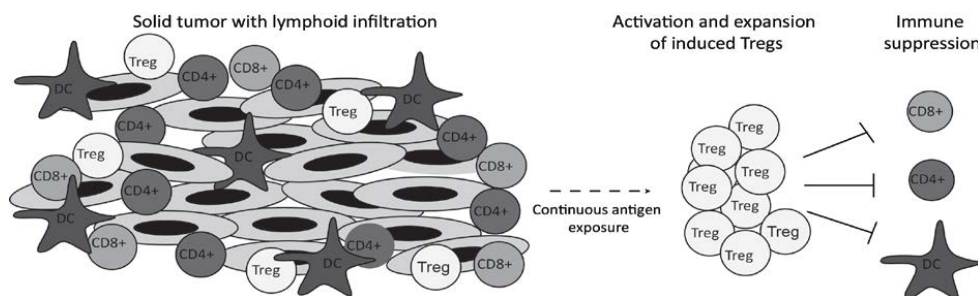
FIGURE 4: Regulatory T cells are induced in the tumor microenvironment and suppress anti-tumor immune responses.

3.1.4.3 *Tregs in cancer*

There is growing interest in and intense research on the role of Treg-based therapy. With regards to treatment of autoimmune disease and graft-versus-host disease, the transfer of expanded and pure Treg populations is under investigation. However, our main focus has been on Tregs in tumor immunology. Tumors can recruit Tregs to the tumor site, in addition to converting naïve and effector T cells into Tregs. Accumulating evidence indicates that Tregs are enriched in the blood, malignant effusions, draining lymph nodes and tumor tissues of various malignant diseases [36-38]. Many studies have been conducted on Tregs and various

types of solid tumors. The presence of Tregs in colorectal cancer patients was increased in the vicinity of tumor invaded tissue, as compared to normal tissue [39]. It has also been shown that the prevalence of CD4$^+$CD25^{++} T cells inside the prostate was significantly higher in cancer tissue compared with benign tissue [40]. Furthermore, studies have shown a strong correlation between the presence and amount of Tregs in gastric, hepatocellular, breast, colorectal cancer and melanomas, and poor clinical outcome and prognosis [38;41]. Specifically, decreased ratios of CD8$^+$ T cells to FoxP3$^+$CD25$^+$CD4$^+$ Treg cells in tumors correlate with poor prognosis.

The presence of elevated Treg numbers in cancer patients can prevent protective anti-tumor immunity, and recent clinical trials propose that reducing Tregs can be clinically beneficial [37]. With the aim to target Tregs for immune potentiation purposes, efforts have been made to find cell surface molecules that are predominately expressed by Tregs or can specifically modulate Treg function. In mice there has been some success in augmenting tumor-specific immunity with the use of monoclonal antibodies targeting CD25 (a depleting antibody), CTLA-4 (a blocking antibody) and GITR (an agonistic antibody). In addition, small molecules such as cyclophosphamide can deplete Tregs and thus enhance anti-tumor immunity [42]. CTLA-4-specific blocking antibodies are already in clinical use in treatment of advanced malignancies, such as malignant melanoma and non-small cell lung cancer [43-46]. Since many Treg-specific cell surface markers are also expressed by activated effector T cells, there is an urgent need to find a specific Treg marker that sets the population apart from effector T cells. Another means of overcoming Treg induced anti-tumor immunity, is possibly through a combination of monoclonal antibodies that target different molecules to more efficiently control the balance between Tregs and effector T cells towards dominance of effector T cell immunity.

3.2   Cancer immuno-editing

The immune system has the potential to recognize and eliminate primary developing tumors, but it also has the capacity to promote tumor growth [3]. The intricate relationship between developing tumors and the immune system was first described by Ehrlich in 1909 [47], and later modified by Burnet and Thomas in 1957 [48].

Cancer immuno-editing is the process describing the immune system's dual effect on developing tumors, and comprises elimination, equilibrium and escape [49]. On the one hand, the abnormality of a malignant tumor should target it for immune destruction and elimination of the fast-growing cells. However, a tumor cell is also *self*. Protective mechanisms against

autoimmunity could therefore impede antitumor immunity and immune surveillance, allowing tumors to grow limitlessly. Sometimes the growth-and-attack interactions between enlarging tumors and the immune system cause no one to prevail, creating a state of equilibrium, or tolerance. Both the innate and adaptive immune system is implicated in antitumor responses, and in the dynamic interaction with the cancer leading to selection of escaping cells, termed immuno-editing [50].
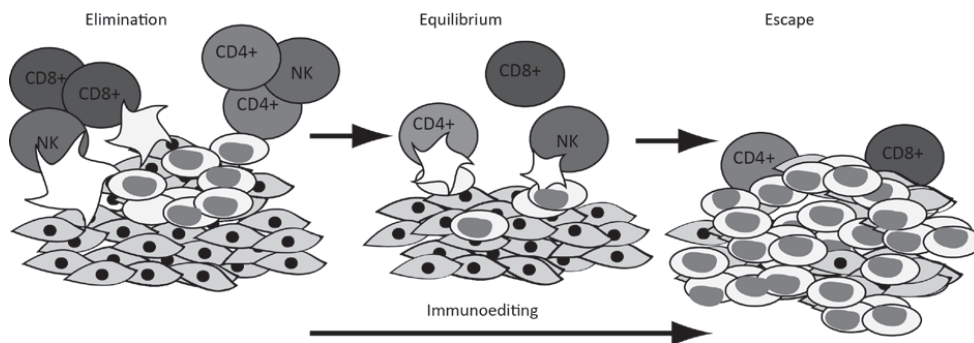
FIGURE 5: Cancer immuno-editing describes the intricate relationship between a developing tumor and the immune system, and comprises elimination, equilibrium and escape. This dynamic process gives rise to a selection pressure that leads to a survival benefit for clones with immune escaping properties.

3.2.1   Tumor development

Cancer develops as a result of an accumulating amount of altered genes that are involved in tissue homeostasis, cell survival and cell death. Mutated genes that facilitate tumor development can be classified into three types: oncogenes, tumor suppressor genes and DNA repair genes [51]. Oncogenes stimulate cell growth under normal conditions, but mutations and "overexpression" in oncogenes cause continued cell growth in the absence of growth signals. Tumor suppressor genes act oppositely, and inhibit cell growth indirectly through promotion of programmed cell death (apoptosis), or directly by impeding the cells' progression through the cell cycle. When tumor suppressor gene expression is lost as a result of mutations or allelic loss, the cells lose their normal inhibitory growth control. DNA repair genes control the rate of genetic mutation, and their mutation leads to a lack of repair, which consequently induces an accelerated rate of accumulated mutations in oncogenes (activation) and tumor suppressor genes (inactivation).

However, a tumor doesn't merely consist of mutated cells, but constitutes a variety of components including fibroblasts, endothelial cells, extracellular matrix, cytokines, tumor infiltrating immune cells and tumor cells [52]. Together these constituents make up the tumor

microenvironment, and are all vital in the development, progression and treatment of cancer. Active interaction between all these components determines the phenotypic pattern of the tumor, its neovascularization and its ability to metastasize and invade surrounding tissues. The microenvironment is accordingly the focal combat zone during the neoplastic process [1]. Cytokines and chemokines produced by the cells of the microenvironment are key mediators in this complicated interplay that is so crucial for tumor progression.

3.2.2 Immune escape mechanisms

Elimination and equilibrium of tumors is mediated by lymphocytes, and mainly the T cell subset. In several types of cancer, a high number of tumor-infiltrating CD8$^+$ T cells is associated with improved clinical prognosis [36], as a full activation of adaptive immune cells in response to a tumor can eradicate malignant cells . In colorectal cancer the presence of T cells were more accurate in predicting patient outcome, than established prognostic factors [53]. On the other hand, the abundant presence of innate immune cells such as macrophages and neutrophils correlates inversely with outcome, as their presence represents a chronic inflammatory environment.

Unfortunately, the presence of tumor-specific CD8$^+$ T cells rarely limits the tumor growth [1], as the tumors employ efficient means to avoid host immune attacks. One immune escape mechanism is through evasion of immune recognition, which can be achieved by selection of non-immunogenic tumor cell variants to avoid tumor antigen recognition. This is accomplished through several mechanisms, including down-regulation or loss of expression of major histocompatibility complex (MCH) class I molecules, and altered expression and functional activity of adhesive molecules and transport proteins [54]. Furthermore, the expression of non-classical HLA-molecules (HLA-G and HLA-E) belonging to MHC class Ib inhibits NK cell-mediated cytotoxicity. In addition, defects in the maturation process of the antigen presenting dendritic cells can lead to tumor escape.

The adaptive and innate immune cells create a selection pressure leading to the tumor cells surface changes, playing a significant role in "sculpting" the tumors immunogenicity. These changes also influence the sensitivity of tumor cells to the action of killer cells, making tumor cells harder to lysate and kill due to a change in surface ligands.

Another immune escape method is by active suppression of the immune response. Persistent antigen stimulation is often evoked when tumors are unsuccessfully cleared by the immune system. When T cells are constantly exposed to antigens and hence activated, they over-

express negative co-receptors, such as CTLA-4, Programmed Death I (PD1), FasL (CD95L) and B7-H4, leading to a down-regulated immune response [55-58].

Another efficient tumor immune evasion mechanism is through the production of indoleamine 2,3 dioxygenase (IDO) by DC's in tumors and tumor draining lymph nodes. IDO catalyzes the breakdown of the amino acid tryptophan into toxic metabolites, leading to T cell apoptosis and impaired T cell function [59].

Chronic activation in the tumor vicinity also induces aTregs, while nTregs can traffic to cancer tissue, thus repressing anti-tumor immune responses. Myeloid suppressor cells are a subset of innate immune cells that accumulate in tumors and lymphoid organs, and can cause T cell dysfunction through direct cell-cell contact and production of immunosuppressant mediators [60]. In addition, tumor cells themselves can suppress immunity directly through production of immunosuppressive molecules such as TGF-β, soluble Fas ligand, interleukin 10 (IL-10), vascular endothelial growth factor (VEGF) and prostaglandin E 2 (PGE_2) [5;61-63].

The combination of the aforementioned immune suppressive mechanisms that are employed by growing tumors, all contribute to tumor immune evasion, allowing malignant cells to grow limitlessly. This growing understanding of the complex interaction between developing tumors and the immune system is making way for novel therapies utilizing the potential of the body's own defense mechanisms.

3.3   Ovarian cancer

The female ovaries are glands that contain germ cells (eggs), and ovarian tumors represent a range of distinct diseases that share this common anatomical location [64]. Epithelial ovarian cancer (EOC) develops when a normal cell in the ovary transforms and grows uncontrollably. Approximately 85-90% of ovarian tumors originate from epithelial tissue covering the surface of ovaries, from the fallopian tube or the peritoneum (primary peritoneal carcinoma). The remaining 10-15% of ovarian tumors develops from germ cells, the egg-producing cells of the ovary, or stromal cells, the connective tissue cells that holds the ovary together and produces sex hormones. Epithelial ovarian cancer is acknowledged as a highly heterogeneous disease, and can be further subdivided into 4 main histological groups: Serous, mucinous, endometroid and clear cell tumors [65]. EOC can also be separated into two broad categories; type I and type II. Type I tumors constitute 10-20% of all EOC and include low-grade serous, low-grade endometroid, clear cell and mucinous carcinomas. Type I tumors behave in an indolent way, by slow progression and restricted growth to one ovary. They are relatively

resistant to platinum, often constitute solid tumors and harbor p53 wild-type. Type II tumors on the other hand, represent 80-90% of all EOC and include high-grade serous, high-grade endometroid, mixed malignant mesodermal tumors (carcinosarcomas) and undifferentiated carcinomas [66]. They grow more aggressively, respond to platinum, often harbor p53 and BRCA mutations, and are more genomic unstable than their counterparts.
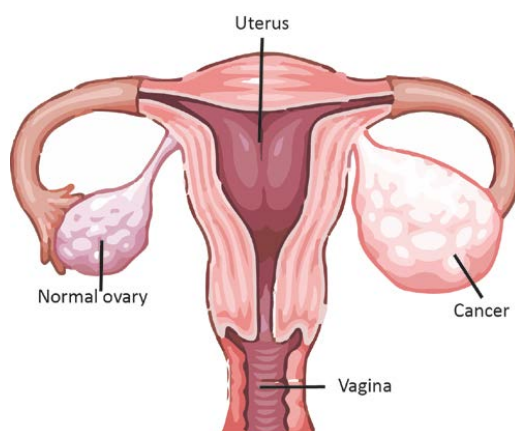
FIGURE 6: Ovarian cancer develops in the female ovarian glands.

EOC is associated with high morbidity and mortality, and is considered among the most fatal malignancies in females [67]. It is an exceedingly metastatic disease distinguished by widespread peritoneal dissemination and ascites. EOC will be the main focus in the ensuing sections.

3.3.1 Epidemiology

Every year, 250.000 women world-wide are diagnosed with ovarian cancer, making it the 6th most common malignancy and the 5th leading cause of malignancy related deaths among females globally. The estimated lifetime risk for a woman developing ovarian cancer is 1 in 54, and this number is reported to be stable [68;69]. Norway has a reported occurrence of approximately 450 new cases annually, with roughly 300 patients dying of the disease every year [70]. The greatest incidence of EOC cases is found in postmenopausal women, in the age group between 40 and 65, with a mean age of 59 years [69;71]. However, the mean age of EOC diagnosis is younger in women with hereditary ovarian cancer syndrome that have mutations in either the breast cancer type 1 (BRCA-1) or breast cancer type 2 (BRCA-2) gene, which affects up to 6% of Norwegian EOC cases. There is a steady increase of new EOC cases until the age of 80 years, when the rate flattens or drops [72].

The five year survival rate is generally poor, ranging from 30 to 92 %, depending on the spread of the disease at the time of diagnosis (Table 2). Common risk factors include early menarche and late menopause, age, obesity, nulligravidity, infertility, endometriosis, polycystic ovarian syndrome and family history [73].

TABLE 2. Ovarian Carcinoma staging according to the International Federation of Gynecology and Obstetrics (FIGO).

Stage	Description	Percentage	Survival
I	Confined to ovaries	24 %	> 90%
II	Confined to pelvis	6 %	70-80%
III	Confined to abdomen and abdominal lymph nodes (intraperitoneal disease)	55 %	20-30%
IV	Distant metastases beyond the peritoneal cavity (liver, lungs)	15 %	< 5%

3.3.2 Ovarian cancer and immunity

Accumulating clinical evidence supports the importance of immuno-editing mechanisms in EOC, both with regards to initiation and progression of the disease.

EOC initiation is believed to be linked to chronic/subclinical inflammation in the reproductive tract, with immune constituents acting as mediators of epithelial transformation. The main hypothesis is that incessant ovulation, when ruptured ovulating follicles traumatize the ovarian surface during ovulation, causes an inflammatory response. This can further be linked to epidemiological evidence that shows a protective effect of multi-parity, oral contraceptive use and breastfeeding, which reduces the number of ovulations through a lifetime [74;75]. The use of anti-inflammatory agents such as NSAIDs and aspirin has also been linked to reduced risk of epithelial carcinomas such as EOC, and will be more closely discussed in subsequent sections. In addition, inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) are associated with an elevated risk of EOC [76]. IL-6 is produced by tumor cells, T cells and macrophages, and stimulates the JAK/STAT, PI3K/Akt and Ras/MEK/ERK pathway. The activation of these pathways is associated with ascites volume, tumor size and decreased survival [77]. However, the role of IL-6 as a biomarker or part of an immune signature is more elusive.

As EOC progresses, the effect of the immune systems become even more clear, and this has been attempted to be used in a diagnostic setting. Several cytokines are upregulated in the plasma and ascites of EOC patients, including IL-6, IL-8, IL-10, VEGF, etc [78]. Attempts are being made to use these cytokines as biomarkers for early detection and pre-surgical diagnosis.

There is strong evidence that supports the association of antitumor immune responses and immune evasive mechanisms in EOC, with increased and reduced survival, respectively. For instance, ovarian tumors that are densely infiltrated with activated CD3$^+$CD8$^+$ cytotoxic T cells are strongly associated with a favorable clinical outcome regardless of stage and OC histology [79;80]. These results suggest that host immunity and immune-surveillance plays an important part in EOC tumor development. Furthermore, the presence of CD3$^+$CD4$^+$Th17 cells, a subset of CD4$^+$ T helper cells, was also associated with improved survival. And the levels of Th17 cells were inversely correlated with Tregs, further emphasizing their potential importance in tumor eradication. On the other hand, the protective effect of NK cells and B cells present in EOC tumors, plasma and ascites, show mixed results regarding prognosis. And in contrast to the amplification of anticancer responses by effector T cells, Curiel et al. have shown that an increased number of CD4$^+$CD25$^+$FoxP3$^+$ Tregs present in ascites fluid from EOC patients correlate with poor patient outcome [81]. The same study also showed that Tregs were more abundant in malignant ascites, in contrast to non-malignant ascites and in peripheral blood. In addition, Sato et al. demonstrated that a low CD8$^+$/Treg ratio decreased survival, while a high CD8$^+$/Treg ratio was associated with increased survival in EOC patients [82]. This suggests that the local tumor region is severely more immunosuppressed than the entire system, in part due to the presence of Tregs [83]. Furthermore, EOC tumors contribute to an immunosuppressive environment through expression of negative cell regulators such as PD-1 and B7-H1, and IDO [84].

The balance of effector and regulatory T cell is influenced by the developing tumor and the tumor microenvironment, as represented by surrounding ascites fluid. The progressively deficient immune response exhibited in the local EOC region and peritoneum, is partly responsible for the poor prognosis of EOC. It is evident that the tumor microenvironment affects the disease progression of EOC patients, making it a possible target for budding therapeutic strategies. New immune regulating strategies that augment the host immunity, while simultaneously preventing local immune-suppression, might thus be important to increase overall survival among EOC patients.

3.3.3 Malignant ascites

Ascites is a pathological accumulation of fluid within the peritoneal cavity that occurs when the body produces more fluid than it can remove from the abdomen. The term malignant ascites is frequently used when the fluid contains malignant cells and has a high level of lactate dehydrogenase [85]. In healthy humans, the peritoneal cavity contains a certain volume

of lubricating peritoneal fluid. This fluid is needed to support organ mobility and to facilitate easy transfer of solutes between adjacent organs and the peritoneum. The precise amount of this peritoneal fluid is strictly regulated through secretion of small molecules from capillaries through the peritoneal membrane, and reabsorption through lymphatic channels.

Excessive amounts of peritoneal fluid develop in EOC patients, when the tumor spreads to the abdominal cavity. The presence of tumor cells leads to disruption of the epithelial lining, increased leakiness of the tumor microvasculature, secretion from tumor cells and obstruction of lymphatic vessels. Tumor cells that have metastasized to the hepatic regions can also block blood flow through the liver, which pushes more fluid into the abdomen because of increased hydrostatic pressure. These processes can all contribute to the development of malignant ascites [86].

In OC patients with advanced stage III to IV disease, the immediate tumor microenvironment is extended from the abdominal cavity in the presence of malignant ascites. Malignant ascites is a complex mixture of soluble components (cytokines, chemokines and growth factors) and a wide range of cell types (free floating tumor cells, immune cells, mesothelial cells, fibroblast and macrophages). The constituents of the malignant ascites together contribute to a tumor microenvironment that promotes inflammation and further drives tumor growth, angiogenesis and fosters tumor infiltration and metastasis, while concurrently impeding anti-tumor immunity. We examined the immune-suppressive role of malignant ascites in **Paper II.**

3.3.4 Diagnosis

There are few, early clinical manifestations of EOC, and they are generally subtle and unspecific, like vague abdominal bloating, abdominal or pelvic pain, dyspepsia, urinary symptoms (frequency and urgency), difficulty eating or feeling full, flatulence etc. Other possible symptoms include fatigue, indigestion, constipation, pelvic masses, ascites fluid, menstrual irregularity, vaginal bleeding or back pain due to ovarian torsion, rupture or metastatic spread [72]. However, most patients are asymptomatic.

The most common physical finding is palpation of a mass during pelvic examination, which is the first line of diagnosis. But early stage tumors are difficult to find because of the deep anatomical location of the ovaries. Pelvic inspection is thus often followed by transvaginal ultrasonography (TVU), for a meticulous evaluation of the pelvic area. TVU can visualize the internal genitalia and detect solid masses and ascites. The challenge lies in distinguishing between normal physiological conditions, inflammation, benign tumors and ovarian cancer,

as the ultrasonographic findings rarely are pathognomic [87]. Unfortunately, percutaneous biopsy is not recommended due to the risk of leaking tumor cells into the peritoneal cavity. Therefore TVU remains inadequate to diagnose EOC [73]. Radiological evaluation with computed tomography (CT) and magnetic resonance imaging (MRI) can be used to demonstrate metastatic disease, but is not specific enough to be used in initial EOC diagnosis [88]. More recently, PET-CT has been introduced as a more thorough detection option for minor metastases, but it is not yet part of the standardized preoperative examination [89].

Several hundred potential biomarkers expressed in EOC have been identified, but no single indicator has been found useful in diagnosis [90]. Cancer antingen-125 (CA125) is a serum glycoprotein that is elevated in > 80% of patients with advanced EOC. However, it is neither specific, nor sensitive enough to be used as a screening tool or as a definitive diagnostic marker. CA125 is increased in other malignancies and benign conditions, and serum values fluctuate during the menstrual cycle [72]. Nevertheless, it can be used to measure treatment success and detect disease relapse in patients with confirmed malignancies. Human epididymis protein 4 (HE4) is another protein that is often overexpressed in EOC, which has a sensitivity equal to CA125, but an increased specificity, as it is less frequently elevated in benign gynecological conditions [91;92]. However, the level of HE4 fluctuates significantly with age, and it is commonly elevated in healthy elderly women [93]. The use of CA125 and HE4 in combination is on the rise, and a treatment algorithm combining the two biomarkers to increase diagnostic accuracy was recently released, namely the "Risk of Ovarian Malignancy Algorithm" (ROME) score [94].

Means of early detection are unfortunately very limited, and most patients present with metastatic disease when the cancer is discovered. The majority has reached FIGO-stage III-IV (International Federation of Genecology and Obstetrics) at the time of diagnosis, with widespread carcinomatosis and accumulated malignant peritoneal fluid, ascites. In the end, only surgery is left as a diagnostic option.

3.3.5 Treatment

Surgery is typically performed to obtain representative tissue for diagnosis, disease staging, and for cytoreduction to remove as much cancer tissue as possible. Optimal surgical debulking improves patient outcome, and is usually followed by adjuvant chemotherapy [95]. In cases where surgery is not possible, due to extensive disease or poor patient conditions, only chemotherapy is attempted.

Primary cytoreductive surgery includes hysterectomy, bilateral salpingoophorectomy, omentectomy and removal of iliac and para-aortic lymph nodes. The degree of cytoreduction has been shown to be the single most important independent prognostic factor for survival [96;97], and centralized surgery has led to better outcomes due to improved competence, experience with advanced surgical procedures and the capability to better handle complications.

Recommended chemotherapy regimens combine 6 cycles of paclitaxel- and platinum-type agents such as carboplatin, in the first-line treatment of EOC patients. And for subgroups with advanced disease or residual tumor following surgery, bevacizumab, a humanized antibody directed against VEGF, is also added to the regime [98-100]. Paclitaxel and carboplatin are efficient in preventing tumor recurrence and improving survival. Paclitaxel works by binding to microtubules, preventing their de-polymerization, which then disrupts mitosis and pro-apoptotic signaling. Platinum-type agents such as carboplatin, are alkylating, and work through chemical cross-linking in DNA which interferes with DNA replication and transcription, thus leading to cell death. For patients with minimal gross disease remaining after surgery, and for patients that can also tolerate aggressive treatment, intraperitoneal drug administration is attempted, and it has significantly improved survival for many [72;101]. However, its use remains limited, partly due to lack of tradition and increased complication rates [102].

Second-line treatment is endeavored in patients with recurrent disease following initial complete response, and in those who did not respond adequately to initial treatment. Relapse rates have been reported up to 70% within 12-18 moths, with most cases being caused by drug resistance. Mechanisms facilitating drug-resistance include increased expression of anti-apoptotic proteins Bcl-2 and XIAP, activation of Akt survival signaling and increased production of pro-inflammatory cytokines such as IL-6, IL-8, thus preventing paclitaxel from inducing apoptosis. Chemotherapy can be tried with a second course of the same regimen, alternatively a different regimen that comprises carboplatin with either gemcitabine or pegylated liposomal doxorubicin (PLD) may be used. Other options include bevacizumab for patients with platinum-resistant tumors (disease recurrence within 6 months), or secondary debulking surgery with an aim to remove recurrent disease or relieve symptoms for palliation [103].

Figure 5. Treatment Course for Ovarian Cancer
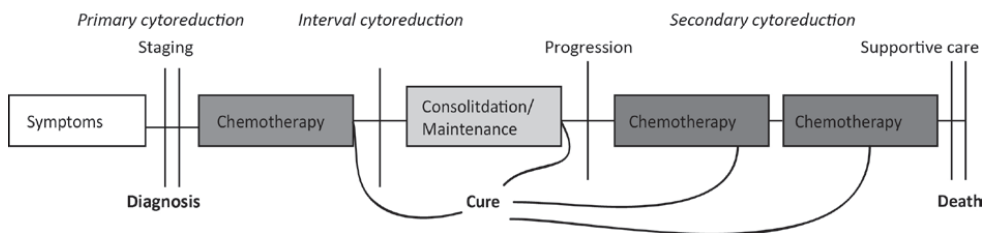
FIGURE 7: Treatment course for ovarian cancer.

Ovarian cancer patients with advanced disease are initially highly responsive to surgery and platinum- and taxane-based chemotherapy, but the majority succumbs to recurrent disease that is resistant to further treatment [79]. Despite efforts to cure ovarian cancer over the past decade, the advances in treatment has done little do reduce the overall survival rate, and established therapies fail to induce a cure at diagnosis [67]. Given the known limitation in current therapies and the association of endogenous immune responses with increased survival in EOC, research in targeted immune-therapy is of utmost importance.

3.4   Colorectal cancer

Colorectal cancer (CRC) is a term used to describe tumors that arise in the large intestines, in either the colon or rectum. About 98% of CRC cases are defined as adenocarcinomas, with tumors arising from glandular tissue that lines the inside of the intestines. Other rare types include squamous cell carcinomas, lymphomas and sarcomas. For the purpose of this Thesis, adenocarcinoma of the colon and rectum will be referred to as CRC. Tumors arising on the right side of the colon (cecum and ascending colon) tend to grow outward from the bowel wall, in an exophytic manner. These tumors rarely cause bowel obstruction, and the most common presenting symptom is anemia and abdominal discomfort. On the other hand, tumors of the left colon and rectum frequently grow circumferential, which may lead to bowel obstruction and thus ileus as a presenting symptom.
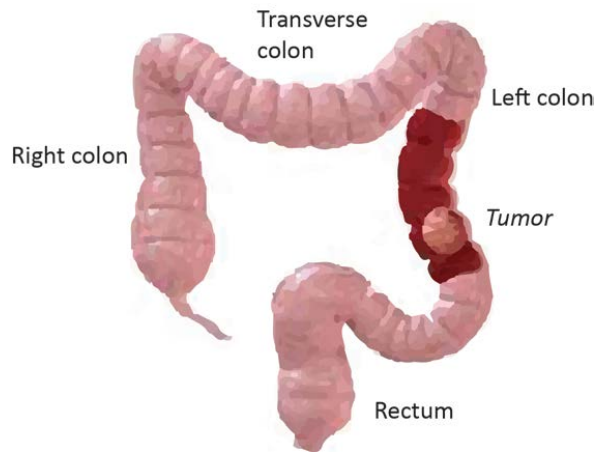
FIGURE 8. Colorectal tumors can develop in the right, transverse or left colon or in the rectum

3.4.1 Epidemiology

CRC is the fourth leading cause of cancer deaths worldwide, with about 3,5 million new cases and approximately 650.000 CRC-related deaths annually [104]. Norway has about 3,500 new cases yearly, and CRC constitutes about 15% of all diagnosed tumors nationally [105]. CRC tumors are subject to staging to ensure appropriate method of treatment and for diagnostic and research purposes. The staging systems for CRC largely depend on the extent of local invasion, the degree of lymph node involvement and whether there are distant metastases. Currently, the TNM system (T: tumor invasion, N: lymphatic node, M: metastasis) is most commonly used. However, there is a need for a new staging system that takes into account recent advances in surgical techniques and the molecular and immunological profile of the tumor.

The 5-year relative survival rate for CRC patients has improved over the last 30 years. Overall the 5-years survival rate is about 40%, but it varies from 13% if the disease presents with distant synchronous metastasis unavailable for surgical removal (stage IV), and up to 90% if the disease is localized at the time of diagnosis (stage I) [70]. CRC tumors can spread by local growth, direct invasion of surrounding tissue (pelvic wall, ureters, vagina and uterus in women, and bladder), and also through lymphatic vessels and blood vessels. Up to 70-80 % of newly diagnosed CRC patients present with localized disease, but between 15-20 % of CRC patients present with hematogenous metastases at the time of diagnosis, also known as synchronous metastases [106]. The majority of these metastases can be found in the liver, but CRC tumors also frequently spread to lungs and the peritoneum. About 35-45 % will develop metachronous liver metastases at a later stage [107].

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3.4.2 Tumor biology

The incidence of CRC is on the rise, and it is believed to be partly due to environmental and modifiable lifestyle factors. Known risk factors for developing CRC include age (> 60 years), diet (low intake of fiber and plant foods (fruits, vegetables, seeds, grains), high intake of red meat and saturated fat), gender (men are at higher risk for developing rectum cancer) obesity, physical inactivity, excessive alcohol consumption, tobacco, bowel inflammation etc [108-111]. However, up to 70-85% of CRC cases occur without a known reason. On the other hand, about 20% of CRC cases are due to familiar factors, even though no known genetic disposition has been found.

In addition to the sporadic and familiar cases of CRC, there are certain hereditary genetic conditions that predispose to CRC. The most prevalent ones are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome. FAP is caused by a mutation in the tumor suppressor and "gate-keeper" gene adenomatous polyposis coli (APC), which inhibits the oncogenic β-catenin signaling in the Wnt-pathway. Inactivation of the APC protein thus leads to uncontrolled growth of colon epithelium [112] and FAP is consequently characterized by colon polyposis. HNPCC is an autosomal dominant inherited disease characterized by mutations in DNA mismatch repair genes (MMR). These mutations prevent proper repair of DNA replication mistakes, thus leading to division of abnormal cells and uncontrolled cell growth.

Most CRC tumors develop through malignant transformation of adenomas, even though only 5-10% of existing adenomas develop into cancer [113]. The aberrant crypts to adenoma to carcinoma sequence, is a multistep development from normal to dysplastic epithelium to carcinoma, which is caused by a multitude of genetic alteration. In addition to the previously mentioned mutations of DNA mismatch repair genes and inactivating mutations or allelic loss of the "gate-keeper" APC, CRC is often associated with activation of the oncogenes Kirsten rat sarcoma viral oncogene homolog (K-ras) and protein kinase B-Raf (BRAF), and inactivation of the tumor suppressor genes tumor protein 53 (Tp53), transforming growth factor-beta (TGF-β) and phosphatase and tensin homolog (PTEN). TGF-β is inactivated in about 1/3 of CRC tumors [114], while PTEN inactivation can be found in up to 20-30% of CRC cases. PTEN acts as a tumor suppressor, through counteraction of the PI3K-Akt-pathway, which in its hyper-activation state can promote malignant growth. Loss of PTEN action results in subsequent reduced apoptosis, stimulated cell growth and increased proliferation [115]. The APC, K-ras and Tp53 mutations are associated with chromosomal instability (CIN),

which causes numerous changes in chromosomal copy number and structure [116]. CIN is found in up to 70% of CRC tumors, making it the most common form of genomic instability in CRC [117]. Microsatellite instability (MSI) is a "hypermutable phenotype" that occurs due to inactivation of DNA mismatch repair genes (MMR) [118]. Microsatellites are tandem repeats of DNA that are particularly prone to errors during DNA replication, and MSI is found in up to 15% of CRC cases, both sporadic and in hereditary HNPCC, which is caused by a mutated MMR [119].   Lastly, epigenetic silencing of gene expression, mostly mediated by aberrant DNA methylation, is a mechanism of gene inactivation through repressed transcription via the promoter region of tumor suppressor genes. CpG (-Cytosine-phosphate-Guanine-) island methylator phenotype (CIMP), refers to widespread hypermethylation of gene clusters termed CpG island loci, and is demonstrated in up to 15% of CRC's [120]. Hypermethylation has also been associated with altered APC function and microsatellite instability. The serrated pathway describes CRC tumor development that is initiated by the presence of protein kinase B-Raf (BRAF) mutation and epigenetic silencing of genes involved in cell differentiation, DNA repair and cell-cycle control [121].
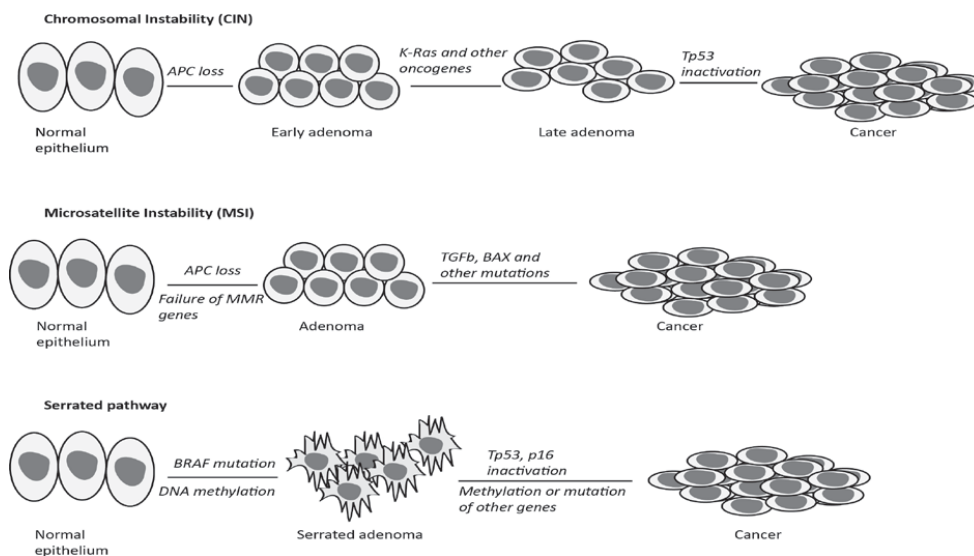
FIGURE 9: Colorectal cancer pathogenesis (adapted from Mundade R, Oncoscience 2014, 1:6)

Studies also show that APC and K-ras mutations regulate expression of cyclooxygenase-2 (COX-2) through down-stream pathways, though the exact mechanisms remain intangible [122]. A study emphasizing this showed that knockout of the COX-2 gene in mice resulted in 60% reduction of APC mutation induced intestinal adenoma [123], while other studies show that

APC can negatively regulate the expression of COX-2 [124]. In addition, the lipid kinase phosphatidylinositol 3-kinase (PI3K) is mutated in its catalytic subunit alpha polypeptide (PI3K-CA mutation) in up to 15-20% of CRC tumors. PI3K activates Akt, which through down-stream mechanisms work to regulate several signaling pathways important for carcinogenesis, such as cell proliferation, adhesion, survival and motility [125]. Up-regulation of PI3K enhances COX-2 activity and PGE_2 synthesis, ensuing in reduced apoptosis in CRC cells [126]. This will be elaborated in more detail in following sections.

It is thought that all of the above pathways can overlap and this genetic diversity is crucial in understanding the tumor biology underlying CRC development.

3.4.3 Cyklooxygenase and prostaglandin E_2 in CRC

The cyclooxygenase (COX) isoenzymes are homodimeric glycosylated proteins located intracellularly on the luminal surface of the endoplasmatic reticulum and in the nuclear envelope. COX exists in 2 main isoforms; the constitutively expressed COX-1 is involved in the physiological production of prostaglandins, prostacyklins and thromboxane to maintain normal homeostasis. It is a housekeeping gene that is essential in the protection of gastric mucosa, platelet aggregation and to preserve the integrity/function of renal microvasculature. On the other hand, COX-2 is only constitutively present in brain, testis and renal parenchyma under regular circumstances, but is increasingly expressed in pro-inflammatory environment and neoplastic tissue, under the influence of mitogens, growth factors and cytokines such as lipopolysaccharide (LPS), IL-1, IL-2 and TNF-a [63]. A third variant also exists, COX-3, which is a splice variant of COX-1[127]. COX-isoenzymes function as the rate-limiting step in the conversion of arachidonic acid, hydrolyzed from cell membrane phospholipids by a phospholipase A_2, to prostaglandin endoperioxide H_2 (PGH_2), the precursor of bioactive lipids such as prostaglandins (PGD_2, PGE_2, PGF_{2a} and PGI_2) and thromboxane A2 (TXA2). Prostanoids normally act in a paracrine and autocrine manner to coordinate intercellular events stimulated by a circulating hormone [128]. PGE_2 is considered the main player in CRC tumorgenesis, as a promotor of colonic adenoma development and progression [129]. PGE_2 activity is enhanced through increased COX-2 induction in a pro-inflammatory and tumor environment, and by the loss of 15-prostaglandin dehydrogenase (15-PGDH), the rate-limiting enzyme in catalyzing degradation of prostaglandins [130].
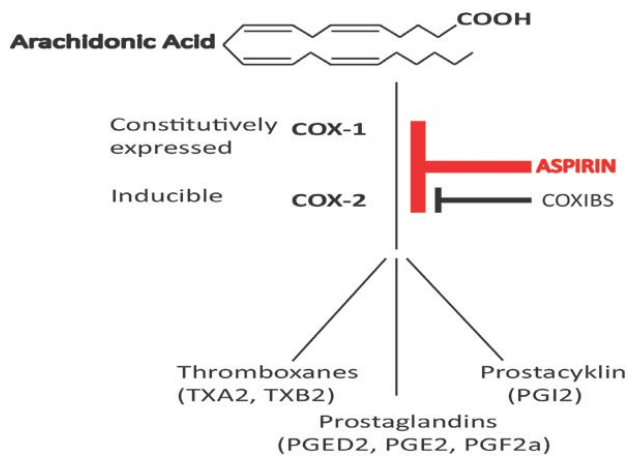
FIGURE 10: COX-enzymes facilitate the conversion of arachidonic acid into bioactive lipids such as prostaglandins, thromboxanes and prostacyklin. This production can be inhibited by NSAIDs, aspirin and COX-2-specific inhibitors.

PGE_2 mediates its biological effect through binding to the prostanoid EP receptor family, which includes four subtypes, EP1-EP4 receptors, belonging to the G-protein coupled receptor family (GPCRs). The binding of PGE_2 to EP activates G-protein downstream signaling through increased intracellular levels of the second messengers cyclic adenosine 3',5'-monophosphate (cAMP) (EP2 and EP4) or phosphinositide (PI) signal transducers (EP3), which in the end induces a decline in adenylyl cyclase/cAMP. EP1 on the other hand, prompts calcium mobilization by activating phospholipase C, and amounts to a "contractile" receptor group.

PGE_2 is an important facilitator of COX-2 associated effects, and its signaling through EP receptors promote tumor growth through a wide range of events, including promotion of proliferation, inhibition of apoptosis, stimulation of tumor invasion, angiogenesis and tumor immune evasion. This multitude of tumor promoting functions emphasizes the importance of COX-2-PGE_2 inhibition as a potential anti-tumor therapy. PGE_2 activates the Ras-MAP kinase cascade, a highly conserved intracellular pathway responsible for neoplastic cell proliferation in many human cancers. PGE_2 also trans-activates epidermal growth factor receptor (EGFR), which is upregulated in colon cancer and increases cell proliferation through activation of its down-stream mediator extracellular signal-regulated kinase (ERK). Furthermore, PGE_2 (through EP1) induces the expression of the intracellular anti-apoptotic mediator nuclear factor kappa B (NK-ķB), and directly inhibits programmed cell death through activation of both PI3-kinase and Wnt-signaling. In addition, COX-2 overexpression

is known to be related to elevated bcl-2 expression, resistance to pro-apoptotic stimuli and delays in G1 transit during cell cycle. Consequently, pharmacological inhibition of COX-2 might inhibit tumor growth and increase apoptosis through these aforementioned pathways. Furthermore, the expression of EGFR on CRC cells also correlates directly with their ability to metastasize and invade surrounding tissue. Crosstalk between COX-2-PGE_2-PKB and EGFR-PI3K/Akt circuits stimulates cell migration and invasion to a further extent than EGFR alone, providing evidence that COX inhibition may reduce the spread of metastatic disease.

COX-2 also induces the angiogenic factors vascular endothelilal growth factor (VEGF), basic fibroblast growth factor (bGFG), transforming growth factor (TGF-1), platelet-derived growth factor (PDGF) and endothelin, which are important for growth and survival of endothelial cells, stimulation of vascular endothelial cell migration and capillary formation[131;132]. Tumor cells need blood vessels to grow beyond 2-3 mm in dimension. PGE_2 is known to mediate the pro-angiogenic effects of COX-2, and several reports indicate that NSAIDs and aspirin have anti-angiogenic effects in CRC through these mechanisms [131;133]. For instance, COX-2 inhibitors decrease VEGF production in fibroblast and tumor cells, and prevent VEGF-induced mitogen-activated protein (MAP) kinase (ERK2) activation in endothelial cells.

Lastly, the Taskén group has showed that COX-2 and PGE_2 are known to induce tumor immune evasion in CRC by contributing to an immunosuppressive environment [39;134]. For instance, CRC tumor cells can produce PGE_2 which directly inhibit the effector T cells [39]. PGE_2 binds to EP2 and EP4 receptors on T cells which leads to increased intracellular cAMP and subsequent activation of the inhibitory pathway PKA – Csk – Lck, which leads to immunosuppression through inhibition of IL-2 production and proliferation[135;136]. PGE_2 also inhibits T cells through cAMP-independent pathways, through suppression of Fyn tyrosine kinase activity and adenylyl cyclase [137]. In addition, PGE_2 induces FoxP3 expression and induction of adaptive Tregs[138], which further inhibit the anti-tumor immune response.
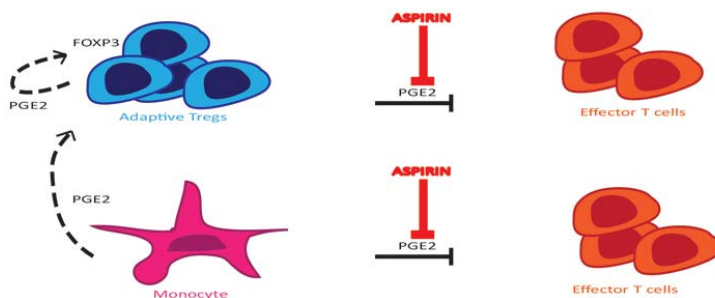
Figure 11: Aspirin inhibits PGE_2-induced tumor progression and angiogenesis, and reduces PGE_2 mediated inhibition of anti-tumor immunity.

COX-2 expression is elevated in nearly 50% of benign polyps and in about 80% of CRC adenocarcinomas. This overexpression is mostly due to abnormal regulation by transcription factors, mRNA stabilization and miRNAs, and not due to amplified gene copies. COX-1 expression on the other hand, is usually found at normal or reduced levels as compared to normal mucosa. Furthermore, elevated levels of COX-2 expression have been associated with decreased survival in CRC patients, and it is also an independent risk factor for metastasis [139;140]. PGE_2 levels are known to be elevated in CRC patient blood samples when compared to healthy donors [39;141-143]. PGE_2 levels are also found to be augmented in colonic adenomas and in carcinomas harbored by individuals with FAP and in sporadic CRC cases [141;144].

3.4.4 Acetylsalisylic acid and CRC

Acetylsalicylic acid, also known by its commonly used sales name, aspirin, is a non-steroidal anti-inflammatory agent (NSAID) and a ubiquitous drug, originally used as an analgesic and antipyretic medicine. Since 400 A.D., plants containing salicylic acid have been given to patients as a pain relief [145]. Today it is among the most commonly used drugs world-wide,

with low toxicity and a well-known safety profile, while also being exceptionally low-cost. In addition to its known anti-inflammatory properties, aspirin is also widely used as a preventive drug against cardiovascular disease due to its platelet inhibitory action. In more recent years, occasional and regular use of aspirin has been associated with lower risk for gastrointestinal and other solid tumors, and its gaining use as a prophylactic cancer remedy.
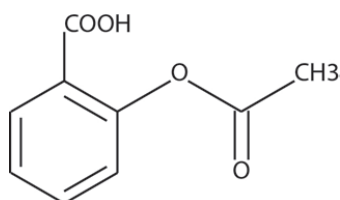
FIGURE 12: The chemical structure of acetylsalisylic acid.

The biological plausibility behind the beneficial use of aspirin in CRC patients is thought to be through COX-1 and COX-2 inhibition. Aspirin and other NSAIDs work by inhibiting COX-induced transformation of arachidonic acid into prostanoids. On the on hand, aspirin inhibits COX-2 and the production of PGE_2. PGE_2 has several known anti-tumor effects, such as promotion of proliferation, survival, migration, invasion, angiogenesis and tumor immune evasion. Looking at the use of aspirin as a primary preventive drug against CRC, its key suppressive effects are only obvious after several years. This may indicate that the primary anti-tumor effect of aspirin is through inhibition of the adenoma to carcinoma sequence, by its known reduction of tumor growth and angiogenesis, whereas reversal of anti-tumor immune evasion would not come into play before the immune system has been exposed to an established tumor. Suppression of COX-1 on the other hand, may lead to reduced metastasis, through inhibition of platelets.
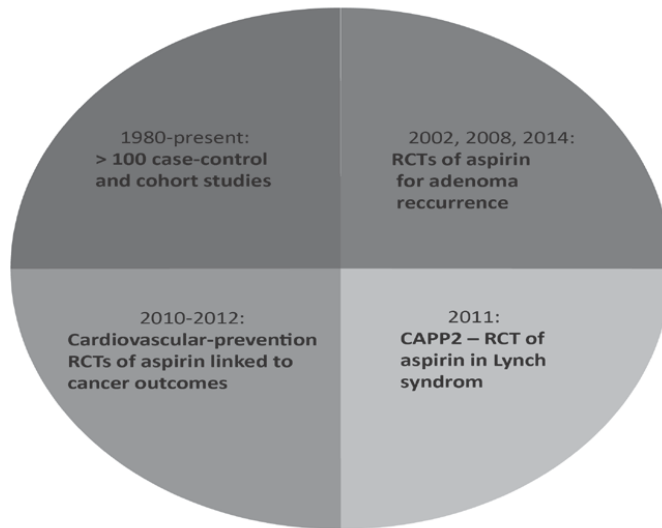
FIGURE 13: Several randomized controlled trials, case-control studies and cohort studies have been conducted to examine the correlation between aspirin use and colorectal cancer related outcomes.

In 1968 it was reported that removal of platelets in rats impaired seeding of metastases, and later studies indicated that aspirin had a similar anti-metastatic effect in animal models [146;147]. These results suggested that platelets play a part in the process of cancer metastasis, and that aspirin may suppress this course. Later, there were the slightly odd findings from cardiovascular trials that revealed that aspirin reduced non-vascular deaths compared with the placebo group. These results led to the hypothesis that aspirin may prevent cancer metastasis and incidence which was supported by a growing body of evidence. In parallel, a number of large placebo-controlled studies were conducted during the 1980's, documenting the benefit of the aspirin inhibitory effect on platelet function and cardiovascular disease. Recently, these trials with aspirin versus control in prevention of vascular events were coupled to national cancer registries and revealed robust statistical effects on reduced cancer metastasis. Further studies were aggregated with longer follow-up time, suggesting that daily aspirin use also reduced cancer incidence and deaths [148-151]. These findings were particularly obvious in cancer of the ventricle, esophagus, breast, prostate, lung, and most importantly for CRC. In addition, several studies have been conducted in animal models and CRC-cell-lines, that show similar results, with reduced adenoma and tumor growth during NSAID treatment [123;152].

Several randomized controlled trials followed, of aspirin, sulindac or COX-2 specific inhibitors (celecoxib and rofecoxib) as secondary prevention in patients with previous CRC

or polyps, or increased risk for these events due to hereditary disease [153-157]. Many of these studies had recurrence of polyps as their chief outcome, and showed a reduction of recurrent polyps in patients receiving aspirin as compared to placebo [156;158]. However, a slight draw-back with looking at polyp recurrence is that it only acts as surrogate endpoint for CRC development. The trials using COX-2 inhibitors were stopped due to adverse cardiovascular effects [159]. In sum, there exists a strong evidential foundation, both experimental and observational, suggesting that daily use of aspirin reduces cancer deaths, distant metastases and also reduces overall cancer incidence, establishing aspirin as primary preventive drug against CRC.

Our chief interest was to study the effect of post-diagnostic aspirin use, to explore its use as an adjuvant remedy. Several studies have been conducted in this regard, looking at the effect of post-diagnostic aspirin use and overall and cancer specific survival in CRC patients. All studies, except one, show a benefit with aspirin use [160-166]. But the recommended dosage and treatment duration remains unknown.

TABLE 3: Summary of publications on aspirin use and survival after diagnosis of colorectal cancer.

Year	First Author	n	Outcome	HR/OR*/RR**	95% CI	P-value
2009	Chan	1279	OS	0.79	0.65 - 0.97	-
			CSS	0.71	0.53 - 0.95	-
2012	Bastiaannet	4481	OS	0.77*	0.63 - 0.95	.015
2012	Reimers	536	OS	0.59*	0.44 - 0.81	.001
2012	Walker	13994	OS	0.91	0.82 - 1.00	-
2012	Liao	964	OS	0.18ᵃ	0.06 - 0.61	< 0.001
			CSS	0.54ᵃ	0.31 - 0.94	0.01
2013	McCowan	2990	OS	0.67	0.57 - 0.79	< .001
			CSS	0.58	0.45 - 0.75	< .001
2014	Cardwell	4794	OS	1,06*	0.94 - 1.19	-
			CSS	1,06*	0.92 - 1.24	-
2014	Reimers	999	OS	0.53**	0.38 -0.74	< 0.001
2014	Goh	726	CSS	0.38	0.17 - 0.84	0.017
2014	Kothari	1487	OS	0.96ᵃ	-	0.86
			CSS	0.60ᵃ	-	0.14

OS, overall survival; CSS, cancer specific survival
ᵃ only patients with mutated-PI3K colorectal cancers

Even though there is strong evidence in support of aspirin as a primary prophylactic agent against CRC, one cannot overlook the potential adverse effects of regular aspirin use over years, with increased risk of gastrointestinal and cerebral bleeding. It is possible to decrease the risk of gastrointestinal bleeding by concomitant use of proton pump inhibitors, but the risk of intracranial bleeding cannot be reduced. One must therefore carefully weigh the benefit against the risk of bleeding before recommending aspirin to healthy individuals that

have a low risk for developing CRC. This dilemma in particular, has led to a continuing debate on the use of aspirin in the healthy population and to the ongoing research on the use of aspirin as a potential secondary preventive measure to prolong survival in patients with established CRC tumors. We examined this effect in a Norwegian population with a registry-based study (**Paper III**).

In a secondary preventive setting, where the tumor is already established, the benefit of taking aspirin rapidly exceeds the potential harms, as the patients are at high risk of developing recurrent disease. As an adjuvant remedy, the most important protective effect of aspirin (in addition to the COX-1-mediated platelet inhibition) is believed to be facilitated through COX-2-PGE_2 suppression of anti-tumor immune response. PGE_2 is secreted by several important players in the tumor microenvironment, such as regulatory T cells, dendritic cells and tumor cells, and this PGE_2 suppresses the anti-tumor effector T cells directly, as described previously.

3.4.5 Diagnosis

Patients that seek medical advice for symptoms associated with CRC are initially subject to a case history recording. Common presenting symptoms include anemia, bloody stools, weight loss, night sweat, loss of energy and diffuse abdominal symptoms. However, some patients also present with acute symptoms due to intestinal perforation as a result of obstructive ileus. The case history is followed by a clinical examination which consists of an abdominal and rectal palpation to assess for masses. Rectal exploration can recognize up to 70% of all rectal tumors and 30% of colon tumors, and often includes a Fecal Occult Blood Testing (FOBT). FOBT is a simple, cheap and noninvasive diagnostic test which indirectly checks for gastrointestinal bleeding through enzymatic or immunological assessment of hemoglobin in feces. An important limitation for FOBT is its low sensitivity at detecting early-stage lesions, and its low specificity as ingestion of certain foods (red meats, fruits, vegetables) and medicines (non-steroidal anti-inflammatory drug) lead to false positive results [167]. Future stool-based biomarker analysis will most likely be based on analysis of DNA for known mutations such as *APC*, *K-Ras* and *p53*, epigenetic markers such as MSI and/or by measuring unfragmented long-form DNA (L-DNA). Recently a large population-based study revealed that a fecal DNA panel consisting of 21 mutations detects a greater proportion of CRC than FOBT without compromising specificity [168;169]. Other commonly used cancer biomarkers include serum detection of carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19-9). CEA is commonly elevated in CRC; however it is not eligible for screening purposes, due

to insufficient sensitivity and specificity, but it can be used to monitor disease progression. CEA levels often decrease post-operatively, and increase during relapse. In addition, increased CEA levels correlate with poor disease outcome. Other proposed tumor markers includes cancer antigen 19-9 (CA 19-9), which is the second most investigated gastrointestinal tumor marker. Unfortunately, CA 19-9 has an inferior sensitivity to CEA, and is thus less commonly applied for diagnostic purposes.

If the notion of CRC is strengthened through clinical examination and/or biomarker testing, the next diagnostic step for confirmation of disease is through examination with an endoscope; sigmoidoscopy and/or colonoscopy. Endoscopic examination allows for localization and biopsy of suspected lesions, and has a sensitivity and specificity for tumor and polyp recognition of 92-97%. However, endoscopy is an interventional method with known side effect such as patient discomfort, bleeding and intestinal perforation. Recently, non-invasive procedures have been introduced, such as noninvasive virtual colonoscopy which allows for 3D visualization of the large intestine. However, these non-interventional procedures do not allow for biopsies which are often needed to confirm the presence of malignant cells. FOBT and flexible sigmoidscopy is also recommended as a feasible screening option for CRC, but will not be more closely discussed in this Thesis [170]. If a malignant tumor is confirmed, further pre-operative examination with ultrasonography, CT or MRI is conducted to look for metastatic disease.

3.4.6 Treatment

Depending on the established tumor stage, the recommended treatment regimens differs. In low-risk carcinomas, local procedures such as endoscopic mucosal resection or a laparoscopic segment resection are often sufficient enough. Early-stage tumors are commonly treated with radical hemicolectomy with lymph node resection (curative (R0) surgical resection) and no additional treatment. On the other hand, tumors that invade the serosa or have spread to local lymph nodes have a higher risk of recurrence, and should thus be treated with adjuvant remedies in addition. Rectum cancer patients often receive several rounds of radiation therapy, which has led to significant increase in survival over the last years. In patients presenting with tumor load exceeding surgical options, neo-adjuvant chemotherapy is attempted for tumor down staging [171], followed by surgery if successful.

Adjuvant chemotherapy is consistently offered to patients with stage III disease, and it is considered in some instances to stage II patients (tumor perforation during surgery or less

than 8 lymph nodes examined in the resected tissue). First line chemotherapy consists of fluoruracil (5-FU) and calsiumfolinate/leucovorin (folinic acid administered as calcium) in combination with oxaliplatin. 5-FU is an antimetabolite which irreversibly inhibits thymidylate synthase (TS), required for the conversion of deoxyuridine monophosphate (dUMP) into thymidine monophosphate (dTMP). 5-FU causes a scarcity in dTMP, leading to cell death in rapidly dividing malignant cells. Calsiumfolinate is an adjuvant which works synergistically with 5-FU, as it stabilizes the 5-FU-TS complex, thus enhancing the toxicity of 5-FU [172]. Oxalaplatin is a platinum-type alkylating agent which interferes with DNA replication and transcription through chemical cross-linking in DNA, causing cell death [173]. This combination regime is known as the FLOX-protocol, and is considered the gold standard of care in the adjuvant setting, having led to significantly improved survival for CRC patients. Patients between 70 and 75 years are only given 5-FU and calsiumfolinate (FLV-protocol), and in older patients the need and tolerance for chemotherapy is considered individually. Another option is the FLIRI-protocol, where FLV is administered with irinotecan, a topoisomerase inhibitor which prevents DNA from unwinding[174].

In patients that present with concomitant metastatic disease or poor performance status, an addition of a biological agent that enhances the effect of cytotoxic therapy is considered. These include bevacizumab, an angiogenesis inhibitory antibody that targets VEGF, which may be added to the first line therapy regimen in patients expected to tolerate this therapy. Other options include the addition of cetuximab/panitumab, an antibody targeting EGFR-receptors, and which shows an enhanced benefit in patients harbouring K-Ras wild-type tumors [174].

Patients with locally advanced rectum tumors (10-15% of cases) that present with short margins to the surgical dissection plane are subject to a combination of neoadjuvant chemo- and radiation therapy. The concomitant radiation-sensitizing chemotherapy consist of one of two possibilities: oral capecitabine which is a prodrug that is enzymatically converted to 5-FU, or Nordic FLV which is administered intravenously.

A leap in surgical technology has pushed the boundaries of what is rendered possible with regards to cytoreductive primary surgery and resection of metastatic disease. Several case series have shown that patients with liver metastasis can achieve long-term survival when liver metastases are resected [175], and it is recommended to attempt curative resection of CRC liver metastases (CRLM). These liver-directed therapies in metastatic colorectal cancer

includes hepatic resection/hepatectomy, systemtic chemotherapy, portal vein embolization (PVE), two-stage hepatectomy, hepatic artery infusion, radiofrequency ablation (RFA), microwave ablation (MWA) and cryoablation. A recent review reported that 15-30% of CRLM patients may be eligible for curative resection, which results in an improved 5-years survival between 30 and 60% [176]. We have analyzed the results of ten years of CRC resections in Oslo (**Paper IV**).

4   Aims of the study

The aims of this Thesis were to explore tumor immune modulating properties at an experimental level, and CRC treatment options at an observational level.

A limitation in the study of Tregs is the lack of a distinct Treg biomarker that sets the population apart from other T cells. FoxP3 is a lineage-specific transcription factor that directs many of the distinct properties of the Treg subsets. However, in humans, FoxP3 is not uniquely expressed in Tregs alone, and its presence in the nucleus makes FoxP3 unavailable for isolation and measurements of live Tregs.

Secondly, we wanted to look at OC patients for our investigation of anti-tumor immunity. Previous studies have been made where the presence of Tregs was shown to correlate with poor patient outcome regardless of FIGO staging, and a parallel study was conducted in our lab to assess the function of ascites-derived T cells and Tregs [2]. Here, we also wanted to investigate immune modulating properties of soluble factors in the tumor vicinity, using cell-free components of ascites (**Paper II**). Ascites fluid produced by OC constitutes a valuable compartment for the exploration of the tumor microenvironment, as tumor cells, immune cells and proteins are freely suspended in the fluid.

In addition to exploring the immune modulatory properties of tumors at a molecular level, we wanted to expand our view of new treatment options for CRC patients. We studied CRC patients in general with regards to post-diagnostic aspirin use. Aspirin is a NSAID that inhibits tumor development through several mechanisms, including modulation of anti-tumor immune responses, and its use is associated with reduced CRC incidence, mortality and metastasis. Our main focus was to explore the use of aspirin as a possible post-diagnosis remedy (**Paper III**). Patients with CRC that present with metastases have decreased survival rates, but recent attempts to treat liver metastases with more aggressive surgical procedures have led to improvements. We have examined results of surgical practice in CRC patients with liver metastasis over a ten years period (**Paper IV**).

The work in this Thesis attended to some of the issues raised. Specifically our aims were to:

I.   Search for and characterize a specific Treg biomarker and if possible establish and test new methods for Treg isolation based on their expression of distinct surface markers (**Paper I**).

II.   Investigate the immune suppressive properties of the OC tumor microenvironment, as represented by cell-free ascites fluid.

III.   Examine the effect of post-diagnostic aspirin use on survival in CRC patients, in a retrospective registry-coupled cohort study.

IV.   Study the impact of new surgical treatment strategies applied to CRLM patients on improved survival.

5   Synopsis of publications included

Paper I

CD147 (Basigin/Emmprin) identifies FoxP3⁺ CD45RO⁺CTLA4⁺-activated human regulatory T cells.

In this paper we examined CD147 as a potential novel Treg biomarker. A proteomic approach was used to yield a list of proteins with a preferential expression on CD4$^+$CD25 (Tregs) vs. CD4$^+$CD25$^-$ (effector T cells). 28 proteins in total appeared to be uniquely expressed on Tregs, including Foxp3, and out of these, 11 were located in the membrane compartment. 4 of the 11 membrane proteins solely expressed on Tregs, CD147 (Basigin/Emmprin), CD148 (receptor-type tyrosine-protein phophatase-eta), CD71 (transferrin receptor protein 1) and CD95 (tumor necrosis factor receptor superfamily member 6), were also known to have extracellular domains. We further characterized these by flow cytometry (Fcm) analysis and showed that their expression correlated with CD25$^+$. CD147 displayed the strongest correlation with CD25 expression, with 59.7% of CD25$^+$ T cells staining positive for CD147.

CD147 divided CD4$^+$CD25$^+$ T cells in two clear subpopulations and was thus chosen for further analysis. The CD147 and Treg correlation was additionally confirmed when the CD4$^+$CD25$^+$CD147$^+$ subset expressed significantly higher levels of Foxp3 than the CD4$^+$CD25$^+$CD147$^-$cells. Increasing CD147 expression correlated with rising levels of the Treg markers CD25, GITR, ICOS, CTLA-4 and CD38. CD147- also correlated with the naïve Treg marker CD45RA, while CD147$^+$ and CD147^{++} were CD45RO$^+$. These results indicate that CD147 represents an activated Treg subset.

CD147 expression corresponded with the CD4+ CD25$^+$CD127$^-$ T cells, demonstrating that CD147 identifies a distinct Treg subpopulation. Additionally, isolation of Tregs based on CD147$^+$CD127$^-$ yielded a higher amount of FoxP3$^+$ cells, as compared to the established method based on CD25$^+$CD127^{low} expression, thus ascertaining CD147 as a novel marker for Treg isolation.

In CD25 enriched populations, CD147$^+$CD25$^+$ or CD147$^+$Foxp3$^+$ cell numbers were independent of T cell activation. In the CD25 depleted population the double positive cell populations increased significantly upon T cell activation. After stimulation the CD25$^-$CD147$^-$cells expressed high levels of CD25 and CD147, thus obtaining a CD25$^+$CD147$^+$

phenotype, and providing a source for CD147^{+}FoxP3^{+} cells. The functional difference between CD147^{+} and CD147^{-} Tregs became apparent through their different suppressive mechanisms. CD147^{+}CD25^{++} Tregs significantly inhibited T cell proliferation more efficiently than CD147^{-}CD25^{++} Tregs, against both CD4^{+}CD25^{-}CD147^{+/-} T cells.

The cytokine production also differed between CD4^{+} FoxP3^{+} T cells based on their CD147 expression. CD4^{+}FoxP3^{+} and CD4^{+}FoxP3^{-} populations were split in three subsets based on their CD147 expression (CD147^{-}, CD147^{+} and CD147^{++}) to study their cytokine producing potential. Irrespective of FoxP3 expression, CD147^{+} cells produced the most IL-2, IFN-γ, TNF-α and IL-17, while CD147^{++} showed a limited ability to produce any of the cytokines tested. Lastly, CD147 expression directly related to T cell subsets as defined by CD45RA and FoxP3. Naïve Tregs are CD45RA^{+}FoxP3^{+} and express low levels of CD147. Highly suppressive and activated Tregs are CD45RA^{-}FoxP3^{+} and express high amounts of CD147 (CD147^{++}), while the cytokine-producing non-suppressive population is CD45RA^{-}FoxP3^{+} and express moderate amounts of CD147 (CD147^{+}).

These findings establish CD147 as a direct marker for activated Tregs within the CD4^{+}FoxP3^{+} subset, providing potentially important means to manipulate Tregs in immune regulation.

Paper II
Characterization of immunosuppressive properties of malignant ascites in ovarian carcinoma

The main objective of this study was to explore contact-independent immune suppressive mechanisms by humoral factors in malignant ascites from ovarian carcinoma that target effector T cells

We assessed the effect of cell-free ascites on autologous T cell function and showed that it was suppressive in a dose-dependent manner. These findings were further confirmed when ascites supernatant also inhibited allogeneic T cell proliferation. Ovarian cancer cells that were isolated from malignant ascites were further cultured and the produced supernatant was subsequently incubated with PBMC without demonstrating any suppressive ability. This indicated that the suppressive factor in ascites is probably not produced by ovarian tumor cells. As a control we also cultured cells from the OC cell-line SKOV-3 in a similar manner, and the culture mediums from these cells were again not able to suppress allogeneic T cell proliferation.

In a further attempt to evaluate the suppressive properties of malignant ascites supernatant, we co-cultured allogeneic T cells and ascites in the presence of different inhibitors aimed at immune-modulating molecules, some which are known to be elevated in malignant ascites from OC patients, including IL-6, PGE$_2$, IL-10, IL-8, CTLA-4, PD-1, B7-DC, B7-H1c(blocking antibodies), PI3K-delta (inhibitor). Among these factors, only the presence of anti-PGE$_2$ did to some extent increase the proliferation, though not significantly. Through a more biochemical approach, we subjected cell-free ascites from ovarian cancer patients to different pre-treatments such as heating, acetone precipitation and proteolysis. We found the inhibitory factor(s) to be sensitive to proteases and denatured by heat and acetone.

In sum, we revealed that ascites supernatant displays global inhibitory activity against allogeneic and autologous T cells, and that this suppression is alleviated by heat and proteolytic treatment. In addition, we showed that this suppressive factor is neither secreted by ovarian cancer cells themselves, nor to be found in acetone precipitate. Lastly, we found that the inhibitory activity is most likely not caused by the sole functions of neither IL-6, PGE$_2$, IL-10, IL-8, CTLA-4, PD-1, B7-DC, B7-H1 nor PI3K.

Paper III

Impact of Aspirin as Secondary Prevention in an Unselected Cohort of 25,644 Patients with Colorectal Cancer – A Population-Based Study

Regular use of aspirin (acetylsalicylic acid) has been associated with reduced incidence and mortality of colorectal cancer (CRC). However, the use of aspirin as primary prevention in the general population is still being debated due to the risk of serious hemorrhagic side effects. In contrast, the use of aspirin as secondary prevention in patients with CRC may be more justified from a risk-benefit prospective. This study was conducted to examine the association between aspirin use after diagnosis of CRC with CRC-specific survival (CSS) and overall survival (OS) in the largest cohort examined to date.

An observational, population-based, retrospective cohort study was undertaken by linking patients diagnosed with CRC from 2004 through 2011 (Cancer Registry of Norway) with the use of aspirin in the same patients (The Norwegian Prescription Database). The registries used cover more than 99% of the Norwegian population, and include all cases in an unselected manner. Exposure was defined as having received prescription for more than 6

months of aspirin after diagnosis of CRC. Multivariate Cox proportional hazard analyses were used to model survival. The main outcome measures of the study were CSS and OS.

In total, 25,644 patients fulfilled our inclusion criteria in the study period and 6,119 of them were defined as exposed to aspirin after the diagnosis of CRC. The median follow-up was 3.0 years. Among aspirin exposed cases (n = 6,119), a total of 2,088 (34.1%) deaths were recorded of which 1,172 (19.2%) were CRC-specific. Among non-exposed aspirin cases (n = 19,525), a total of 9,683 (49.6%) deaths were recorded of which 7,528 (38.6%) were CRC-specific. In multivariate analysis, aspirin exposure after the diagnosis of CRC was independently associated with improved CCS (hazard ratio [HR], 0.84; 95% confidence interval [CI], 0.78-0.90) and OS (HR, 0.94; 95% CI, 0.89-1.00).

Adjustments were made for age, gender, differentiation grade, disease stage, tumor localization, surgery, and the use of possible confounding drugs such as metformin, beta blockers, ACE-inhibitors, statins and NSAIDS/coxibs. In sum, our results indicate that exposure to aspirin after the diagnosis of CRC is independently associated with improved CSS and OS.

Paper IV
Aggressive treatment of patients with metastatic colorectal cancer increases survival: a scandinavian single-center experience.

We examined overall and disease-free survival in 239 patients in this study cohort of patients with metastatic colorectal cancer. Stratification of the data was carried out according to whether the patients received neoadjuvant chemotherapy, the number of resections and the surgical technique applied. In this report we show that patients that had a high tumor load and were initially rendered inoperable, were able to achieve a life expectancy and oncologic outcome similar to patients that were primary resectable, after having received neoadjuvant chemotherapy for tumor downstaging. Furthermore, we were able to show that disease recurrences develop with a similar time interval after the primary tumor and subsequent metastases (11±1 months), and that the overall and disease-free survival is comparable after every resection. These results substantiate recent findings in the biology of tumor development and metastases, and suggest that disease relapse may in fact represent an equivalent progress of metastases that reach a detectable state at different time points, as opposed to a continuing tumour development that rapidly acquires augmented malignant potential. In accordance with these findings, we also observed that later recurrences

represented a shift in target organs, without substantially changing clinical outcome. In addition, we report that neither the presence of positive resection margins nor the presence of resectable extrahepatic disease at the time of the first liver resection, has an inverse correlation with overall survival. These results are in line with emerging evidence in support of a novel classification system in metastatic CRC. Metastatic CRC has an unfortunate forecast, but it is becoming increasingly evident that surgical treatment has obvious patient benefits, and strategies to make patients resectable and accessible for surgery should be pursued.

6   Discussion

Despite substantial development in the field of cancer research in recent years, the prognosis of patients with solid tumors with metastases remains nearly unchanged. In addition we are experiencing a rise in cancer incidences worldwide, particularly with colorectal cancer. The development of tumor immunotherapy as a therapeutic strategy has undoubtedly provided promising results in recent years. However, to excel at prevention, early detection and treatment of malignancies, it is of utmost importance to understand the development of tumor formation, its progression into systemic disease and interaction with host immunity. An introduction to the field has been presented in previous sections. In the following, our results will be discussed with emphasis on how future patients may benefit from findings presented in the included papers.

6.1   Defining a regulatory T cell (Treg) subset

The importance of regulatory T cells in a vast number of diseases, ranging from autoimmune disease and allergy to tumors, has been established in a large number of publications during the last 20 years. However, to target Tregs for therapeutic purposes, either to enhance or diminish their activity, has proved difficult due to the lack of a specific, Treg-defining surface marker. Another obstacle is the heterogeneity and stability of the Treg population, as they have proven to be a rather functionally and phenotypically diverse subset.

In **Paper I** of this Thesis, we set out to find a surface marker that distinguishes the Treg population from activated CD4^{+} T cells, through a proteomic approach (combination of subcellular fractionation, proteomics and flow cytometry). This enabled us to identify a set of membrane proteins with extracellular domains that were highly expressed on the cell surface of Tregs as compared to activated T cells. Among these, the most interesting one was the immunoglobulin superfamily member CD147, or extracellular matrix metalloproteinase inducer (Emmprin) or Basigin.

CD147 has previously been shown to be expressed by activated CD4^{+} T lymphocytes in the periphery, and to correlate with increasing amounts of CD25 and HLA-DR [177]. CD25 (IL-2 receptor) is a well-known activation marker for T cells, while HLA-DR has been proposed as a marker for an effector Treg subset with an increased suppressive capability. Furthermore, HLA-DR Tregs may represent a terminally differentiated subset in the activated Treg pool.

This is in line with our findings, as we show that CD147 can be used to identify human FoxP3 Tregs with an activated phenotype.

Miyara et al. [30] were among the first to functionally divide the Foxp3 Treg compartment into three subgroups; resting Tregs (rTregs), activated Tregs (aTregs) and cytokine-secreting non-suppressive FoxP3$^+$ cells (cytokine-secreting FoxP3$^+$). Phenotypically, these cells can be distinguished based on their expression of the naïve T cell marker CD45RA and FoxP3 as CD45RA$^+$FoxP3$^+$ rTregs, CD45RA$^-$FoxP3^{++} aTregs and CD45RA$^-$FoxP3$^+$ cytokine-secreting FoxP3$^+$ cells. CD45RA$^-$FoxP3$^+$ cytokine-secreting FoxP3 cells are non-suppressive, and are believed to belong to the Th17 family due to their expression of transcription factor retinoid acid receptor(RAR)-related orphan receptor C (ROR-C), and their secretion of IL-17, IL-2 and TNF-a. Both rTregs and aTregs exhibit a suppressive phenotype, and upon activation the rTregs are able to proliferate and convert to aTregs. In **Paper I** of this Thesis, we demonstrate that CD147 may mark the switch from a naïve to an activated state, and thus contribute to the identification of an activated and highly suppressive CD45RO$^+$ Treg subset. We show that CD147 splits the FoxP3 population and categorizes the aforementioned CD45RO$^+$ subsets; aTregs correspond to CD147^{++}, rTregs with low expression of CD147$^-$, and the non-suppressive FoxP3$^+$ T cell is equivalent to the cytokine-producing non-suppressive CD147$^+$ subset. Although these three subsets expressed different amounts of FoxP3 protein, exhibited distinctive cytokine-producing potential and suppressive ability, they expressed comparable amounts of FoxP3 transcripts. This substantiates the significance of defining and delineating Tregs subset dissimilarities at the protein level and the necessity to better describe their functions. Our results demonstrate by direct comparison that the level of CD147 staining directly aligns with the CD45RA/FoxP3 defined subsets and that the level of CD147 expression (CD147$^-$, CD147$^+$, CD147^{++}) correlates with the activation status and function of human FoxP3 cells.

A complicating matter in the research on human Tregs, is the functional heterogeneity between different FoxP3$^+$ subsets and more dynamic expression of FoxP3 itself. It has previously been shown that the difference between non-regulatory and functional FoxP3 Tregs may be linked to the methylation status of the Foxp3 gene, which is incompletely demethylated in the non-suppressive cytokine producing CD45RA$^-$FoxP3$^+$ non-regulatory T cells, but is completely demethylated in FoxP3$^+$ Tregs with suppressive activity (aTregs and rTregs). In line with this, we demonstrated that the Treg-specific demethylated regions (TSDR) of the FoxP3 gene were > 90% demethylated in CD147^{++}CD25^{++} activated Tregs

(aTregs), $CD147^{++}CD25^-$ activated effector T cells and $CD147^-CD25^{++}$ resting Tregs (rTregs). In contrast, the TSDR in $CD147^+CD25^-$ naïve T cells were about 75% methylated. This epigenetic status marker indicates stable expression of FoxP3 in the $CD147^{++}CD25^{++}$ activated Treg lineage, and is partly helpful when assessing Treg function and when isolating cells.

Tregs employ a number of mechanisms to exercise their suppressive functions, including IL-2 consumption, secretion of suppressive cytokines and cell-to-cell contact-dependent mechanisms. CTLA-4 is a known negative co-stimulatory T cell molecule that is upregulated on activated T cells and on Tregs. CTLA-4 is also described to play a key role in regulating Treg suppressive activity, and high CTLA-4 expression correlates with activated, highly suppressive human Tregs. In **Paper I** of this Thesis, we show that there is a strong positive association between accumulative CD147 expression and CTLA-4 within the $FoxP3^+$ Treg subset. We also show that CD147 expression correlates with ICOS, which has also been proposed as a Treg marker. In addition, we were able to show that the CD147 expression correlated with the suppressive function of Tregs, using a CFSE assay, which is the preferred assay for Treg-inhibiton-assessment. These findings further support that CD147 can be a useful marker to target and study functional subsets of human Tregs.

Our Treg work revealed CD147 as a potential novel biomarker for activated Tregs. Surface markers that allow identification of phenotypically and functionally homogeneous Tregs subsets are crucial, both for functional analysis and therapeutic approaches. As mentioned previously, FoxP3 remains the most renowned Treg marker due to its distinctive expression in Tregs. However, both targeting of Tregs for interventional purposes and purification of Tregs for functional assays must be based on surface markers, rendering FoxP3 an impractical option. A common approach, also partly used in our study, has been through a combination of positive selection using CD25 and negative selection using CD127. However, this approach does not enable us to distinguish between the three aforementioned Treg subsets. In contrast, CD147 may be a better molecule for isolation of Tregs, as it helps to divide Tregs into their functional subsets, with aTregs being directly selected as $CD25^+CD147^+$ and rTregs as $CD25^+CD147^-$ cells. Future experiments looking into Treg function could perhaps be based on $CD147^+$Treg isolation instead of solely relying on $CD25^+$ based methods.

Furthermore, the fact that CD147 represents an activated Treg subset with exceedingly suppressive properties makes it an interesting subject of study in clinical settings such as cancer, autoimmunity and tolerance to autotransplants. However, the distinctive Treg marker that sets Tregs completely apart from activated T cells still remains to be discovered, as CD147 is known to also be expressed on activated T cells. Explicit targeting of Tregs for interventional purposes thus continues to be a challenge.

6.2 Anti-tumor immunity in ovarian carcinoma

A large number (>1/3) of ovarian cancer patients present with malignant ascites at diagnosis, and mostly all develop ascites during relapse. The presence of malignant ascites relates to peritoneal spread of the disease, and is thus associated with poor outcome.

Malignant ascites acts as a reservoir of soluble elements and cellular components which constitute a pro-inflammatory and tumor-promoting microenvironment for the malignant cells. In fact, ascites represents a rich tumor-friendly microenvironment which both promotes the growth and motility of tumor cells, and also contributes to inhibiting the response of chemotherapy. In other words, the ascites embodies a chief source of morbidity for OC patients.

The fact that ascites is immunosuppressive is something that was first discovered in the 1980's. Several groups assessed the inhibitory effect of ascites on immune activation using different assays [178-181]. We confirmed ascites induced-suppression of T cells with a CFSE analysis, something which has not been done before, and we discovered that the suppression was equal against *self* and *non-self* cells, in a concentration-dependent manner. These findings substantiate the fact that the tumor microenvironment is highly immunosuppressive in ovarian carcinoma patients. Despite the presence of antitumor immune cells, the immune system is unable to eradicate the tumor. As mentioned in the introduction, tumors employ a large number of mechanisms to achieve immunological escape. Among these are the recruitment and induction of Tregs, which are known to be present in the malignant ascites from OC patients, and the presence of which is known to correlate badly with patient outcome [81;182]. Tregs suppress other cells through several mechanisms, including cell-to-cell contact, up-regulation of inhibitory molecules such as CTLA-4, and through secretion of immune inhibitory molecules such as PGE_2 and IL-10 [23;32;182]. After assessing whether ascites contains a soluble factor which is able to suppress T cell proliferation, we attempted to investigate the source of this inhibitory molecule. Our results indicate that ovarian cancer

tumor cells are not responsible for secretion of the inhibitory factor. This was tested both with cell-lines (SKOV-3) and inherent tumor cells isolated from ascites. As tumor cells were excluded as a potential source, some other cell-type which is abundant is ascites must be the responsible, and Tregs might possibly be the source.

As the presence and possible origin of the inhibitory molecule was confirmed, further investigations were warranted. A recent multiplex profiling of cytokines in ascites demonstrated increased expression of several factors, including pro-inflammatory molecules such as IL-6, IL-8, IL-10. The presence of both IL-6 and IL-10 is also associated with poorer disease outcome in OC patients [183;184]. IL-6 is known to promote tumor growth, invasion, migration, and angiogenesis and promote chemo resistance [185], while IL-10 is known to inhibit T cell proliferation, hamper dendritic cell maturation and inhibit T cell co-stimulatory molecules. It seems that the presence of IL-10 in ascites may contribute to tumor cells evading host immunological surveillance [186]. Furthermore, it has been shown that the concentration of IL-6, IL-8 and IL-10 are significantly higher in the ascites of ovarian cancer patients compared to the levels in serum, and correlated with poor prognosis and lack of response to therapy. Furthermore, B7-molecules are known to be present in malignant ascites, and they are known as potential targets for immune modulating therapies [56;57;84;187]. Another interesting target is PD-1, which has also been implicated in negative regulation of immune cells in ovarian carcinoma patients [188], and which can now be targeted with checkpoint inhibitors.

We reported that cell-free ascites suppress T cell proliferation in a concentration-dependent manner. We tried to abolish ascites-induced suppression by blocking different immune inhibitory molecules which are known to be present in malignant ascites, including IL-6, IL-8, IL-10, PGE_2, CTLA-4, B7-H1, B7-DC, PD-1 and PI3K. PI3K was chosen as it is a known mediator of the PGE_2-patwhay [126;189]. Of the molecules tested, only antagonizing of PGE_2 showed some tendency to reduction in ascites-induced suppression, although none of the effects were significant. However, it is interesting to note that the aforementioned molecule that did show some effect is implicated in Treg-modulating pathways. It might be that it is not sufficient to target the molecules individually, but that a combined treatment leading to synergistic effect could be needed to achieve a proper reduction of ascites-induced immunosuppression.

Furthermore, we characterized a possibly suppressive factor in ascites as heat sensitive, partly in line with Sheid and Boyce [181]. Other studies reveal the factor to possess heat-stable properties [178;179]. In addition, we found suppression to be reversed after proteolytic enzyme digestion. On the contrary, a previous study revealed the inhibitory substance to be unaffected by protease treatment. A successful isolation attempt has previously been made with TCA precipitation, where the factor stayed soluble in TCA and showed an augmentation of suppressive activity [178]. Our attempts to purify the factor with acetone precipitation however revealed that the factor is not to be found in the acetone precipitate. We also examined the supernatant, subsequent to heat-inactivation, a combined treatment that also reversed ascites' suppressive action.

The use of malignant ascites from ovarian carcinoma patients as a model to study anti-tumor immunity has both its benefits and disadvantages. On the one hand, malignant ascites contains all the constituents of the tumor microenvironment in a solution, including tumor cells, immune cells, fibroblast, cytokine etc. Cells in suspension are easily accessible for both isolation and further experiments, in particular with flow cytometry, which is the main experimental method used in this Thesis. Flow cytometry is based on laser detection of cells and molecules in suspension that are labelled with fluorescent antibodies targeting either surface or intracellular antigens.

However, the ascites samples received for this study were quite heterogeneous in their nature both with regards to cell numbers, cell distribution and protein content. In order to perform reproducible experiments, it is required that the protocol remains somewhat constant. However, due to the variation in both cell count and dispersal, it was more often than not impossible to get adequate number of cells required for the experiments. In addition, on several occasions the ascites was not suitable for use in experiments, due to extensive coagulation most likely caused by high fibrin content. However, despite the heterogeneous nature of the malignant ascites, their immunosuppressive properties remained persistent throughout various experiments.

Previous studies report characterization and isolation of inhibitory factor(s) in malignant ascites. However, the results from such characterizations vary, with some data being consistent with our own, while other findings are contradictory to what we found. The confirmation that ascites contains suppressive substance(s) is clinically relevant as it contributes to the understanding of how ovarian tumors can develop in the presence of host

immunity, and could possibly provide a much needed target for novel OC therapy. OC continues to be one of the most lethal malignancies, with an almost unchanged outcome in recent years. The future discovery of a specific immunosuppressive substance(s) might constitute the basis for future immune modulating therapy, where manipulation of host immunity can be used in treatment of OC patients.

6.3 Improving treatment of colorectal cancer

With increasing CRC incidences world-wide, there is an urgent need for improved surgical procedures and new chemotherapy and targeted therapy protocols, when the disease is established, but there is also a need to look into potential chemoprevention, both to reduce disease-development and progression.

6.3.1 Aspirin

Chemoprevention is an emerging science which refers to the use of agents that inhibit, delay or reverses carcinogenesis. Aspirin is among the most promising agents for CRC patients, with strong evidence from animal studies, experimental studies, observational studies and randomized controlled trials to support its use.

In our cohort of 25,644 patients diagnosed with colorectal cancer, we had 6,109 patients that used aspirin regularly (> 3 prescriptions = 6 months regular use). The regular aspirin users had an average adherence of 0.99 defined daily dose (DDD), which virtually equals 1 tablet/day during the time of follow-up. We have conducted the largest study of its kind to this date, and the quality of the data used is among the best in the world.

In **Paper III** of this Thesis, we report that regular use of aspirin following diagnosis of CRC, results in a 16 % improved survival from colorectal cancer, and a 6 % increased overall survival. These findings are much in line with previous reports[160-163;165;166;190-192]. However, we were not able to stratify the data on known tumor molecular profiles such as PI3K-CA or K-Ras mutations or according to COX-2-expression levels, due to the nature of the registry material. It is well-known that COX-2 transcription is up-regulated in both human adenomas and CRC, which probably relates to APC mutations. Previous observational studies have looked at the benefit of aspirin use after CRC diagnosis in patients with COX-2 overexpression and PI3K-CA mutations. Chan et al. showed that regular aspirin use was associated with a lower risk of colorectal cancer-specific mortality in patients harboring tumors with COX-2 overexpression, and another study by Liao et al., published in 2012, showed increased survival with post-diagnostic aspirin use in patients with mutated-PI3K-CA

colorectal cancer [160;192]. On the other hand, Reimer et al. could not find a survival benefit neither with COX-2 over-expression nor PI3K-CA mutation, and Kothari et al. did not confirm an advantage of aspirin use in CRC patients harboring PI3K-CA mutations either [162;193]. However, these studies had significant weaknesses with regard to study size, which should be taken in to account when interpreting the results. Conversely, our study offers a large sample, but has limitations due to a lack of opportunity to adjust for these possibly predictive factors. It may be that aspirin is only or mostly useful for a selected group of CRC patients, with a particular molecular profile. But we were not able to look into that in our study. However, our study included more than twice as many study subjects as the previous studies, making our findings quite valuable. In addition, we were able to stratify the effect of aspirin to important factors such as differentiation grade, tumor site and stage.

In our study we mainly assessed the effect of aspirin on CRC- and overall survival. However, we also adjusted for the use of NSAIDs and coxibs, in addition to other drugs that are known to affect CRC prognosis. The VICTOR trial used the COX-selective inhibitor, rofecoxib (VIOXX), which showed no benefit of its use after CRC diagnosis regardless of PI3K-mutation status. However, adjuvant aspirin use was shown to be beneficial in PI3K-CA mutant CRC in the same study [194]. The COX-2-specific inhibitor, celecoxib, was shown to promote caspase- and proteasome-dependent degradation of β-catenin, a crucial oncogenic transcription factor, in colon cancer cells, independent of COX-2 expression [195]. Furthermore, sulindac (non-selective COX inhibitor) induced tumor regression in FAP patients, but didn't change the level of major prostaglandins in rectal mucosa [196]. Several in vitro studies also suggest the presence of COX-independent mechanisms, as both non-selective (NSAIDS, sulindac) and COX-2-selective inhibitors (celecoxib and rofecoxib, the latter with the highest COX-2 selectivity), have been shown to suppress proliferation rate, alter cell cycle distribution and induce apoptosis of colon cancer cell lines regardless of their COX-2 status. In addition, several studies show that the more COX-selective the NSAID/drug is, the more limited the anti-cancer effect becomes. Conventional NSAIDS such as sulindac and aspirin, reduce the number of intestinal polyps in FAP patients more efficiently than by COX-2-selective inhibitors such as celecoxib [197;198].

In addition to the COX-2 mediated CRC-protective effect conferred by aspirin, comes inhibition of COX-1 on platelets, which is believed to reduce metastasis. COX-1 leads to accumulation of thromboxane A2 (TXA2), which promotes platelet activation and

aggregation. Activated platelets can aggregate circulating tumor cells, and thus conceal them from recognition and killing by natural killer cells.

Aspirin has a short half-life (about 2-3 hours at low dose, and 10-15 hours with high dose) when administered *in vivo*. At low doses aspirin nearly completely inhibits the capacity of COX-1 to activate TXA2, due to irreversible inhibition of COX-1 and the limited ability of anucleated platelets for *de novo* protein synthesis. This leads to profound inhibition of platelet function throughout the dose intervals (24h). COX-2 inhibition on the other hand, requires higher doses. However, much remains unknown about the exact mechanisms underlying the anti-tumor effects of aspirin. Most likely it is a combined effect of COX-1 and COX-2 inhibition, but one cannot dismiss the possibility that the CRC protective effect of aspirin is due to COX-independent mechanisms.

In Norway, enteric coated aspirin tablets are available on prescription either as 75 mg or 160 mg. We refrained from using the dose in survival analysis because of several reasons. For instance, almost 20% of the aspirin exposed cases had received prescriptions for both doses. Furthermore, the indication for the different doses differs, with 75 mg being prescribed for cardiovascular protection, and 160 mg to prevent cerebral strokes. The latter group is often older and has more comorbidity. These differences could introduce biases that could account for the differences in outcome. Also, our study was not designed to assess a dose-response-relationship, and thus we are careful to give any strong recommendations to dose.

In addition, we stratified the aspirin exposed cases according to whether they began their aspirin use prior to CRC diagnosis and continued with it afterwards (pre- and post), or whether they solely used it after CRC diagnosis (post). Our results show that patients using aspirin pre- and post-diagnosis, have the most benefit of aspirin use (CSS HR, 0.75; 95% CI, 0.69–0.82) and OS HR, 0.85; 95% CI, 0.80-0.91). However, it is important to note that the analysis has limitations, as we were only able to assess aspirin use from 2004. But the results remained the same when we stratified the material to look at aspirin use from 2004 and CRC diagnosis from 2005. In sum, our results show an advantage with pre- and post-diagnosis aspirin use, when compared with sole post-diagnosis aspirin use. On the other hand, McCowan, Chan and Bastiaanet et al. [160;161;166], show that patients taking aspirin prior to CRC diagnosis had a slightly less profound effect of aspirin on disease-specific and/or overall survival.

We addressed our hypothesis with an observational cohort study based on data linkage between two national registers of documented high quality, validity, completeness and comparability [199]. Norway is privileged with a national health system with universal coverage, and all patients are identified through a distinctive 11-digit individual identification number. The ID-numbers provide a trustworthy means of tracking patients, and are a crucial foundation for the several nation-wide databases that collect health information on all inhabitants. For hospitals, pathological laboratories and general practitioners, it is mandatory to fill in structured template reports to the Cancer Registry of Norway (CRN), with information on localization, extent of disease and treatment. National Statistics Office (Statistics Norway) provides the CRN with information on cause of death notified on death certificates [199]. All dispensed prescriptions are registered electronically, and automatically included into the Norwegian Prescription Database (NorPD). The NorPD has been functional since 2004, and includes information on patient (encrypted personal ID, birthdate, gender, place of residence), prescriber-ID and drug (strength, pharmaceutical form, pack size, number of packs, ATC-code, DDD, intended use, prescribed dose, price and dispensing date). However, drugs sold over the counter are not registered in the NorPD. Furthermore, NorPD undergoes monthly quality checks to ensure the validity of the data. The purpose of the NorPD has been to i) serve as resource for conducting record-linkage studies and ii) provide a sound evidence base for national decision-making with regards to drug utilization.

Our study has several great advantages related to the data sources. For instance, unlike the reports by Chan et al. and Liao et al., that based the assessment of aspirin use on patient questionnaires, our study extracted data on aspirin use from a register with almost 99% coverage, where medication use is based on the established Anatomical Therapeutic Chemical (ATC) classification system, with daily defined dose (DDD) as a reliable unit of measurement [200]. This prevents recall bias and is a much more dependable and unbiased source of information. One of the advantages with using registry data is that it is a method of assured quality [199;201]. Furthermore, the previously mentioned studies included selected study subjects (female nurses and male physicians), which may lead to selection bias due to inherent differences between the study subjects and the general population, which may not be accounted for in the study design. Our data on the other hand, included data on all patients diagnosed with CRC in the Norwegian population, all included in an unselective manner. This makes it easier to interpret and generalize the findings for the whole population, and not just for a highly selected subgroup. Furthermore, the assessment of both diagnosis

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(morphology and topography) and cause of death, in the CRN is based on a transnational taxonomy method, the International Classification of Diseases for Oncology, 3rd edition (ICD-O3) and International Classification of Diseases, 10th edition (ICD-10), which further emphasizes the high quality and validity of the data.

On the other hand, some are reluctant to base clinical decisions on sole observational studies. For instance, it is virtually impossible to know if the observed effect is due to some unknown or unadjusted for confounding factor.

Our results are in line with previous reports regarding the potential benefits of aspirin to increase CRC-specific and overall survival in CRC patients. Considering the significant effect we report, and the limited side-effects of long-term aspirin use, especially when compared to conventional cancer remedies, the potential benefit of aspirin in this patient group is enormous. Also, from a cost-perspective, the use of aspirin is minimal compared to conventional chemotherapy. This further substantiates our findings. Aspirin use cannot be recommended on a general basis to all CRC patients quite yet, as more data is needed to further evaluate which patient group that benefits the most. And further evidence is needed from randomized controlled trials.

6.3.2   Surgery

An increasing number of patients diagnosed with colorectal cancer are presenting with either synchronous or metachronous metastases, with the liver being the main target organ. The reason for this is most likely because all venous blood from the intestine drain through the portal circulation to the liver, making the liver into a filter that prevents tumor cells from passing further. Hepatic disease accounts for two-thirds of CRC deaths, emphasizing the importance of multidisciplinary and multimodality treatment options for colorectal liver metastases (CRLM) [202]. Metastatic disease was considered incurable just a few years ago, and patients with untreated liver metastasis until recently had an expected survival rate of < 6 months. Today however, patients presenting with synchronous liver metastasis are in some instances able to achieve survival rates comparable to patients presenting solely with localized disease. In fact, recent year's advances in surgical treatment have led to an increase in curable rates from 10% to 20-25% [203]. The definition of resectability has also shifted from a focus on tumor characteristics (tumor number and size), to determination of whether both intrahepatic and extrahepatic disease can be completely resected and whether such an approach is appropriate from an oncologic standpoint for a given patient.

CRLM patients today have improved expected survival due to advances in modern chemotherapy and other adjuvant treatment remedies such as RFA and portal vein ligation (PVL), to mention a few. Treatment modalities that render patients eligible for future resection are strongly recommended, however if the patient is still deemed unresectable then other treatments that may lengthen disease-free and overall survival should be pursued. Even though cure cannot be achieved in all CRLM patients, it is highly beneficial to convert the cancer from rapidly progressing to a slow progressing or chronic state. This approach has improved the quality of life and time of survival in those that are not curable [204]

The anticipated 5-year survival rate after resection for CRLM is approximately 35%, and patients with disease initially determined to be anatomically unresectable can be offered neoadjuvant downsizing followed by resection of CRLMs to reach a comparable survival rate of > 50% [176]. These findings are in line with our results, showing that patients receiving neoadjuvant chemotherapy to downsize the tumor load reached an overall long-term survival comparable to that of primary resectable patients. Furthermore, neoadjuvant chemotherapy serves incidentally as a tool for selecting patients with an optimal post-operative prognosis, as patients progressing during ongoing treatment most likely will not benefit from surgical treatment. These results support an aggressive treatment approach to metastatic CRC, as suggested by others. However, it is important to remember that morbidity increases with duration of neoadjuvant therapy, due to hepatotoxic effects of both oxalaplatin and fluorouracil [205], and in some instances other treatment modalities such as ablation and PVE should be considered.

In **Paper IV** of this Thesis, we present data from a time period at Oslo University Hospital where several novel management strategies were applied to CRLM patients in an attempt to improve treatment and thus survival. These include neoadjuvant and adjuvant chemotherapy, two-stage surgery with portal vein embolization, laparascopic surgery, surgical techniques adapted from liver transplantation, technique and surgical tools adapted to limit blood loss, radio frequency ablation and re-resection for disease recurrence. In our study with 239 patients with resectable liver metastasis, we compared outcome with respect to overall and disease-free survival in different subgroups based on established risk factor and the new modalities. Despite the small size of our cohort, the heterogeneity of the disease and between patients, we believe our material is interesting with respect to discussions regarding optimal treatment of CRLM.

Approximately two-thirds of patients have recurrence following resection of colorectal metastases. Our findings indicate that a second and third resection of recurring CRLMs should be considered when possible because the survival is the same after each resection. Our results are in line with previous reports that also indicate that resection should be assessed in patients with extrahepatic recurrences [206]. Extrahepatic metastatic spread has previously been considered an end-stage-disease. However, in recent years, combined liver and lung resection has produced long-time survivors. But it is important to note that among extrahepatic metastatic sites, lung metastases have a superior survival to metastases located in pedicular lymph nodes or the peritoneum. In the material presented in **Paper IV**, six patients presented with concomitant liver and lung metastasis, and only one of them died of the progression of the lung manifestation that could not be handled surgically. In addition, it is interesting to note that patients resected for recurrent disease of the lung following hepatic resection, had comparable and perhaps even better survival compared to those repeatedly resected for recurrent disease of the liver. Accordingly, both pulmonary and non-pulmonary extrahepatic disease should be considered for surgical resection, as long as it is resectable or may become resectable after downsizing. In more recent years, a "liver first" approach, where liver metastases are removed initially, followed by primary tumor resection, has been promoted, but this was not considered in our material [207].

The findings presented in this paper can be interpreted in light of two common ways to view systemic cancer development. On the one hand is the linear model, in which the primary tumor is regarded as the locomotive behind tumor advancement. The linear model advocates that development of malignant cells happens locally at the primary tumor site, and that these cells are further released in to the systemic circulation at a later time point. It is believed that as the tumor and disease progresses, it acquires increasingly aggressive biological features. On the other hand is the parallel model, which perceives the development of tumors as a corresponding course between the primary tumor and distant metastases. In light of this model, if the metastasizing process occurs at an early time point during the development of the primary tumor, the disease may not progress and become more aggressive at later stages [208;209]. The primary tumor may in fact possess aggressive metastasizing properties from the very commencement of its advance. And the primary tumor and its metastases may develop in parallel, gain different mutations and become more or less aggressive than the primary tumor due to varying conditions in the tumor microenvironment.

Hypothetically, in the parallel model, all the metastases that eventually will be found are already present at the time of diagnosis. Metastases in various organs may be manifestations of a process that has taken years to reach a level of detection, and as such, metastases may not be a sign of an explosive metastatic spread [210]. In **Paper IV** of this Thesis we report mean time intervals from primary tumor to liver metastases and from liver metastases to presentations of recurrences in patients with resectable CRLM. The disease-free interval between each event is the same, and even though it is not firm evidence of the disease not becoming more aggressive, it is a curious finding. Furthermore, if the metastases that eventually present are already there at resection of primary disease, these patients may in fact have a better chance of cancer-specific survival after removal of every "new" disease recurrence as this may not represent firsthand disease manifestations, but the last remaining. In line with this, "recurrent disease" may be an incongruity as the disease may not be recurring, but continues to deliver earlier established metastases growing in parallel and reaching a size that allows detection at different time points following primary surgery.

In reality it is most likely that metastases develop on both a linear and parallel scale and that metastatic disease is systemic or multifocal in its nature and may encompass unrecognized foci at the time of surgery, irrespective of the presentation at the time of diagnosis. This interpretation/view substantiates an aggressive treatment approach to induce resectable patients and perform repeated resections that continue to reduce tumor load.

6.4 Perspectives

There is a continuous interplay between a developing tumor and the immune system. In **Paper I** of this Thesis, we discovered a novel Treg surface marker that identifies a highly suppressive and activated subset. However, a totally specific Treg marker that sets the population completely apart from activated T effector cells, still remains to be found. This is vital to be able to target Tregs specifically for interventional purposes. In addition, we attempted to unravel some of the soluble mechanisms conveying immunosuppression in the tumor microenvironment in ovarian carcinoma. There is still a need to fully understand these mechanisms, to be able to target them for future immune-therapeutically purposes.

Furthermore, in **Paper III**, we looked at the effect of post-diagnostic aspirin use in CRC patients, and found that it conveys a significant survival benefit. In light of these findings, we intend to do a randomized controlled trial in CRC patients with liver metastasis, which will

be randomly allocated in to receiving aspirin or placebo, following surgery, and followed-up for 3 years.

In **Paper IV**, we highlight the effect of implementation of novel treatment strategies with respect to disease-free and overall survival in patients directly subjected to liver surgery or after downsizing to resectable CRLMs. Our report adds arguments to the ongoing discussions in the field, and substantiates the importance of aggressive treatment in these patients.

7 Conclusions

I. The trans-membrane protein CD147 defines a subset of activated, highly suppressive regulatory $CD4^+CD25^+FoxP3^+$ T cells, and CD147 Treg subsets corresponds to CD45RA-based classification of Treg subsets

II. Cell-free ascites from ovarian carcinoma inhibits T cell function in a dose-dependent manner, against *self* and *non-self*. However, the soluble mediator of immune inhibition in ascites is not secreted from intrinsic OC tumor cells or OC tumor cell-lines. Furthermore, the inhibitory effect of ascites was not abolished when targeting known immune-modulating factors known to be present in malignant ascites, such as IL-6, IL-8, IL-10, PI3K, B7-DC, B7-H1, CTLA-1 and PGE_2.

III. Post-diagnostic aspirin in CRC patients is a strong and independent predictor for increased CRC-specific and overall survival. Furthermore, patients using aspirin before and after CRC diagnosis, have the most benefit with regards to survival.

IV. Pre-operative chemotherapy is beneficial for patients with high tumor load of CRLM, as we show that these patients that receive neoadjuvant chemotherapy achieve oncologic results and life expectancy comparable to those that are primary resectable with low tumor load. Furthermore, following multiple resection, patients with recurrent CRLM achieve equal estimated survival as after primary resection. Recurrent disease in these patients present with similar time intervals as the primary tumor and subsequent metastasis. These findings indicate that recurrent disease may not be a sign of increasingly aggressive and progressive metastatic disease.

8 References

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