TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 1009

MEDICA - ODONTOLOGICA

FACTORS AFFECTING CANCER BEHAVIOR WITH SPECIAL REFERENCE TO LYMPHATIC VESSELS, MACROPHAGES, EGFR, AND PIM-1 IN COLORECTAL CANCER

by

Annika Ålgars

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2012

From the Department of Oncology and Radiotherapy, Turku University Hospital;
the MediCity Research Laboratory and the Department of Medical Microbiology and Immunology, University of Turku;
the National Graduate School of Clinical Investigation;
Turku Doctoral programme of Clinical Sciences.

Supervised by
Professor Sirpa Jalkanen, MD, PhD
MediCity Research Laboratory
Department of Medical Microbiology and Immunology
University of Turku
Turku, Finland

Docent Raija Ristamäki, MD, PhD
Department of Oncology and Radiotherapy
Turku University Hospital
University of Turku
Turku, Finland

Reviewed by
Professor Ari Ristimäki, MD, PhD
Department of Pathology
Helsinki University
Helsinki, Finland

Docent Minna Tanner, MD, PhD
Department of Oncology and Radiotherapy
Tampere University Hospital
University of Tampere
Tampere, Finland

Dissertation opponent
Professor Caj Haglund, MD, PhD
Department of Surgery
Helsinki University Hospital
Helsinki University
Helsinki, Finland

ISBN 978-951-4965-6 (PRINT)
ISBN 978-951-4966-3 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland, 2012

To my Family and Friends

Our greatest glory is not in never failing, but in rising up every time we fail

~ Ralph Waldo Emerson~

ABSTRACT

Annika Ålgars

Factors affecting cancer behavior with special reference to lymphatic vessels, macrophages, EGFR, and Pim-1 in colorectal cancer

From the Department of Oncology and Radiotherapy, Turku University Hospital;
the MediCity Research Laboratory and the Department of Medical Microbiology and Immunology, University of Turku;
the National Graduate School of Clinical Investigation;
Turku Doctoral programme of Clinical Sciences.
Annales Universitatis Turkuensis.
Turku, Finland 2012.

The behavior and prognosis of cancer is affected by several factors, including alterations in the cancer cells and changes in the tumor microenvironment.

The aim of the present study was to investigate new predictive and prognostic factors located in the cancer cells (*EGFR* gene copy number, EGFR, oncogene *pim-1*) or tumor microenvironment, including lymphatic vessels (CLEVER-1, podoplanin), macrophages (CD68, CLEVER-1), and T-lymphocytes (CD3) in colorectal cancer. Furthermore, the molecular characteristics of lymphatic vessels (CD73, LYVE-1, podoplanin) as well as the lymphocyte and dendritic cell trafficking in lymphatics were studied.

The results indicate that high Pim-1 expression, a high number of $CD68^+$ macrophages peritumorally, and in early stage disease, a high number of CLEVER-1^+ macrophages and vessels peritumorally are factors associated with a favorable disease outcome in colorectal cancer. In contrast, in stage IV disease, a high number of CLEVER-1^+ macrophages both intra- and peritumorally are associated with poor survival. *EGFR* gene copy number measured by silver *in situ* hybridization predicts anti-EGFR treatment response in metastatic colorectal cancer more accurately than the routinely used *KRAS* analysis. In addition, lymphatic vessels exhibit marked phenotypic heterogeneity in healthy and cancer tissues and the function of CD73 in lymphatics differs from blood vessels as demonstrated in this study.

In summary, colorectal cancer prognosis is affected distinctly at different stages of disease and varies depending on the location of lymphatic vessels and macrophages, whereas a high Pim-1 expression is associated with a favorable survival. Moreover, *EGFR* gene copy number is a promising new predictive marker in metastatic colorectal cancer patients with wild type *KRAS*.

Keywords: Lymphatic vessels, macrophages, colorectal cancer, prognostic markers, *EGFR* gene copy number

TIIVISTELMÄ

Annika Ålgars

Syövän käyttäytymiseen vaikuttavat tekijät, joista erityishuomion kohteena imutiet, makrofagit, EGFR ja Pim-1 kolorektaalisyövässä

Syöpätautien klinikka, Turun yliopistollinen keskussairaala; MediCity tutkimuslaboratorio ja Lääketieteellinen mikrobiologia ja immunologia, Turun Yliopisto; Valtakunnallinen kliininen tutkijakoulu; Turun kliininen tutkijakoulu.
Annales Universitatis Turkuensis.
Turku, Suomi 2012.

Syövän käyttäytymiseen ja ennusteeseen vaikuttavat monet tekijät, muun muassa muutokset syöpäsoluissa sekä kasvainta ympäröivässä mikroympäristössä.

Tutkimuksen tavoitteena oli tutkia uusia prediktiivisiä ja prognostisia ennustetekijöitä syöpäsoluissa (*EGFR* geenikopiomäärä, EGFR, onkogeeni *pim-1*) sekä syöpäkasvaimen mikroympäristöön kuuluvissa imuteissä (CLEVER-1, podoplaniini), makrofageissa (CD68, CLEVER-1) ja T lymfosyyteissä (CD3) kolorektaalisyövässä. Lisäksi tutkittiin imuteiden molekulaarisia ominaisuuksia tarkemmin (CD73, LYVE-1, podoplaniini) kuten myös lymfosyyttien ja dendriittisolujen liikennöintiä imuteissä.

Tutkimustulokset osoittavat että korkea Pim-1 ekspressiotaso, suuri peritumoraalinen $CD68^{+}$ makrofagimäärä sekä varhaisen vaiheen taudissa suuri CLEVER-1^{+} peritumoraalinen makrofagimäärä ovat hyvän ennusteen tekijöitä kolorektaalisyövässä. Metastaattisessa taudissa sen sijaan suuri määrä CLEVER-1^{+} makrofageja, sekä intra- että peritumoraalisesti, liittyy huonoon tautiennusteeseen. *EGFR* geenikopiomäärä, EGFR proteiinipitoisuuden ohjaaman hopea *in situ* hybridisaatiomenetelmän avulla määritettynä, ennusti vastetta anti-EGFR hoidolle metastaattisessa kolorektaalisyövässä tarkemmin kuin nykyisin rutiinisti käytössä oleva *KRAS* määritys. Lisäksi havaittiin että imutiet ovat monimuotoisia imutiemarkkeri ekspressionsa suhteen sekä normaali- että syöpäkudoksissa. CD73 molekyylin funktio imuteissä poikkesi selvästi molekyylin funktiosta verisuonissa.

Yhteenvetona voidaan todeta että kolorektaalisyövän ennusteeseen vaikuttavien tekijöiden merkitys vaihtelee taudin levinneisyysasteen sekä imuteiden että makrofagien sijainnin perusteella. Korkea Pim-1 ilmentyminen on yhteydessä hyvään kolorektaalisyöpäennusteeseen. Lisäksi *EGFR* geenikopiomäärä osoittautui lupaavaksi uudeksi prediktiiviseksi ennustetekijäksi *KRAS* villintyypin metastaattista kolorektaalisyöpää sairastavilla potilailla.

Avainsanat: Imusuonet, makrofagit, prognostiset tekijät, *EGFR* geenikopiomäärä, kolorektaalisyöpä

TABLE OF CONTENTS

1 INTRODUCTION 13
2 REVIEW OF THE LITERATURE 14
2.1 Characteristics of cancer development and behavior 14
2.1.1 General aspects of cancer biology 14
2.1.2 Maintenance of proliferative signaling and reprogramming of energy metabolism 14
2.1.2.1 EGFR pathway 15
2.1.3 Loss of suppressive capacity 15
2.1.4 Unlimited replicative potential of cancer cells 17
2.1.5 Angiogenesis 17
2.1.6 Properties enabling invasion and metastatic dissemination of cancer cells 17
2.1.6.1 Lymphangiogenesis 18
2.1.6.2 Leukocyte and cancer cell trafficking 26
2.1.7 Cancer and inflammation 28
2.1.7.1 Tumor initiation 29
2.1.7.2 Transcription factors 29
2.1.7.3 Cytokines 30
2.1.7.4 Chemokines 31
2.1.7.5 Antitumoral immune response 31
2.1.7.6 Tumor escape phase 32
2.1.7.7 Metastatic phase 32
2.1.7.8 Colorectal cancer and inflammation 33
2.1.7.9 Tumor associated macrophages in cancer 34
2.1.7.10 Tumor associated macrophages in colorectal cancer 37
2.1.7.11 Immunotherapeutic treatment options 38
2.2 Oncogene *Pim-1* 38
2.2.1 Pim-1 expression, function, and regulation 38
2.2.2 Pim-1 in cancer 40
2.3 CD73 41
2.3.1 Expression and enzymatic role of CD73 41
2.3.2 CD73 in lymphocyte adhesion and transmigration 43
2.3.3 CD73 in vasculature 43
2.3.4 CD73 in cancer 44
2.4 Colorectal cancer 45
2.4.1 Incidence, risk factors, and staging system 45
2.4.2 Molecular features of colorectal cancer development 47
2.4.3 Prognostic factors in colorectal cancer 48
2.4.4 Colorectal cancer treatment 49
2.4.4.1 Adjuvant therapy for stage II and III disease 49
2.4.4.2 Treatment of rectal cancer 50
2.4.4.3 Treatment of metastatic stage IV disease 51

2.4.5 Predictive markers in colorectal cancer 54
2.4.5.1 Predictive markers for anti-EGFR therapy 55
3 AIMS OF THE STUDY 57
4 MATERIALS AND METHODS 58
4.1 Patient materials and tissue samples 58
4.2 Animals 59
4.3 Antibodies and immunohistochemistry kits 60
4.4 Methodology used in the studies 61
4.4.1 Immunohistochemistry (paraffin-embedded samples) 61
4.4.2 Immunofluorescence stainings 61
4.4.3 Evaluation of immunohistochemical stainings 62
4.4.4 Silver and fluorescence *in situ* hybridization 62
4.4.5 Enzyme assays 62
4.4.6 Lymphocyte migration assays 63
4.4.7 Intravenous lymphocyte homing assay 63
4.4.8 FITC painting 64
4.4.9 Skin explant culture 64
4.4.10 Statistical methods 64
4.5 Ethics 64
5 RESULTS 66
5.1 CD73 in lymphatics (I) 67
5.1.1 CD73 expression on lymphatics is heterogenous 67
5.1.2 Nucleotide metabolism differs between lymphatic and vascular endothelium 67
5.1.3 CD73 on lymphocytes affects their migration 67
5.1.4 LFA-1 is not involved in lymphocyte migration via afferent lymphatics 68
5.1.5 CD73 does not affect the homing of lymphocytes under normal conditions 68
5.1.6 Dendritic cell trafficking in the afferent lymphatics does not involve CD73 68
5.2 Lymphatics, macrophages, and $CD3^+$ T lymphocytes in colorectal cancer (II) 68
5.2.1 Lymphatic vessels are heterogenous 68
5.2.2 Intratumoral lymphatics - a sign of poor prognosis 68
5.2.3 Peritumoral lymphatics - a sign of good prognosis 69
5.2.4 CLEVER-1^+ vessel and macrophage numbers correlate with each other 69
5.2.5 CLEVER-1 is a marker for type 2 macrophages 69
5.2.6 The location of macrophages is crucial for CRC outcome 69
5.2.7 Subtype of macrophages influences recurrence site 70
5.2.8 $CD3^+$ lymphocytes in Stage III colorectal cancer 70
5.2.9 A new prognostic score is associated with CRC survival 70
5.3 *EGFR* gene copy number as a predictive marker for anti-EGFR treatment in metastatic colorectal cancer (III) 70
5.3.1 EGFR protein expression correlates with *EGFR* GCN 70

5.3.2 *EGFR* GCN predicts responsiveness to anti-EGFR treatment 71
5.3.3 A high *EGFR* GCN is associated with improved survival 72
5.4 Pim-1 in colorectal cancer (IV) 72
5.4.1 Pim-1 protein expression is heterogenous in CRC 72
5.4.2 Radiation therapy alters subcellular Pim-1 expression levels 73
5.4.3 High Pim-1 expression is associated with a favorable CRC outcome 73
6 DISCUSSION 75
6.1 CD73 in lymphatics 75
6.2 Lymphatics in colorectal cancer 76
6.3 Tumor associated macrophages in colorectal cancer 79
6.4 *EGFR* GCN in metastatic colorectal cancer 81
6.5 Pim-1 in colorectal cancer 83
7 SUMMARY AND CONCLUSIONS 85
8 ACKNOWLEDGEMENTS 87
9 REFERENCES 90
ORIGINAL PUBLICATIONS 103

ABBREVIATIONS

ADP	adenosine diphosphate
AJCC	American Joint Committee on Cancer
ALI	acute lung injury
AMP	adenosine monophosphate
AP	activated protein
APC	adenomatous polyposis coli
ATP	adenosine 5´-triphosphate
BAD	BCL2 antagonist of cell death
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CCL	chemokine (C-C motif) ligand
CCR	chemokine receptor
CD	cluster of differentiation
Cdc25A	cell division cycle 25 homolog
CIN	chromosomal instability
CIMP	CpG island methylator phenotype
CISH	chromogenic *in situ* hybridization
CLEC	C-type lectin-like receptor
CLEVER	common lymphatic endothelial and vascular endothelial receptor
C-myb	myeloblastosis oncogene
C-myc	v-myc myelocystomatosis viral oncogene homolog
COX	cyclooxygenase
CR	complete response
CRC	colorectal cancer
C-TAK1	Cdc25C associated kinase
CXCL	chemokine (C-X-C motif) ligand
DFS	disease-free survival
DNA	deoxyribonucleic acid
DPD	dihydropyrimidine dehydrogenase
DSS	disease-specific survival
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ERCC	excision cross-complementing gene
ErbB	erythroblastic leukemic viral oncogene homolog
FISH	fluorescence *in situ* hybridization
FOLFIRI	5-fluorourasil/leucovorin/irinotecan
FOLFOX	5-fluorourasil/leucovorin/oxaliplatin
Foxc2	forkhead transcription factor 2
FOXP3	forkhead box P3
5-FU	5-fluorourasil
GCN	gene copy number
GM-CSF	granulocyte macrophage colony stimulating factor

GTP	guanosine triphosphate
Gy	gray
HEV	high endothelial venules
HER	human epidermal growth factor receptor
HIF	hypoxia-inducible factor
ICAM	intercellular adhesion molecule
IF	immunofluorescence
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
KRAS	Kirsten-ras
LEC	lymphatic endothelial cells
LV	leucovorin
LFA	lymphocyte function associated antigen
LPS	lipopolysaccharide
LVD	lymphatic vessel density
LVI	lymphatic vessel invasion
LYVE	lymphatic vessel endothelial hyaluronan receptor
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
M-CSF	macrophage-stimulating-factor
MDSC	myeloid-derived suppressor cells
MEK	mitogen-activated kinase kinase
MMP	matrix metalloproteinase
MMR	mismatch repair
MR	macrophage mannose receptor
mRNA	messenger ribonucleic acid
MSI-H	microsatellite instability-high
MSI-L	microsatellite instability-low
MSS	microsatellite stable
NTPDase	nucleoside triphosphate diphosphohydrolase
NF-κB	nuclear factor-κB
NK	natural killer
NP	neuropilin
NSAID	non-steroidal anti-inflammatory drug
NuMA	nuclear mitosis apparatus
OS	overall survival
PD	progressive disease
PFS	progression-free survival
PI3K	phosphoinositide kinase 3
Pim-1	proviral integration site MuLV
PR	partial response
Prox-1	prospero-related homeodomain transcription factor
PSA	prostate specific antigen
PT	peritumoral
PTEN	phosphatase and tensin homolog

RECIST	response evaluation criteria in solid tumors
REMARK	REporting recommendations for tumor MARKer prognostic studies
ROC	receiver operating characteristic
SCC	squamous cell carcinoma
SD	stable disease
SISH	silver *in situ* hybridization
Slp-76	lymphocyte cytosolic protein 2
SOCS	suppressor of cytokine signaling protein
SPARC	secreted protein acidic and rich in cysteine
STAT	signal transducer and activator of transcription
TAM	tumor associated macrophage
TGF	transforming growth factor
Tie	tyrosine kinase with immunoglobulin-like and EGF-like domains
TME	total mesorectal excision
TNF	tumor necrosis factor
TNM	Tumor Node Metastasis classification of malignant tumors
Topo-1	topoisomerase-1
TP	thymidine phosphorylase
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell
TS	thymidylate synthase
VAP-1	vascular adhesion protein-1
VCAM	vascular cell-adhesion molecule
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VLA	very late antigen
XELOX	capecitabine/oxaliplatin
UICC	Union for International Cancer Control

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles. The publications are referred to in the text by Roman numerals (I-IV).

I *Ålgars A, *Karikoski M, *Yegutkin G, Stoitzner P, Niemelä J, Salmi M, Jalkanen S**.** Different role of CD73 in leukocyte trafficking via blood and lymph vessels. *Blood*. 2011; 117(16):4387-4393.
* Authors equally contributed to this work

II Ålgars A, Irjala H, Vaittinen S, Huhtinen H, Sundström J, Salmi M, Ristamäki R, Jalkanen S. Type and location of tumor infiltrating macrophages predict survival of colorectal cancer patients. *Int J Cancer* 2011; Sep 22, Epub.

III Ålgars A, Lintunen M, Carpén O, Ristamäki R, Sundström J. *EGFR* gene copy number assessment from areas with highest EGFR expression predicts response to anti-EGFR therapy in colorectal cancer. *Br J Cancer*. 2011; 105(2):255-262.

IV Ålgars A, Ristamäki R, Kujari H, Laine J, Sundström J, Pyrhönen S, Jalkanen S. Upregulation of Pim-1 in colorectal cancer – a sign of favorable disease outcome. Submitted.

The original publications have been reproduced with the permission of the copyright holders.

1 INTRODUCTION

The development of cancer is a complex process requiring several molecular alterations. However, cancer behavior and prognosis does not merely depend on changes in the precancerous and cancer cells themselves, but include the interaction of the surrounding host microenvironment, as well.

The knowledge regarding the risk factors and molecular changes involved in the development of colorectal cancer (CRC) has evolved over the years. In addition, new treatment strategies and use of prognostic and predictive markers have improved the outcome of the disease. We are gradually stepping into an era of personalized medicine in the field of CRC, Kirsten ras (*KRAS)* testing being a proof of that. *KRAS* gene mutation analysis is today used as a marker in routine clinical practice to predict responsivenss to anti-epidermal growth factor receptor (EGFR) therapy. Other promising predictive markers are waiting for robust validation prior to establishment in this field.

CRC and inflammation are clearly interrelated. Inflammatory cells are present in practically all malignant tumors and they may either work in a harmful protumoral or protective antitumoral fashion. Inflammation has been shown to increase the risk for CRC, whereas the densities of inflammatory cells, including macrophages, in the microenvironment of established CRC tumors seem to influence CRC prognosis. However, the role of macrophage subpopulations in CRC is still largely unknown. CRC, similar to other solid tumors, induces lymphangiogenesis, at least in part due to growth factors excreted by tumor associated macrophages (TAM). CRC cells may disseminate through the lymphatic vessels to the local lymph nodes, which has a strong negative prognostic impact. No treatment option available today has the ability to prevent this event. There is less knowledge available in the field of cancer-related lymphangiogenesis than in the field of tumor-associated angiogenesis due to the fact that lymphatic endothelial cell specific markers have not been previously available. The influence of lymphatic vessel density on CRC prognosis has been investigated, but the results have been inconsistent and no firm conclusion on the prognostic value of the lymphatic vessel density can as yet be drawn. Molecules involved in leukocyte or cancer cell trafficking in the lymphatics are sparsely known, which is in striking contrast to the blood vessels. In blood vessels, for instance CD73 is involved in lymphocyte trafficking and has anti-inflammatory effects. In addition, CD73 seems to promote tumor growth. The function of CD73 in the lymphatics is not known.

The behavior and prognosis of cancer is affected by a very complex signaling network including various molecules with often interrelated, sometimes dual functions. Oncogenes play an important role in carcinogenesis and tumor progression. Interestingly they may have tissue specific effects, which might be completely opposite between different malignancies. More knowledge is needed in the field of factors affecting the course of malignant diseases or responsiveness to specific therapies.

2 REVIEW OF THE LITERATURE

2.1 Characteristics of cancer development and behavior

The development of cancer is a complex process requiring the involvement and alteration of numerous molecules within cancer cells as well as in the tumor microenvironment. Prognosis of cancer is likewise affected by molecular features of the tumor and its surrounding. This review of the literature will discuss different aspects of tumorigenesis and factors affecting cancer behavior with emphasis on lymphangiogensis and inflammation in colorectal cancer. Prognostic and predictive markers in CRC are also addressed.

2.1.1 General aspects of cancer biology

Malignant transformation occurs frequently in a portion of the cells in the human body. Fortunately these very seldom give rise to the development of cancer. Certain capabilities are required for a cancer cell to survive in the body as well as to acquire characteristics enabling tumor formation and metastatic spread of the malignant cells. Notably, not only the characteristics of the cancer cells are important but also the surrounding non-malignant cells interacting with the cancer cells play a crucial role in the development and behavior of malignant tumors.

To improve our understanding of the complex biology of cancer Hanahan and Weinberg have proposed six hallmarks of cancer (Hanahan & Weinberg, 2000), which have been broadened by two enabling characteristics and two emerging hallmarks in year 2011 due to increased knowledge in this field. The hallmark capabilities include: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death. The enabling characteristics are: tumor-promoting inflammation, genomic instability, and gene mutations. Deregulating cellular energetics and avoiding immune destruction are referred to as emerging hallmarks (Hanahan & Weinberg, 2011).

2.1.2 Maintenance of proliferative signaling and reprogramming of energy metabolism

Opposite to normal cells, cancer cells are capable of sustaining proliferative signaling in various ways, e.g. by producing growth factors, becoming hyper-responsive by increasing the number of growth factor receptors at their cell surface, or by constitutive activation of downstream pathways of growth factor receptors (Hanahan & Weinberg, 2011; Lemmon & Schlessinger, 2010).

Cancer cells alter their energy production and seem to favor aerobic glycolysis also in the presence of oxygene instead of mitochondrial oxidative phosphorylation (metabolic switch, Warburg effect). The aerobic glycolysis generates adenosine 5´-triphospate (ATP) inefficiently, but provides glycolytic intermediates (e.g. acetyl-

coenzyme A and nicotinamide adenine dinucleotide phosphate-oxidase) needed for macromolecule production to meet the demand of active cell proliferation. Activated oncogenes and inactivated tumor suppressor genes are linked with the metabolic switch occuring in cancer. The increased uptake and usage of glucose in many human malignant tumors can be visualized by 18F-fluorodeoxyglucose positron emission tomography scanning (Hanahan & Weinberg, 2011; Jones & Thompson, 2009; Vander Heiden *et al.* 2009).

2.1.2.1 EGFR pathway

EGFR [also known as human epidermal growth factor receptor (HER) 1] is a cell membrane receptor consisting of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic domain. The cytoplasmic part contains a tyrosine kinase domain and a carboxy terminal region with tyrosine autophosphorylation sites. EGFR belongs to the erythroblastic leukemia viral oncogene homolog (ErbB) family consisting of four members: HER1 (EGFR, ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Ligands binding to EGFR include epidermal growth factor (EGF), transforming growth factor (TGF) α, amphiregulin, epiregulin, betacellulin, heparin-binding EGF, and epigen (Saif, 2010). The binding of ligands to the extracellular part of the receptor activates the intracellular kinase domain. The activated kinase phosporylates intracellular proteins, thereby activating signaling pathways downstream from the receptor (Bass, 2011). Amplifications and mutations of genes within a pathway may cause abnormal signaling and thus by disrupting the normal regulated cell division, cellular proliferation, differentiation, and migration contribute to the development of cancer. As an example, an activating v-raf murine sarcoma viral oncogene homolog B1 (*BRAF)* gene mutation (V600E), which is found in less than 10% of the colorectal tumors (Price *et al.* 2011; Van Cutsem *et al.* 2011), leads to a constitutive activation of the downstream pathway of EGFR (Michaloglou *et al.* 2008). *KRAS* gene mutations representing the same pathway are found more frequently (in about 40% of the cases) in CRC (Amado *et al.* 2008; Douillard *et al.* 2010; Karapetis *et al.* 2008; Peeters *et al.* 2010; Qiu *et al.* 2010; Van Cutsem *et al.* 2011). These mutations disable guanosine triphosphatase (GTPase) activity and cause *KRAS* to accumulate in the active GTP-bound conformation. A simplified EGFR signaling pathway is presented in **Figure 1**.

2.1.3 Loss of suppressive capacity

As a response to atypical harmful events in a cell, suppressor genes normally react by inhibiting cell proliferation. Defects in these tumor suppressor genes contribute to cancer progression by allowing uncontrolled tumor growth. The loss of cell-to-cell contact inhibition further increases tumor growth. One factor attributing to the prevention of cancer development is programmed cell death (apoptosis), which is induced by cell stress.

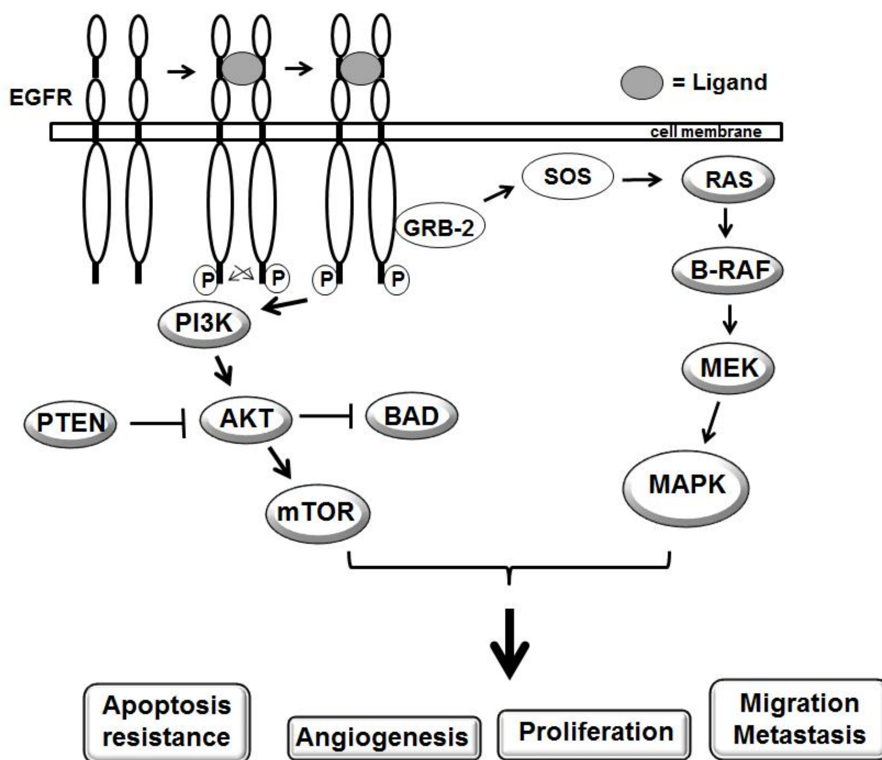


Figure 1 EGFR signaling pathway. Upon ligand binding EGF receptors form dimers and get activated with subsequent intracellular kinase domain activation. This leads to autophosphorylation and activation of downstream signaling pathways like *RAS*/*RAF*/MEK/MAPK and the PI3K pathway.
Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog; BAD, BCL2 antagonist of cell death; GRB-2, growth factor receptor bound protein 2; MAPK, mitogen activated protein kinase; MEK, mitogen activated kinase kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide kinase 3; PTEN, phosphatase and tensin homolog; SOS, son of sevenless.

Tumor cells are, however capable of evading apoptosis in several ways, for example by losing the tumor protein 53 suppressor function, by downregulating proapoptotic factors, and by increasing the expression of survival signals or antiapoptotic regulatory proteins (Hanahan & Weinberg, 2011). Furthermore autophagy, a lysosomal degradation pathway, works as a survival pathway for cancer cells by helping them to tolerate various types of stress. Autophagy may also work in an opposite fashion by eliminating damaged cells and thus prevent tumorigenesis (White & DiPaola, 2009). Necrotic cell death, in contrast to apoptosis and autophagy, releases proinflammatory signals into the tissue microenvironment, thereby recruiting protumoral inflammatory cells favoring tumor growth (Hanahan & Weinberg, 2011; Vakkila & Lotze, 2004). The loss of caretaker gene function (genes that encode genome stabilizing products) attributes to the development of genomic instability especially in inherited cancers. Our knowledge regarding the development of genomic instability in sporadic cancer is still limited (Negrini *et al.* 2010).

2.1.4 Unlimited replicative potential of cancer cells

In contrast to normal cells, cancer cells owe an unlimited replicative potential. Most cancer cells express telomerase, which elongates and thereby maintains the protective telomeric deoxyribonucleic acid (DNA) at the ends of chromosomes and causes senescence (nonproliferative but viable state of the cell) and provides resistance to apoptosis. In premalignant lesions telomere deficiency, on the other hand, seems to work in a protumoral fashion by increasing genomic alterations of the cells (Chin *et al.* 2004; Hanahan & Weinberg, 2011; Raynaud *et al.* 2010).

2.1.5 Angiogenesis

Angiogenesis occurs during fetal development, in adults during the female reproductive cycle, and wound healing. In addition, angiogenesis is induced during cancer progression to meet the increasing need for oxygen and nutrients of the growing tumor. Neovasculature is also needed for waste and carbon dioxide removal from the tumor. An important angiogenesis inducer is vascular endothelial growth factor (VEGF) A, which can be upregulated by e.g. hypoxia or various oncogenes. In addition, bone-marrow derived cells like macrophages, mast cells, neutrophils, and myeloid progenitors support angiogenesis by secreting proangiogenic growth factors or alternatively the bone marrow derived progenitor cells may get integrated as pericytes or endothelial cells into neovasculature. The blood vessels formed through pathological tumor-associated neoangiogenesis are leaky, enlarged, irregular, and characterized by abnormal blood flow, arterio-venous shunting, and microhemorrhages. The loosely joined endothelial cells facilitate the spread of malignant cells. Endogenous inhibitors of angiogenesis are also present in the body, for instance endostatin, angiostatin, and thrombospondin-1 (Hanahan & Weinberg, 2011; Potente *et al.* 2011).

2.1.6 Properties enabling invasion and metastatic dissemination of cancer cells

In order to invade and metastasize cancer cells undergo a multi-step procedure involving local invasion, intravasation into blood and lymphatic vessels, survival in the circulation, extravasation, formation of micrometastasis, and finally growth into a macroscopic tumor. Cancer cells need to weaken their cell-to-cell adhesion properties by e.g. E-cadherin down-regulation and undergo epithelial-mesenchymal transition (EMT). During EMT cancer cells become spindly shaped, have increased motility, are apoptosis resistant, and express matrix-degrading enzymes. Stromal cells, like macrophages in the tumor microenvironment contribute to the invasion and metastasis events, as well. Cancer cells may also invade through so called collective or amoeboid invasion mechanisms. The growth of a macroscopic metastasis in a new site requires adaptation of the cancer cells to the new environment. The growth into a clinically detectable metastasis can happen promptly (e.g. CRC, lung cancer, pancreatic cancer) or be proceeded by a long latency period (e.g. breast cancer, prostate cancer) (Hanahan & Weinberg, 2011; Nguyen *et al.* 2009).

2.1.6.1 Lymphangiogenesis

Lymphatic vasculature

Lymphatic vessels are important regulators of tissue fluid homeostasis. They transport extravasated protein rich fluid and macromolecules from the tissues back to the blood stream. Lymphatics are found in all vascularized tissues with some exceptions, like the brain and bone marrow. The lymphatic system includes lymphatic capillaries, precollectors, collecting lymphatic vessels, lymph nodes, efferent lymphatic vessels exiting the lymph nodes, as well as the thoracic duct and right lymphatic duct connecting with the venous system. Lymphatic capillaries are blind-ended small vessels with only one layer of lymphatic endothelial cells. They lack smooth muscle cells and pericytes, are connected to the surrounding extracellular matrix by anchoring filaments, and have button-like discontinuous junctions making them highly permeable. Lymphatic capillaries absorb approximately one tenth of the interstitial fluid extravasated from the arterial capillaries and transport it via precollector vessels and collecting lymphatic vessels back to the venous circulation (right and left subclavian veins). The collecting lymphatic vessels differ from the lymphatic capillaries in several ways: they are surrounded by a basement membrane, pericytes, and smooth muscle cells, have continuous inter-endothelial junctions and luminal valves that prevent the backflow of the lymph fluid, and are not involved in fluid adsorption from the surrounding tissues. The lymphatic vessels that transport lymph to and the ones leaving the lymph nodes are referred to as afferent and efferent lymphatic vessels, respectively. The molecular phenotype of lymphatic capillaries and collecting lymphatic vessels differ from each other also. Lymphatic vessel hyaluronan receptor (LYVE) 1, podoplanin, prospero-related homeodomain transcription factor 1 (Prox-1), and VEGF receptor (VEGFR) 3 are expressed by mature lymphatic capillaries, whereas collecting lymphatic vessel valves express Prox-1, forkhead transcription factor C2 (Foxc2) protein, and VEGFR-3 in adulthood (Achen *et al.* 2005; Norrmén *et al.* 2011; Tammela & Alitalo, 2010).

In addition to the functions mentioned above lymphatic vessels absorb dietary fats and fat-soluble vitamins in the small intestine. Disorders involving lymphatic insufficiency are characterized by accumulation of subcutaneous fat. Mice lacking Prox-1 on their endothelium or *prox-1* heterozygotic mice, that survive until adulthood, become obese and develop chylous ascites implicating that lymphatics are important in the peripheral tissues also for lipid trafficking and metabolism (Norrmén *et al.* 2011; Tammela & Alitalo, 2010). The lymphatics are also involved in immune cell trafficking. Antigens, antigen presenting cells, and leukocytes are transported via afferent lymphatic vessels to the lymph nodes, where the antigens are trapped and trigger an adaptive immune response

Lymphangiogenesis under normal conditions

Lymphangiogenesis occurs during embryonic development and is orchestrated by several molecules, including Prox-1, VEGFR-3, VEGF-C, neuropilin (NP) 2, as well as molecules responsible for blood-lymphatic vessel separation e.g. podoplanin, C-type lectin-like receptor (CLEC) 2, spleen tyrosine kinase, lymphocyte cytosolic protein 2 (Slp-76), T-synthase, angiopoietin-like protein 4, homeobox protein Meis1 and sprouty-related EVH1 domain-containing proteins 1 and 2 (Tammela & Alitalo, 2010).

The initial budding and differentiation of lymphatic endothelial cells (LEC) require Prox-1 induction in the cardinal vein by the homeobox transcription factor SOX18 (Breiteneder-Geleff *et al.* 1997; Petrova *et al.* 2002; Tammela & Alitalo, 2010). Prox-1, in turn upregulates VEGFR-3 (Petrova *et al.* 2002). Mutations in the *SOX18* gene cause hypotrichosis-lymphedema-telangiectasia characterized by lymphedema of the legs, telangiectasia, and hairloss (Irrthum *et al.* 2000).

After the budding and differentiation phases endothelial cells start to migrate in a direction of a VEGF-C gradient. The VEGF-C receptor VEGFR-3 is required for the development of the cardiovascular system and is present on both vascular and lymphatic endothelia during early embryonic development. Later on VEGFR-3 is downregulated in blood vessels and is mainly expressed on lymphatic endothelial cells with the exception of fenestrated blood vessels in e.g. the thyroid gland and pancreas. Of the VEGFR-3 ligands, VEGF-C is similarly to VEGFR-3 required for proper lymphangiogenesis, in contrast to the other ligand VEGF-D (Tammela & Alitalo, 2010). However, the inhibition of VEGF-C/VEGFR-3 signaling does not seem to affect the stable lymphatic vasculature in adults (Karpanen *et al.* 2006). VEGF-C and VEGF-D signaling through VEGFR-3 and to a lesser extent via VEGFR-2 are the most important lymphangiogenic growth factors. VEGFR-2 and VEGFR-2/VEGFR-3 heterodimers, which bind fully processed VEGF-C and –D play a role in lymphatic maturation. Mice with homozygous deletion of VEGF-C lack lymphatic vasculature, whereas VEGF-C heterozygous mice have severe lymphatic hypoplasia (Karkkainen *et al.* 2004). An inactivating point mutation of the VEGFR-3 gene causes Milroy disease, characterized by lymphatic vessel hypoplasia of the skin (Irrthum *et al.* 2000).

NP-2 is a VEGF-C and VEGF-D co-receptor expressed in lymphatic vessels and veins. NP-2 may collaborate with VEGFR-3 and thereby enhance the sensing of VEGF-C and VEGF-D growth factor gradients involved in the initial lymphatic sprout elongation (Tammela & Alitalo, 2010).

Essential for the blood-lymphatic vessel separation to occur is podoplanin, which is during early development expressed in the cardinal vein and later on lymphatic endothelial cells. Podoplanin activates the CLEC-2 receptor on platelets which in turn activates spleen tyrosine kinase and adaptor protein Slp-76, resulting in platelet aggregation at the sites of lymphatic-venous connections (Norrmén *et al.* 2011; Tammela & Alitalo, 2010).

The remodeling and maturation of the lymphatic vessel network involve several molecules e.g. angiopoietins, ephrins, FoxC2, adrenomedullin, and apoptosis stimulating protein p53. The angiopoietin growth factors and their receptors tyrosine kinase with immunoglobulin-like and EGF-like domains (Tie) 1 and 2 are important for lymphatic vessel maturation, whereas FoxC2 seems to control the differentiation of lymphatic capillary *versus* collecting lymphatic vessel phenotype (Norrmén *et al.* 2011). Loss of function mutations of *FoxC2* causes a syndrome called lymphoedema-distichiasis characterized by a double row of eyelashes, varicose veins, and late onset lymphoedema. These patients have abnormal lymphatic capillaries covered by ectopic smooth muscle cells and basement membrane as well as lymphatic collecting vessels with valve defects leading to lymph backflow (Mellor *et al.* 2007).

Lymphangiogenesis occurs in addition to embryonic development in adults during wound healing and corpus luteum development. In adulthood lymphangiogenesis occurs mainly by sprouting from pre-existing vessels (Norrmén *et al.* 2011; Tammela & Alitalo, 2010).

Lymphatic endothelial markers

In adulthood, lymphangiogenesis is also induced during pathological conditions like inflammation, cancer, and transplant rejection. The discovery of specific lymphatic endothelial markers has made it possible to study the role of lymphatics more precisely, resulting in evolving knowledge in these fields. The most widely used markers are LYVE-1, podoplanin, Prox-1, and VEGFR-3. Additional lymphatic markers include common lymphatic endothelial and vascular endothelial receptor 1 (CLEVER-1, also called Stabilin-1, FEEL-1, MS-1) and macrophage mannose receptor (MR).

LYVE-1 functions as a receptor for the extracellular matrix glycosaminoglycan hyaluronan, which is involved in cell migration and differentiation (Banerji *et al.* 1999). In adults LYVE-1 is highly expressed on endothelial cells of lymphatic capillaries in contrast to the collecting lymphatic vessels and thoracic duct (Norrmén *et al.* 2011). LYVE-1 is, however, not an exclusive lymphatic marker since it is also expressed in the sinusoids of the normal liver (Mouta Carreira *et al.* 2001) and spleen. Furthermore, subpopulations of macrophages have been found to express LYVE-1, like human placental macrophages (Böckle *et al.* 2008) and $CD68^+$ macrophages of the normal human choroid (Schroedl *et al.* 2008) as well as $CD11b^+$ macrophages in the inflamed cornea (Maruyama *et al.* 2005) and trachea (Baluk & McDonald, 2008) in mice. LYVE-1 can be downregulated in inflammatory conditions and in some tumor-associated lymphatic vessels (Van der Auwera *et al.* 2006). Moreover, hylauronan on tumor cells appears to bind to LYVE-1 on lymphatics and thereby potentially mediate lymphatic spread of cancer cells (Du *et al.* 2011).

Podoplanin is a transmembrane glycoprotein, originally found in the kidneys (Breiteneder-Geleff *et al.* 1997), where it controls the shape of visceral glomerular epithelial cells (podocytes) (Matsui *et al.* 1999). Podoplanin expression is seen on lymphatic capillaries, not blood vessels, making it a specific and valid lymphatic marker (Breiteneder-Geleff *et al.* 1997; Breiteneder-Geleff *et al.* 1999). Besides lymphatic vessels podoplanin is expressed in the heart, lung, skeletal muscle, placenta, myofibroblasts of the breast and salivary glands, mesothelial cells, osteoblasts, keratinocytes of the skin, esophagus and cervix, follicular dendritic cells and stromal reticular cells of lymphoid organs, as well as alveolar type I cells (Schacht *et al.* Am J Pathol 2005). Podoplanin induces platelet aggregation and is capable of promoting tumor cell migration and invasion in an EMT independent manner (Wicki & Christofori, 2007).

The homeo-domain protein Prox-1, which controls lymphatic endothelial cell identity, is localized in the nuclei of lymphatic endothelial cells, thereby somewhat complicating the use of this marker, especially the analysis of lymphatic vessel density by light micropsopy. Prox-1 expression is not found in blood vessels, but is expressed by non-endothelial cells in e.g. the heart, liver, pancreas, brain, and the eye lens (Van der Auwera *et al.* 2006).

VEGFR-3 (FLT4, fms-like tyrosine kinase 4) is a cell surface receptor tyrosine kinase activated by the ligands VEGF-C and VEGF-D. Besides lymphatic vessels and fenestrated blood vessels in some organs, VEGFR-3 is expressed on blood vessel endothelium in chronic wounds (Paavonen *et al.* 2000) as well as tumor-associated blood vessels in e.g. angiosarcomas (Partanen *et al.* 1999), breast cancer (Valtola *et al.* 1999), and other malignancies.

CLEVER-1 is a transmembrane protein expressed under normal conditions by sinusoidal non-continuous endothelium in the liver, spleen, adrenal cortex, bone marrow, and lymph nodes, as well as high endothelial venules (HEVs) in secondary lymphatic tissues. CLEVER-1 is present both on afferent and efferent lymphatic vessel endothelium (Irjala *et al.* 2003b) and is also found on vascular endothelia of continuous origin during pathological angiogenic conditions, like wound healing, chronic inflammation, and malignant diseases. CLEVER-1 is in addition expressed by a subset of type 2 macrophages, where it is induced by interleukin (IL) 4 and dexametason (Goerdt *et al.* 1993; Politz *et al.* 2002). CLEVER-1 positive macrophages are found in the placenta and the lungs under normal conditions as well as at sites of inflammation, wound healing, and cancer (Goerdt & Orfanos, 1999). CLEVER-1 on macrophages mediates the uptake and clearance of the ligands acetylated low density lipoprotein and secreted protein acidic and rich in cysteine (SPARC). SPARC is a component of the extracellular matrix functioning as a regulator of tissue remodeling, developmental processes, angiogenesis, obesity, diabetes, wound healing, and cancer progression (Kzhyshkowska, 2010; Kzhyshkowska *et al.* 2006). Recent data show that practically all human placental macrophages express CLEVER-1 and they are present throughout the body in a very early phase of the fetal development (week 11 onward). Placental CLEVER-1 is also capable of scavenging acetylated low density lipoprotein (Palani *et al.* 2011).

MR is a heavily glycosylated scavenger molecule clearing endogenous glycoproteins. MR is expressed both on afferent and efferent lymphatic vessels and is absent from blood vessels, including HEVs. In addition to lymphatics, MR is expressed by type 2 macrophages (Irjala *et al.* 2001). The use of MR in marker studies is complicated by the lack of anti-MR antibodies functioning in paraffin-embedded tissue samples.

Lymphangiogenesis during inflammation

Lymphangiogenesis that takes place at the sites of inflammation is needed for removal of excess interstitial fluid resulting from increased plasma leakage by inflamed blood vessels. The inflammatory cytokine tumor necrosis factor (TNF) α seems to be important for lymphangiogenesis induction during inflammation. The blocking of TNF-α signaling has been shown to reduce lymphangiogenesis during *mycoplasma pulmonis* infection in mice even though the lymphatic endothelial cells lacked TNF type 1 receptors, indicating the involvement of other mediators induced by TNF- α, like the prolymphangiogenic growth factor VEGF-C (Baluk *et al.* 2009). Indeed, in a similar chronic infection caused by *mycoplasma pulmonis* VEGF-C and –D expressed by airway epithelial cells, macrophages, neutrophils, and dendritic cells promoted lymphangiogenesis via VEGFR-3 signaling. The blocking of VEGFR-3 signaling prevented lymphangiogenesis resulting in lymphedema and airway obstruction (Baluk *et al.* 2005).

Cancer associated lymphangiogenesis
Neovascularization is required for tumor growth beyond the size of a few cubic millimeters (Folkman, 1995). Lymphangiogenesis on the other hand is not needed for tumor growth but is thought to be essential for tumor spread and for regulating tumor behavior. Lymphatic vessels provide a convenient route for cancer cell dissemination to the sentinel lymph node, which often represents the initial step of cancer metastasis. The spread of cancer to the regional lymph nodes impairs the survival of the patients and usually acquires a more aggressive treatment approach. The metastatic seeding of the malignant cells can occur either through pre-existing lymphatic vessels or via newly formed lymphangiogenic vessels. Tumor-associated lymphangiogenesis is triggered by promoting factors excreted by the tumor cells themselves or alternatively factors derived from the surrounding tumor microenvironment. In a study by Skobe *et al.* the expression of the key lymphangiogenic growth factor VEGF-C by the tumor cells increased tumor-associated lymphangiogenesis, the incidence of lymph node metastasis, and distant metastasis. VEGF-C expression did not increase angiogenesis in the breast cancer tumors studied, indicating that the increased distant metastasis observed occurred via the lymphatic system (Skobe *et al.* 2001b). The findings are in line with one study analyzing a squamous cell carcinoma (SCC) mouse model, where a high VEGF-C expression was found to associate with an increased risk for sentinel lymph node metastasis, which in turn elevated the risk for further metastasis to the distant lymph nodes as well as other distant sites. Interestingly, VEGF-C induced lymphangiogenesis both in the tumor area and within the sentinel lymph node even prior to lymph node metastasis formation (Hirakawa *et al.* 2007). In addition, VEGF-D has been shown to induce lymphatic vessel formation and increase lymph node metastasis (Stacker *et al.* 2001).

In some studies merely an increase in VEGF-C or VEGF-D expression, not the number of lymphatic vessels, has been found to correlate with increased metastasis, suggesting that not only enhanced lymphangiogenesis, but also the modulation of lymphatics (e.g. enlargement of collecting vessels, formation of intercellular gaps), as a response to VEGF-C and VEGF-D stimulation, facilitates tumor metastasis (Tammela & Alitalo, 2010). Endothelial specific adhesion molecule (ESAM) and endoglin were both found to be up-regulated in CRC related lymphatic vessels in a gene expression profile study comparing normal and tumor associated LECs, underlining the importance of qualitative changes in the lymphatics in addition to changes in their distribution and density during tumorigenesis (Clasper *et al.* 2008).

Macrophages may also promote pathological lymphangiogenesis, either by secreting VEGF-C and VEGF-D (Ji, 2011) or alternatively macrophages have the ability to differentiate into lymphatic endothelial cells (Kerjaschki *et al.* 2006; Maruyama *et al.* 2005). Macrophages are recruited to the tumor microenvironment by tumor derived chemoattractants like macrophage colony-stimulating factor (M-CSF) 1 and VEGF (Ji, 2011; Skobe *et al.* 2001a). The inhibition of M-CSF, which under normal conditions is required for differentiation of monocyte lineage cells, has been demonstrated to reduce lymphangiogenesis, tumor growth, and metastasis in e.g. a mouse sarcoma model (Kubota *et al.* 2009).

A positive association between a high lymphatic vessel density (LVD) and lymphovascular invasion (LVI) as well as an association between a high LVD and/or the presence of LVI with an elevated risk for lymph node metastasis and worse survival has been demonstrated for instance in breast cancer (Nakamura *et al.* 2005; Schoppmann *et al.* 2004). But since some studies fail to show a similar connection the subject is still somewhat unclear (Royston & Jackson, 2009).

Tumor associated lymphatics can be found either intra- or peritumorally. The amount of intratumoral lymphatic vessels reported in different studies show considerable variation. In some studies very few intratumoral lymphatics were observed e.g. in breast cancer (Schoppmann *et al.* 2004), whereas in several other studies lymphatics were present also intratumorally, for instance in breast cancer (Irjala *et al.* 2003a; Nakamura *et al.* 2005), head and neck SCC (Irjala *et al.* 2003a; Maula *et al.* 2003), papillary thyroid carcinoma (Hall *et al.* 2003), clear renal cell carcinoma (Horiguchi *et al.* 2008), and melanoma (Dadras *et al.* 2003; Straume *et al.* 2003).

Some studies indicate that the intratumoral lymphatics are dysfunctional and therefore the preferable route for cancer cell metastasis would be the functional peritumoral lymphatics (Padera *et al.* 2002). In contrast, other studies point out that in particular the intratumoral lymphatics seem to be important for the metastatic spread of cancer cells to the regional lymph nodes (Hall *et al.* 2003; Irjala *et al.* 2003a) and consequently be associated with poor survival (Horiguchi *et al.* 2008; Maula *et al.* 2003). Ki-67 positive intratumoral lymphatics have been found in melanoma studies demonstrating that active lymphangiogenesis does indeed occur also within the tumors (Dadras *et al.* 2003; Straume *et al.* 2003). Contradictory results regarding the prognostic significance of intratumoral lymphatics have been reported for instance in melanoma. Straume *et al.* observed an improved survival for melanoma patients with either high LVD intra- or peritumorally (Straume *et al.* 2003), whereas the presence of intratumoral lymphatics associated with a worse survival in another study (Dadras *et al.* 2003).

An association between a high number of peritumoral lymphatic vessels and a favorable disease outcome has been reported in head and neck carcinoma (Maula *et al.* 2003), whereas in contrast a high LVD both peri- and intratumorally was associated with lymph node metastasis in a papillary thyroid carcinoma study (Yasuoka *et al.* 2005).

The prognostic impact of CLEVER-1 positive lymphatic vessels in breast cancer and SCC of the head and neck has been studied by Irjala *et al.* who observed CLEVER-1 positive vessels intratumorally in all head and neck SCC patients that developed regional lymph node metastasis (finding statistically non-significant). In breast cancer, however, CLEVER-1 positive vessel density did not associate with lymph node involvement (Irjala *et al.* 2003a). In contrast, a recent study that examined the relationship between CLEVER-1 positive vessels and various clinicopathological variables in breast cancer patients reported the following: the density of CLEVER-1 positive blood vessels correlated positively with inflammatory cell and CLEVER-1 positive macrophage numbers, whereas the density of CLEVER-1 positive lymphatic vessels correlated positively with lymph node status, density of inflammatory cells, and hormone receptor status of the tumor (Ammar *et al.* 2011). In summary, the issue regarding the

localization of the lymphatic vessels and its importance for tumor cell spread is still controversial and unsolved (Ji, 2006).

Lymphangiogenesis in colorectal cancer

Early regional lymph node metastasis occurs frequently in CRC. In fact, almost 40% of the newly diagnosed CRC patients have regional lymph node involvement and thus belong to the Stage III disease group (Howlader *et al.* 2011). Lymph node metastasis affects survival negatively and therefore stage III patients are treated with postoperative adjuvant therapy to reduce the recurrence risk of the disease and to improve survival. For instance an advanced T category of the primary tumor, the presence of LVI (Günther *et al.* 2005), and a poor tumor differentiation grade are factors that have been shown to be associated with an increased risk for lymph node metastasis.

The number of lymphatic vessels has been demonstrated to be elevated in CRC as compared to normal gut tissue, both in early and advanced CRC (Gao *et al.* 2006; Li *et al.* 2011b; Liang *et al.* 2006; Lu *et al.* 2007a; Omachi *et al.* 2007; Parr & Jiang, 2003; Sundlisaeter *et al.* 2009). Debate regarding the existence as well as functionality of intra-tumoral *vs* peritumoral lymphatic vessels has been on-going in the field of CRC just like in other malignancies. The relationship between the number of lymphatic vessels and risk for lymph node metastasis has also been a subject of controversy. As an example, podoplanin expression measured with quantitative real-time PCR has been found higher in tumors of patients with lymph node metastasis than those with node negative disease. However, in the same study LYVE-1 expression did not differ between the two stage groups (Lu *et al.* 2007b). In contrast, Sundlisaeter *et al.* found no difference in number of podoplanin positive lymphatic vessels between stage II and III CRC (Sundlisaeter *et al.* 2009). The impact of LVD on lymph node involvement and to a lesser extent on disease outcome in CRC has been investigated in several studies, as well. The results reported show conflicting results and therefore the role of lymphatic vessels in CRC is not clear. The association of LVD and LVI with lymph node metastasis and survival in CRC according to previously published results are shown in **Table 1.**

Several studies have attempted to clarify the association between the vascular endothelial growth factors (VEGF-A, VEGF-C, and VEGF-D) and their receptors with CRC-associated lymphangiogenesis. The VEGF-C level has been shown to be higher in CRC tissue than in corresponding normal tissue (Akagi *et al.* 2000; George *et al.* 2001; Li *et al.* 2011b) and a higher expression has been found in the peritumoral region than intratumorally (Lin *et al.*; Lin *et al.* 2011). VEGF-C associates with presence of LVI (Akagi *et al.* 2000; Lin *et al.* 2011), lymph node involvement (Akagi *et al.* 2000; Li *et al.* 2011b; Lin *et al.* 2011; Zhong *et al.* 2009), and LVD (Li *et al.* 2011b; Zhong *et al.* 2009) according to some reports. On the other hand, some studies have not found an association between VEGF-C expression level and lymphatic spread (George *et al.* 2001). The results regarding the effect of VEGF-C expression on CRC survival are likewise conflicting, since for instance in a study by Li *et al.* patients with VEGF-C positive tumors had shorter disease-free survival (DFS) and overall survival (OS) than patients with VEGF-C negative tumors (Li *et al.* 2011b), whereas others have failed to confirm such an association (Akagi *et al.* 2000; Lin *et al.* 2011).

Table 1. Association of LVD and LVI with colorectal cancer prognosis.

Study	Stage of disease	Number of patients	Lymphatic marker used	Method used	LVD assessed	LVI assessed	Region of interest: Peri- vs intratumoral	Proliferative marker used	Proliferative lymphatic vessels detected	Association of LVD with node positivity	Association of LVI with node positivity	Association of LVD with survival	Association of LVI with survival
Liang *et al.* Virchows Arch, 2006	I, III	87	Podoplanin	IHC	Yes	Yes	Both	Ki-67	No	Yes (PT)	Yes* (PT)	N/A	N/A
Liang *et al.* Ann Surg Oncol, 2006	I, II, III	419	Podoplanin	IHC	No	Yes	Not reported	No	N/A	N/A	Yes	N/A	Yes
Matsumoto *et al.* Dis Colon Rectum 2006	I, II, III, IV	106	Podoplanin	IHC	Yes	Yes	Both	No	N/A	No	Yes	Yes* (IT)	Yes
Saad *et al.* Mod Pathol, 2006	I, II, III, IV	90	D2-40 (podoplanin)	IHC	Yes	Yes	Intratumoral	No	N/A	Yes* (IT)	Not reported	N/A	N/A
Omachi *et al.* Cancer Letters, 2007	I, II, III, IV	64	Podoplanin	IHC	Yes	Yes	Intratumoral	Ki-67	Yes	No	Not reported	No	N/A
Kaneko *et al.* Dis Colon Rectum, 2007	I, III	268	Podoplanin	IHC	Yes	Yes	Intratumoral	No	N/A	Yes*	Yes	N/A	N/A
Duff *et al.* Colorectal Dis, 2007	I, II, III, IV	30	LYVE-1	IHC	Yes	Yes	Both	No	N/A	No	Not reported	N/A	N/A
Ishikawa *et al.* Cancer, 2008	I, III	71	LYVE-1	IHC	No	Yes	Both	No	N/A	N/A	Yes* (PT)	N/A	N/A
Yan *et al.* World J Gastroenterol, 2008	I, II, III	132	D2-40 (podoplanin)	IHC	Yes	No	Both	No	N/A	Yes (PT)	N/A	Yes (IT)	N/A
Longatto-Filho *et al.* Virchows Arch, 2008	I, II, III, IV	120	D2-40 (podoplanin)	IHC	Yes	Yes	Both	No	N/A	No	Yes (PT)	N/A	N/A
Zhong *et al.* Cancer Biol Ther, 2009	I, II, III, IV	50	Podoplanin	IHC	Yes	No	Not reported	No	N/A	Yes	N/A	N/A	N/A
Li *et al.* J Gastrointest Surg, 2011	I, II, III, IV	147	D2-40 (podoplanin)	IHC	Yes	No	Intratumoral	No	N/A	Yes	N/A	Yes (IT)	N/A
Akishima-Fukasawa, Histopahtology, 2011	I, III	111	LYVE-1	IHC	No	Yes	Both	No	N/A	N/A	Yes*	N/A	N/A

Abbreviations: *, significant association also in multivariate analysis; IHC, immunohistochemistry; LVD, lymphatic vessel density; LVI, lymphatic vessel invasion; IT, intratumoral; PT, peritumoral; N/A, not applicable

Affects patient outcome negatively [boxed cells]
Association found also in multivariate analysis*

Elevated VEGF-A protein levels are in a similar fashion to VEGF-C seen in CRC tissue as compared to normal intestinal tissue (George *et al.* 2001), with the highest expression present in the peritumoral region as well (Lin *et al.* 2011). Patients with high VEGF-A expressing tumors seem to have more commonly lymph node involvement (George *et al.* 2001) and a worse 5-year survival (Lin *et al.* 2011) than patients with low VEGF-A expressing tumors.

The results regarding VEGF-D expression in CRC tissue and the association of it with lymphatic spread have been conflicting. Elevated VEGF-D levels were observed in CRC tissue and high VEGF-D expression correlated with increased lymph node metastasis, elevated risk for tumor recurrence as well as decreased DFS and OS in one study (White *et al.* 2002). In contrast, another study observed the highest VEGF-D levels in normal gut tissue and found no association between VEGF-D expression and lymphatic spread of CRC (George *et al.* 2001).

The role of chemokine receptors in lymphatic spread of CRC has in addition been investigated. A high chemokine receptor (CCR) 7 expression measured by immunohistochemistry (IHC) at the invasive front of CRC primary tumors associated positively in multivariate analysis with lymph node metastasis, distant metastasis, and poor survival in a study by Günther *et al.* (Günther *et al.* 2005).

2.1.6.2 Leukocyte and cancer cell trafficking

Lymphocytes recirculate continuously from the blood to the tissues and back to the blood circulation via the lymphatic vessels and lymph nodes. This trafficking of leukocytes is essential for the immune system in order to function properly and is regulated by homing associated adhesion molecules, chemokines, and enzymes. Cancer cells utilize the same routes and in part the same molecules as trafficking leukocytes for metastatic spread. During the last twenty years several molecules have been implicated in leukocyte trafficking. However, only some selected ones are discussed below.

Trafficking in blood vessels

The multistep process by which leukocytes migrate mainly through postcapillary venules into peripheral tissues and through the high endothelial venules to the lymph nodes is referred to as the extravasation cascade. The trafficking of leukocytes is regulated by sequential interactions of molecules expressed on endothelial cells and leukocytes. During normal conditions lymphocytes extravasate to secondary lymphoid tissues like peripheral lymph nodes, whereas during inflammation the leukocytes are capable of migrating to the sites of inflammation in the peripheral tissues. The multistep extravasation cascade starts with the tethering (secondary leukocyte capture) and rolling of the leukocytes on the blood vessel endothelium. Molecules involved in these steps are the E- (expressed by inflamed endothelial cells), P- (expressed by inflamed endothelial cells and platelets), and L-selectins (expressed by leukocytes), which interact with P-selectin glycoprotein ligand 1 as well as other ligands. Thereafter chemokines and their receptors on the leukocytes activate integrins resulting in arrest and firm adhesion of the leukocytes to the vascular endothelium. The firm adhesion is mediated by integrins binding to adhesion molecules belonging to the immunoglobulin

superfamily. Transmigration through the blood vessel wall (endothelial cell barrier, basement membrane, pericyte sheaths) is the final step in the leukocyte extravasation cascade. Prior to transmigration, which can occur either through a paracellular or transcellular route leukocytes crawl (lateral migration) on the vascular endothelium, which involves the integrin MAC1 on leukocytes and its ligand ICAM (intercellular adhesion molecule) 1 on the endothelium. Several molecules are involved in the leukocyte transmigration process, e.g. the integrins lymphocyte function associated antigen (LFA) 1, MAC1, very late antigen (VLA) 4, the immunoglobulin superfamily member PECAM1, and CD99 on leukocytes as well as the immunoglobulin superfamily members ESAM, JAM-A, JAM-B, JAM-C, PECAM-1, and ICAM-2 on endothelial cells (Jalkanen & Salmi, 2008; Ley *et al.* 2007; Salmi & Jalkanen, 2005).

CLEVER-1 on vascular endothelium has also been shown to participate in the leukocyte extravasation cascade by being involved in the rolling and transmigration of peripheral blood mononuclear cells (Salmi *et al.* 2004). The blocking of CLEVER-1 by antibodies *in vivo* was demonstrated to inhibit lymphocyte, monocyte, and granulocyte trafficking through the blood vessels to the sites of inflammation in a study by Karikoski *et al.* The normal immune responses of the host were not notably affected by the antibody therapy (Karikoski *et al.* 2009). In addition, CLEVER-1 on placental macrophages mediates their adhesion to the vascular endothelium and transmigration through the vessel wall (Palani *et al.* 2011). Besides the molecules mentioned above ectoenzymes like vascular adhesion protein-1 (VAP-1) and CD73 are involved in the leukocyte extravasation cascade. VAP-1 is a semicarbazide-sensitive amine oxidase expressed on endothelial cells, adipocytes, and smooth muscle cells. VAP-1 is also present in a soluble form in the plasma. The VAP-1 enzyme deaminates amines in a two-step catalytic reaction generating an aldehyde, ammonium, and hydrogen peroxidase end-products. VAP-1 plays a role in leukocyte rolling, firm adhesion, and transmigration. In VAP-1 deficient mice leukocyte rolling is faster and the adhesion and transmigration of leukocytes are impaired. VAP-1 is involved in the leukocyte adhesion cascade and leukocyte migration both as an adhesion molecule and via its catalytic activity (Jalkanen & Salmi, 2008; Salmi & Jalkanen, 2005; Salmi & Jalkanen, 2011). Another ectoenzyme, named CD73 is also involved in leukocyte adhesion and transmigration, which is discussed in detail in chapter 2.3.2.

Trafficking in lymphatic vessels

Naïve T cells recirculate mostly between the blood and secondary lymphoid organs, e.g. lymph nodes, which they enter via HEVs. In contrast, effector T cells and memory T cells migrate into non-lymphoid tissues and into sites of inflammation and infection returning thereafter back to the lymph nodes via the afferent lymphatic vessels. The same route is used by dendritic cells, granulocytes, macrophages, and antigens, whereas the majority of the lymphocytes utilize the HEVs for this purpose. The only leukocyte subpopulation capable of leaving the lymph nodes via the efferent lymphatic system, comprising the lymphatic sinusoids and efferent lymphatic vessels, are the lymphocytes. The trafficking of leukocytes in the lymphatics has been less studied than in the blood vessels but nevertheless some molecules participating in this event have been discovered, for instance MR, CLEVER-1, sphingosine 1 receptor, and CCR7-

CCL21. MR mediates lymphocyte binding to sinusoidal endothelial cells at the site of lymphocyte exit from the lymph nodes *in vitro*. L-selectin on lymphocytes is involved in this event (Irjala *et al.* 2001). A study using $MR^{-/-}$ mice revealed that MR on afferent lymphatics is involved in the trafficking of B and T cells to the draining lymph nodes and demonstrated further proof for the role of MR in lymphocyte binding to the endothelium of lymphatic sinuses (Marttila-Ichihara *et al.* 2008). CLEVER-1 is another molecule mediating the binding of lymphocytes to lymphatic endothelium in the lymph node sinusoids and participating in the trafficking of lymphocytes in the lymphatic vessels (Irjala *et al.* Eur J Immunol 2003). In addition, *in vitro* work show that endothelial CLEVER-1 is involved in leukocyte transmigration in the lymphatics (Salmi *et al.* Blood 2004). The blocking of CLEVER-1 by antibodies *in vivo* was demonstrated to prevent lymphocyte trafficking to the lymph nodes via the afferent lymphatic vessels (Karikoski *et al.* 2009). C-C chemokine receptor 7 (CCR7) expressed by activated dendritic cells and T lymphocytes is important for proper trafficking of these immune cells via afferent lymphatic vessels to the draining lymph nodes. The binding partner of CCR7 is chemokine C-C motif ligand (CCL) 21 on the lymphatics (Britschgi *et al.* 2010; Debes *et al.* 2005). The exit of lymphocytes from lymph nodes is regulated by the lysophospholipid sphingosine 1-phosphate receptor (Mandala *et al.* 2002; Rosen *et al.* 2003).

Tumors are capable of utilizing the same trafficking mechanism as leukocytes for malignant cell invasion to the lymphatics and migration via afferent lymphatics to the lymph nodes. VEGF-C secreted by tumor cells increase CCL21 secretion by the afferent lymphatic endothelial cells and lymph nodes, which in turn enhances the chemotaxis of CCR7 expressing tumor cells toward the lymphatics (Issa *et al.* 2009). In addition, the adhesion molecules MR and CLEVER-1 on lymphatic endothelial cells mediate malignant cell adhesion to the lymphatics and thereby control their trafficking (Irjala *et al.* 2003a). Cancer cell migration to draining lymph nodes is impaired in $MR^{-/-}$ mice (Marttila-Ichihara *et al.* 2008).

2.1.7 Cancer and inflammation

Chronic inflammatory conditions, triggered by microbes, autoimmune, or inflammatory diseases increase cancer risk at the sites of inflammation. Tobacco smoking, asbestosis, or obesity may also cause chronic inflammation and thereby increase cancer risk (Grivennikov *et al.* 2010). By contrast, an initial genetic alteration can give rise to an inflammatory microenvironment that promotes tumor growth. These two opposite pathways are referred to as the extrinsic (the former) and intrinsic (the latter) pathways linking cancer and inflammation to each other (Mantovani *et al.* 2008).

Most malignant tumors are infiltrated by immune cells and cancer related inflammation has been shown to affect all parts of tumorigenesis. The immune cells in the tumor microenvironment comprise cells representing the innate (macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, natural killer (NK) cells, dendritic cells) and adaptive immune system (B and T lymphocytes), the most abundant cell types being the T-cells and TAMs. The immune cells may function, either anti- or pro-

tumorally and interestingly only the NK cells, which act in an antitumoral fashion, lack this dual role (Grivennikov *et al.* 2010). The phases of tumor-host immune system interaction (immunoediting) in cancer include: the elimination phase, which aims at tumor eradication, the latent equilibrium phase, and finally the escape phase, when tumor cells evade the immune responses (Dunn *et al.* 2004).

2.1.7.1 Tumor initiation

During tumor initiation reactive oxygen species and reactive nitrogen intermediates produced by inflammatory cells induce DNA damage, genomic instability, and mutations in cells nearby. Mutagenesis may inactivate mismatch repair enzymes thereby further increasing mutagenesis and eventually lead to inactivation of tumor suppressor genes. Inflammation induced epigenetic mechanisms may also contribute to tumor initiation (Grivennikov *et al.* 2010).

2.1.7.2 Transcription factors

Genetic alterations in cancer cells activate transcription factors like nuclear factor-κB (NF-κB), signal transducer and activator of transcription (STAT) 3, and activated protein (AP) 1 in the tumor cells. Transcription factors are proteins that control the transcription of genetic information from DNA to messenger ribonucleic acid (mRNA) by binding to specific DNA sequences. The transcription factors regulate the production of inflammatory mediators (e.g. chemokines and cytokines) and cyclooxygenase (COX) 2 of the tumor cells. Prostaglandins produced by COX-2, chemokines, and cytokines recruit inflammatory cells to the tumor microenvironment and activate their transcription factors. As a consequence, the inflammatory cells start to produce inflammatory mediators too (Mantovani *et al.* 2008). Cancer related chronic inflammation is sustained through this positive feedback loop.

NF-κB and STAT-3, which are activated in most cancers, including CRC, activate genes involved in cell proliferation, growth, angiogenesis, survival, invasiveness, motility and, as already mentioned, the production of chemokines and cytokines. NF-κB, the key regulator of genes involved in cancer-related inflammation, has an important role both in controling the innate and adaptive immune responses as well as the inflammation linked carcinogenesis. Bacteria, viruses, necrotic cell products, and inflammatory cytokines activate NF-κB on inflammatory cells, which once activated produce growth and survival signals, inflammatory cytokines, and angiogenic factors. The activation of NF-κB on cancer cells, on the other hand, leads to increased expression of proteases [e.g. matrix metalloproteinase (MMP) 9], apoptosis inhibitors, and cell cycle genes (e.g. cyclin-D1) thereby promoting tumor formation and progression. Interestingly, NF-κB in epithelial cells, not inflammatory cells, has been shown to oppose tumor formation in certain circumstances, like chemically induced skin and liver cancers. NF-κB has been reported to be associated with therapy induced senescence and according to two recent lymphoma studies, mutations in the NF-κB pathway leads to upregulation of the *BCL2* oncogene and thus increased tumor cell survival. In contrast, in those tumors, where the prosurvival signal originates from e.g. *BCL2* overex-

pression independent of NF-κB, NF-κB enhances tumor sensitivity to cytotoxic chemotherapy and works in an opposite antitumoral fashion (Chien *et al.* 2011; Jing *et al.* 2011; Klein & Ghosh, 2011).

2.1.7.3 Cytokines

Cytokines are small cell-signaling proteins involved in intercellular communication. They are produced by many cell types including tumor cells and inflammatory cells and can exhibit either protumoral, antitumoral, or dualistic effects.

TNF-α, a key regulator of inflammation and host defence has the ability to act in an antitumoral fashion. It can be used in the treatment of melanoma and soft tissue sarcomas by using an isolated limb perfusion technique. In contrast, by activating the transcription factor NF-κB, TNF-α generates tumor promoting effects via NF-κB mediated upregulation of genes involved in tumor cell proliferation, invasion, survival, angiogenesis, and metastasis. The other members of the TNF superfamily mediate mainly antitumoral effects (Aggarwal *et al.* 2012). IL 6 has a complex role in cancer. It mediates tumor cell proliferation by activating the *RAS*/*RAF*/MEK/MAPK pathway and activates STAT3 thereby promoting cell cycle progression. Furthermore IL-6 increases angiogenesis, mediates anti-apoptotic effects, and promotes immune escape of tumor cells (Ara & Declerck, 2010). IL-17 produced by Th17 cells can work both in an anti- or protumoral manner. By recruiting neutrophils to the tumor microenvironment, by promoting the cytolytic effects of NK cells and cytotoxic T lymphocytes, and maturation of dendritic cells IL-17 generates antitumoral effects. The promotion of angiogenesis and the creation of a chronic inflammatory state in the tumor microenvironment are factors linking IL-17 on the other hand to tumor promotion (Murugaiyan & Saha, 2009). IL-12 and IL-23, members of the IL-12 family of proinflammatory heterodimeric cytokines, are mainly produced by dendritic cells, phagocytes, and activated antigen presenting cells. IL-12 activates cytotoxic T cells and NK cells, promotes Th1 adaptive immunity, and induces interferon (IFN) γ production thus generating antitumoral effects. IL-23 exerts dual roles in the tumor microenvironment by increasing the production of IFN-γ and IL-12 by activated T cells and by activating Th17 cells. TNF-related apoptosis-inducing ligand (TRAIL), mainly produced by NK cells and activated T cells, is a member of the TNF superfamily. TRAIL binds to death receptors 4 or 5 and induces apoptosis thereby generating antitumoral effects. IL-10 is an activator of STAT3 and inhibitor of NF-κB with opposing roles on tumor development. It acts mainly in an antitumoral manner by inhibiting tumor development and progression. It modulates apoptosis, exerts anti-angiogenic effects, and is furthermore capable of mediating antitumor activity of regulatory T cells (Tregs). The tumor promoting effects are mainly mediated via STAT3. Transforming growth factor (TGF) β functions in contrast, as a mediator of the immunosuppressive activity of Tregs. TGF-β may act in an antitumoral fashion, as well (Lin & Karin, 2007).

The involvement of cytokines in various malignancies has been investigated. For instance IL-6, IL-11, IL-23, TNF-α, and IL-1β seem to be involved in colon cancer progression, IL-11 in gastric cancer, TNF-α and IL-6 in hepatocellular carcinoma

(Grivennikov *et al.* 2010), IL-6 in multiple myeloma, Hodgkin`s lymphoma, breast cancer and Kaposi sarcoma, IL-17 in cervical cancer, non-small cell lung carcinoma, and fibrosarcoma (Lin & Karin, 2007).

2.1.7.4 Chemokines

Chemokines are soluble heparin binding proteins which are important regulators of cancer related inflammation and cell migration. They are divided into four subgroups: chemokine (C-X-C motif) ligand (CXCL), CC chemokine ligand (CCL), CX_3CL and CL, and bind to chemokine receptors on epithelial and endothelial cells, cancer associated fibroblasts, and cancer cells. The most abundant chemokine receptor expressed by cancer cells is CXCR4 enabling the migration of the malignant cells towards the ligand CXCL12. Chemokines may be induced by e.g. NF-κB, hypoxia inducible factor (HIF) 1α as well as other transcription factors. Chemokines are involved in EMT, cell proliferation, cell motility, and apoptosis inhibition, thereby promoting tumor growth, invasion, and metastasis. Chemokines recruit TAMs, myeloid-derived suppressor cells (MDSCs), and Tregs thus creating an immunosuppressive tumor microenvironment. On the contrary, chemokines are able to inhibit tumorigenesis in the early phases of cancer through senescence induction. Recruitment of tumor infiltrating lymphocytes and dendritic cells to the tumor microenvironment by chemokines functions also in an antitumoral manner. CCL-2 can activate NK cells, which oppose tumor growth. In addition, chemokines can either promote or inhibit angiogenesis by affecting endothelial cells directly or alternatively in an indirect fashion by recruiting angiogenic growth factor secreting inflammatory cells. The inflammatory cells may also acquire endothelial cell phenotypes and get incorporated into the neovasculature (Mukaida & Baba, 2012). Especially CCL-2 seems to play an important role in CRC (Erreni *et al.* 2011).

2.1.7.5 Antitumoral immune response

The immune system (both the innate and adaptive) and in particular the $CD8^+$ cytotoxic T lymphocytes, $CD4^+$ Th_1 helper T cells, and NK cells are important for preventing tumor development and progression. Evidence for this has emerged from e.g. murine models and the observation that in some human cancers a high density of inflammatory cells is associated with a good prognosis (Hanahan & Weinberg, 2011). The increased risk of malignancies observed in immunosuppressive patients further supports this (Roithmaier *et al.* 2007). The immune system can function in an antitumoral fashion by opposing viral infections, by performing timely pathogen elimination and resolution of inflammation, or by eliminating tumor cells.

The immune system reacts against tumor specific or tumor-associated antigens, which are presented as peptides to cytotoxic $CD8^+$ T cells by antigen presenting cells e.g. dendritic cells. Factors contributing to the development of a proinflammatory tumor microenvironment include granulocyte macrophage colony stimulating factor (GM-CSF), tumor antigens, products of dying cells, macrophages and dendritic cells which present antigens and respond to danger and stress signals, as well as various immunoregulatory or cytotoxic cytokines (IFN-α, IFN-β, IFN-γ, Fas ligand, TRAIL,

IL-12) (Finn, 2008; Grivennikov *et al.* 2010). IFN-γ, an important antitumor effector, upregulates tumor immunogenicity, promotes the production of $CD4^+$ Th_1 T cells and cytolytic T cells, and activates cytotoxic characteristics of macrophages. Perforin and TRAIL are important cytolytic molecules involved in immunosurveillance (Dunn *et al.* 2004). NK cells and cytotoxic T lymphocytes acquire their cytotoxic effects via TRAIL, perforin, granzyme B, or death-inducing Fas ligand, Th1 cells via IFN-γ production, and Th17 cells via IL-17A production (Grivennikov *et al.* 2010).

2.1.7.6 Tumor escape phase

Failure of the immune system to control tumor growth is referred to as the tumor escape phase. Several factors contribute to this phase, e.g. cancer cells themselves by expressing immunosuppressive factors like TGF-β, Fas ligand, and indolamine-2,3-dioxygenase. Reduced immunogenicity of the tumor or major histocompability complex (MHC) downregulation, *FAS* gene and TRAIL receptor death receptor 5 gene mutations, over-expression of anti-apoptotic BCL-XL and FLICE like inhibitory protein, as well as the recruitment of immunosuppressive leukocytes by VEGF, IL-1β, and GM-CSF are additional protumorigenic factors of note. The different leukocyte subpopulations are important components of the immunosuppressive machinery for instance type 2 TAMs, which acquire immunosuppressive abilities by expressing e.g. TGF-β, arginase, and IL-10 (Mukaida & Baba, 2012). Tregs, on the other hand, suppress antitumoral effector T-cells by producing TGF-β and IL-10 (Finn, 2008).

2.1.7.7 Metastatic phase

Cancer related inflammation plays a role also in the metastatic phase of cancer. For metastatic spread, tumor cells utilize adhesion molecules (e.g. selectins), chemokines and their corresponding receptors, which are normally involved in leukocyte trafficking. In addition, myeloid cells, T lymphocytes, and cancer cells produce TGF-β, a cytokine regulating EMT and metastasis. The cytokine TNF-α stabilizes Snail, a repressor of E-cadherin transcription during EMT. Proteases, like MMP2 and MMP9 produced by inflammatory cells enable cancer invasion through the extracellular matrix. Prostaglandins, cytokines, and MMPs control the intravasation step, which is directed via specific chemokine receptors towards a chemokine gradient. Once within the circulation metastatic cancer cells are assisted to survive by cytokines, including TNF-α, epiregulin, and IL-6. Interestingly, cancer cells may escape immunosurveillance in the circulation by traveling together as clusters with macrophages or platelets. The circulating cancer cells finally attach to the endothelium in an integrin-dependent manner, which is followed by extravasation (Grivennikov *et al.* 2010).

The immunologic responses in cancer are complex and seem to be affected by the tumor location, tumor type, cytokine profile, and inherent immunity (Dunn *et al.* 2004). Open questions still remain in the interactions between the tumor and the immune system of the host and, therefore immunoevasion has only been proposed to represent an emerging hallmark of cancer at this point (Hanahan & Weinberg, 2011).

2.1.7.8 Colorectal cancer and inflammation

A link between CRC and inflammation is evident. For instance, more than 20% of patients with chronic inflammatory bowel diseases will eventually develop CRC (Terzić *et al.* 2010). Furthermore, the long-term use of either aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to reduce CRC risk in the general population (Chan *et al.* 2005) and among Lynch syndrome patients (Burn *et al.* 2011). Furthermore, prediagnostic NSAID use (Coghill *et al.* 2011) and the use of aspirin in Stage I - III CRC after the diagnosis and primary tumor removal is associated with improved survival in patients with COX-2 overexpressing tumors (Chan *et al.* 2009). The protective role of aspirin and NSAIDs in CRC results from their inhibition of COX-1 and -2 thereby preventing the production of prostaglandins from arachidonic acid. The protumorigenic COX-2 pathway is involved in e.g. cell proliferation, angiogenesis, inhibition of apoptosis, invasion, and immunosuppression. Elevated COX-2 expression is often present in CRC and a high expression is more commonly observed in metastases than primary tumors. A high COX-2 expression associates with poor survival in CRC (Soumaoro *et al.* 2004) like in other malignancies e.g. gastric cancer (Thiel *et al.* 2011). COX-2 has been shown to correlate with VEGF-C expression in CRC (Soumaoro *et al.* 2004) similar to e.g. papillary thyroid cancer (Siironen *et al.* 2006).

Further proof for the connection between CRC and inflammation is provided by numerous studies addressing the relationship between inflammatory cell infiltrates and CRC prognosis. In general, a high number of inflammatory cells both intra- and peritumorally seems to be associated with a favorable CRC outcome. The immune cell subtypes have been studied more in detail in several studies. CD3 is used as a pan-T-lymphocyte marker and a majority of the studies reported today link a high number of $CD3^+$ T cells to improved CRC survival (Roxburgh & McMillan, 2011). For instance, Galon *et al.* investigated CD3, CD8, and CD45RO positive T cells as well as the cytotoxic molecule granzyme B in CRC and showed in three independent patient cohorts that the combination of high peritumoral and high intratumoral $CD3^+$ T cell counts was an independent positive predictor of DFS and OS, even superior to the Tumor (T) Node (N) Metastasis (M) classification of malignant tumors (TNM) (Galon *et al.* 2006). However, in a study by Nosho *et al.* intratumoral $CD3^+$ cell density did not associate with survival in stage I-IV CRC (Nosho *et al.* 2010). Laghi *et al.* found no association between a high peritumoral $CD3^+$ T cell density and survival in stage III CRC either, in contrast to stage II disease, where a high $CD3^+$ T lymphocyte number predicted improved disease-specific survival (DSS) and DFS (Laghi *et al.* 2009). Additional studies investigating the prognostic role of inflammatory cell subtypes in CRC showed an association between a high cell number and good prognosis in 5 out of 6 $CD4^+$ helper T-lymphocyte studies, in 9 out of 9 $CD45RO^+$ memory T-lymphocyte studies, 20 out of 25 $CD8^+$ cytotoxic lymphocyte studies, 4 out of 4 NK cell studies, 4 out of 6 dendritic cell studies, 5 out of 6 eosinophil studies and 4 out of 4 neutrophil studies. Studies elucidating forkhead box P3 $(FOXP3)^+$ Tregs in CRC have been more conflicting (Roxburgh & McMillan, 2011).

Zlobec *et al.* investigated the prognostic impact of CD8^{+} cytotoxic T cells, CD68^{+} macrophages and FOXP3^{+} Tregs in the microenvironment of tumor budding in both mismatch repair deficient and proficient CRC. A high number of all cell types examined, also FOXP3^{+}cells, affected the survival positively. The authors proposed the use of a prognostic score combining the good prognosis markers CD8, CD68, and FOXP3 with the poor prognosis feature tumor budding (Zlobec *et al.* 2011). Another recent study evaluated a combined score, including CD3^{+}, CD8^{+}, and granzyme B^{+} cell densities at the invasive margin of CRC liver metastases. The impact of the score on chemotherapy response, progression-free survival (PFS), and OS were analyzed. A high density of inflammatory cells associated with improved treatment responses as well as longer PFS and OS. FOXP3^{+} T cells cells were also analyzed, but they were very rare and did not associate with treatment responses (Halama *et al.* 2011).

Interestingly, colorectal tumors with a high level of microsatellite instability (MSI-H) and thereby a high mutation frequency are often infiltrated by an excessive number of lymphocytes, which has been shown to be associated with a favorable disease outcome (Swann & Smyth, 2007). One explanation for the detrimental effects observed as a response to adjuvant chemotherapy in patients with MSI-H CRC tumors could be the weakened host immune response caused by the cytotoxic treatment (Nosho *et al.* 2010).

2.1.7.9 Tumor associated macrophages in cancer

Macrophages are important players both in the innate and adaptive immune system. Macrophages phagocytize pathogens, apoptotic, and malignant cells and thus provide prompt protection as a response against foreign intruders. In addition, macrophages regulate the adaptive immune responses (Sica *et al.* 2008). TAMs, that constitute the key component of cancer related inflammation, are recruited from the vasculature as monocytes to the tumor microenvironment by e.g. VEGF, angiopoietin-2, and chemokines (Mukaida & Baba, 2012). TAMs are plastic cells with dual influence on cancer behavior, functioning either anti- or protumorally. Factors in the tumor microenvironment strongly influence the phenotype of TAMs. The so called type 1 (M1) and type 2 (M2) macrophages represent the two extremes of the macrophage polarization gradient. The classification of macrophages into three main categories; classically activated macrophages, regulatory macrophages, and wound-healing macrophages, has also been suggested (Mosser & Edwards, 2008).

Classically activated antitumoral type 1 macrophages have cytotoxic effects on tumor cells. They are induced by IFN-γ and bacterial lipopolysaccharides (LPS) and have a high antigen presenting capacity. Type 1 macrophages produce high amounts of e.g. IL-12 and IL-23, thereby activating T-cell responses and by secreting cytotoxic factors like nitric oxide, reactive oxygen intermediates, and TRAIL they promote the killing of tumor cells (Allavena *et al.* 2008; Mantovani & Sica, 2010; Mosser & Edwards, 2008).

Type 2 macrophages occur naturally in the placenta and in the lungs and are increased in chronic inflammatory diseases like rheumatoid arthritis. Two distinct stimuli are required for the induction of an anti-inflammatory type 2 macrophage phenotype

(Mosser & Edwards, 2008). Type 2 macrophages represent the majority of TAMs in most malignant tumors. TAMs accumulate in the hypoxic regions of tumors and they, in contrast to the type 1 macrophages, promote tumor growth, angiogenesis, and suppress adaptive immunity. The protumoral, immunosuppressive type 2 macrophages are induced by e.g. adenosine, IL-4, IL-10, IL-14, glucocorticoids, TGF-β and they express IL-10, TGF-β, MR, CLEVER-1, CD163, arginase, scavenger receptor A, and downregulate MHC class II and IL-12 expression. The expression of IL-10, TGF-β, and indeoleamine dioxigenase as well as defective activation of NF-κB, are examples of factors contributing to the immunosuppressive effects of type 2 macrophages (Csóka *et al.* 2012; Mantovani & Sica, 2010; Sica *et al.* 2008). Type 2 macrophages induce Tregs (Savage *et al.* 2008) and are not capable of triggering Th1 immune responses, which further increase their immunosuppressive traits.

Transcription factors (NF-κB, AP-1, STAT3) on TAMs regulate proangiogenic genes like *HIF-1α* and *VEGF*. During hypoxia HIF-1α stimulates the expression of CXCL12, an angiogenesis trigger. CXCL12 activates and recruits CXCR4 positive endothelial cells to the tumor microenvironment thus promoting angiogenesis. Furthermore TAMs express the prolymphangiogenic factors VEGF-C, VEGF-D, and VEGFR-3. Macrophages may also secrete enzymes capable of modifying the extracellular matrix (e.g. MMPs, cathepsins) thereby facilitating invasion and metastasis of cancer cells and activation of EMT (Hanahan & Weinberg, 2011).

Factors contributing to macrophage recruitment to the tumor microenvironment, factors affecting the polarization of the macrophages as well as selected type 1 and 2 macrophage characteristics are shown in **Figure 2**.

The impact of TAMs on cancer prognosis has been elucidated in several studies. Most studies have used the pan-macrophage marker CD68 but some studies have investigated the subpopulation of type 2 macrophages, as well. A high number of $CD68^+$ macrophages associated with poor prognosis in a study including classical Hodgkin´s lymphoma patients (Steidl *et al.* 2010), whereas only a borderline association was found in a study by Zaki *et al*. When they examined the subgroup of patients with mixed cellularity Hodgkin`s disease the opposite was found, a high type 1 macrophage number ($HLA\text{-}DR^+CD68^+$) associated with a favorable prognosis (Zaki *et al.* 2011). In follicular lymphoma a high $CD68^+$ macrophage density is associated with poor disease outcome, which interestingly can be circumvented by anti-CD20 rituximab treatment (Canioni *et al.* 2008; Taskinen *et al.* 2010). A high $CD68^+$ macrophage number associated with poor prognosis in intrahepatic cholangiocarcinoma (Hasita *et al.*), lung adenocarcinoma (Zhang *et al.* 2011), uveal melanoma (Bronkhorst *et al.* 2011), and Ewing sarcoma (Fujiwara *et al.* 2011). $CD68^+$ macrophage density does not seem to associate with clinical outcome in breast cancer (Mahmoud *et al.* 2011; Murri *et al.* 2008).

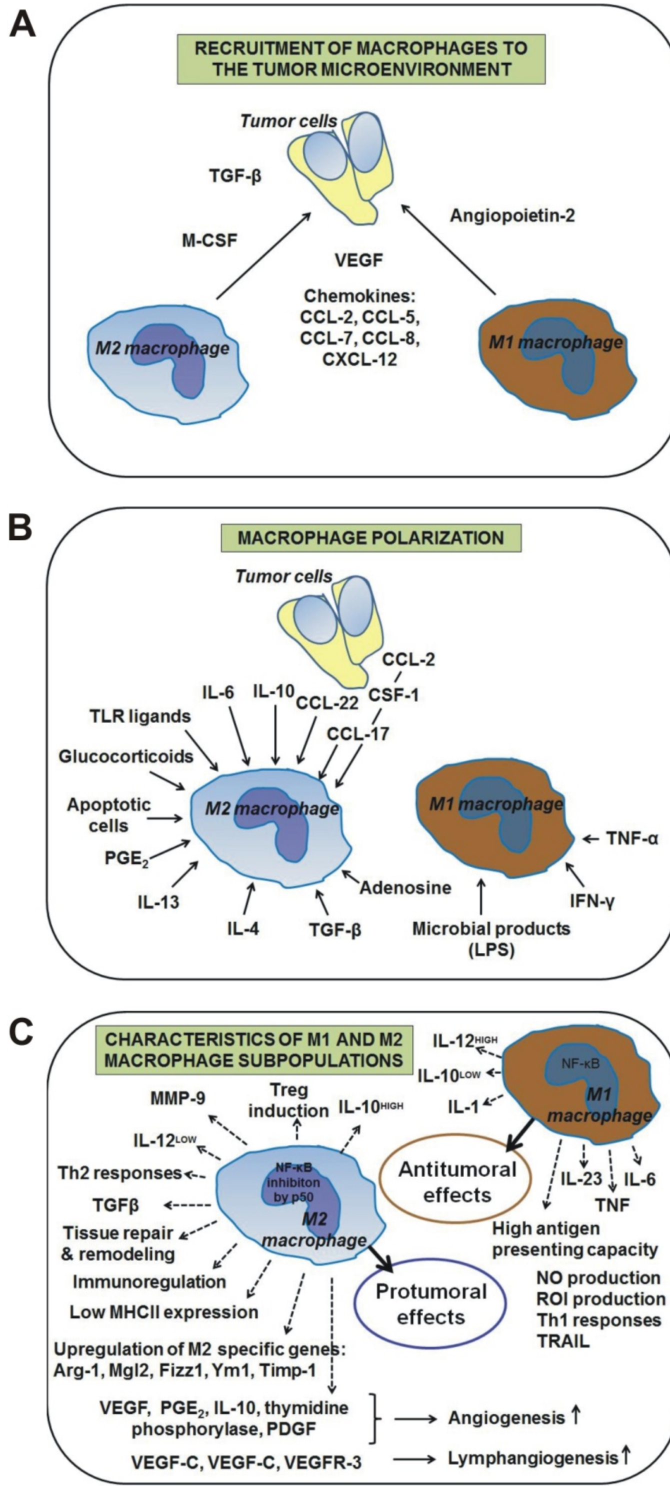


Figure 2 Factors affecting the recruitment, polarization, and characteristics of macrophages. **(A)** Factors contributing to macrophage recruitment to the tumor microenvironment. **(B)** Factors affecting macrophage polarization. **(C)** Characteristics of the type 1 (M1) and type 2 (M2) subtypes of macrophages. Abbreviations: MHC II, major histocompatibility complex II; NO, nitric oxide; PDGF, platelet derived growth factor; PGE2, prostaglandin E2; ROI, reactive oxygen intermediate; TLR, toll-like receptor.

The prognostic role of type 2 macrophages has also been investigated even though to a lesser extent. Markers used for detection of protumoral type 2 macrophages comprise CLEVER-1/Stabilin-1, CD163 (macrophage scavenger receptor), CD204 (macrophage scavenger receptor 1), CD206 (macrophage mannose receptor, MR). In addition neuropilin 1 is considered a type 2 macrophage marker (Erreni *et al.* 2011). In classical Hodgkin´s lymphoma, a high density of CD163$^+$ type 2 macrophages associated with a shorter OS (Zaki *et al.* 2011). A high CD163$^+$ macrophage count was in a similar fashion associated with a decreased DFS in intrahepatic cholangiocarcinoma (Hasita *et al.* 2010) and uveal melanoma (Bronkhorst *et al.* 2011). In gliomas M-CSF was shown to shift the macrophage balance towards the type 2 macrophage phenotype, which positively associated with tumor growth (Komohara *et al.* 2008). In pancreatic cancer a high number of CD163$^+$ or CD204$^+$ type 2 macrophages was associated with increased LVD and poor prognosis in a study by Kurahara *et al.* whereas CD68$^+$ macrophage number was not (Kurahara *et al.* 2011). Recently one study reported an association between a high CLEVER-1$^+$ macrophage number and poor OS in breast cancer (Ammar *et al.* 2011). To my knowledge no other predictive or prognostic studies have been conducted with CLEVER-1 as a macrophage marker.

2.1.7.10 Tumor associated macrophages in colorectal cancer

A dense inflammatory cell infiltration along the invasive front or within the tumor appears to be a sign of good prognosis in CRC (Klintrup *et al.* 2005; Roxburgh & McMillan, 2011). A high CD68$^+$ macrophage number associates with an improved CRC survival as well, opposite to several other malignancies. Especially the CD68$^+$ macrophages located peritumorally seem to be related to an improved survival (Forssell *et al.* 2007; Lackner *et al.* 2004; Zhou *et al.* 2010) or other favorable disease characteristics (Funada *et al.* 2003; Nakayama *et al.* 2002). The prognostic role of intratumoral CD68$^+$ macrophages resemble the peritumoral ones, with most studies showing a positive impact of a high CD68$^+$ intratumoral count on CRC outcome (Khorana *et al.* 2003; Nagorsen *et al.* 2007; Nagtegaal *et al.* 2001; Tan *et al.* 2005).

Kang and colleagues however, reported opposite to the studies mentioned above an association between a high intratumoral CD68$^+$ macrophage number and tumor characteristics related to poor prognosis (Kang *et al.* 2010). A high number of CD68$^+$ macrophages in the metastatic regional lymph nodes appear to associate with an improved survival in stage III CRC (Oberg *et al.* 2002). By contrast, some studies report a lack of association between CRC outcome and number of CD68$^+$ macrophages (Baeten *et al.* 2006).

The role of CD68$^+$ macrophage density in distant metastatic spread has also been studied. Nagtegaal *et al.* reported that a high number of CD68$^+$ macrophages either intra- or peritumorally was associated with a reduced distant metastasis risk (Nagtegaal *et al.* 2001), whereas Zhou *et al.* observed a reduction in liver metastasis and a longer time interval to the appearance of liver metastasis in patients with a high number of CD68$^+$ TAMs at the invasive tumor front (Zhou *et al.* 2010). To my knowledge only one study has investigated the prognostic impact of type 2 macrophages in CRC before. They used

CD163 as a type 2 macrophage marker and reported a positive prognostic impact of a high CD163$^+$ macrophage number in terms of improved survival (Nagorsen *et al.* 2007).

Interestingly, *in vitro* studies have suggested that direct cell-to-cell contact between macrophages and colon cancer cells inhibit cancer cell growth, whereas when the direct contact between the cancer cells and macrophages is lacking, like in cases with only a few macrophages present, cancer cells become more migratory, loose E-cadherin expression, and translocate β-catenin to the nucleus as a response to mediators secreted by the macrophages (Forssell *et al.* 2007). Recently, a study was conducted to find an explanation for the correlation between a high macrophage number and good prognosis observed in CRC. Both *in vitro* and *in vivo* models showed that CD68$^+$ TAMs in CRC exert antitumoral responses, like increased production of pro-inflammatory cytokines, increased recruitment of type 1 T cells, and enhanced antigen presenting capacities (Ong *et al.* 2012).

2.1.7.11 Immunotherapeutic treatment options

During recent years immunotherapeutic agents have become available for clinical use in the field of cancer treatment. Antibodies against tumor antigens are used in the treatment of e.g. breast and gastric cancer (anti-HER2 antibody, trastuzumab), colorectal cancer (anti-EGFR antibodies, cetuximab and panitumumab; anti-VEGF antibody, bevacizumab), and non-Hodgkin´s B-cell lymphoma (anti-CD20 antibody, rituximab; anti-CD20 radiolabaled antibody, ibritumomab tiuxetan) (Finn, 2008). Sipuleucel-T, a cancer vaccine used in the treatment of castration-resistant prostate cancer is the first autologous cellular immunotherapy approved by the United States Food and Drug Administration. Sipuleucel-T consists of autologous peripheral blood mononuclear cells, including antigen presenting cells, that have been activated *ex vivo* with a recombinant fusion protein comprising a tumor-associated antigen (prostatic acid phosphatase) and granulocyte colony-macrophage stimulating factor. Sipuleucel-T prolongs OS in castrate resistant prostate cancer with about 4 months (Kantoff *et al.* 2010). In melanoma treatment, the monoclonal antibody ipilimumab which blocks the negative T cell regulator cytotoxic T-lymphocyte-associated antigen 4 thereby enhancing T cell activation and proliferation has shown efficacy both as single agent therapy (Hodi *et al.* 2010) and in combination with dacarbazine (Robert *et al.* 2011) in phase III trials. Lenalidomide, a structural analogue of thalidomide used for the treatment of multiple myeloma, exerts one of its actions through the inhibition of inflammatory cytokine production of e.g. monocytes (Dimopoulos *et al.* 2007). Drugs under investigation targeting cancer associated inflammation include COX inhibitors, TNF-α antagonists, anti-cytokine (drug target examples IL-6, IL-1) and anti-chemokine drugs (drug target examples CCL-2, CCR-4, CXCR4), and transcription factor inhibitors among others (Balkwill & Mantovani, 2010).

2.2 Oncogene *Pim-1*

2.2.1 Pim-1 expression, function, and regulation

Proviral integration site MuLV *(pim-1)* is a proto-oncogene, localized on chromosome 6p21 (Nagarajan *et al.* 1986) and originally identified as a proviral insertion site of the

Moloney Murine Leukemia Virus in murine T cell lymphomas (Cuypers *et al.* 1984). *pim-1* encodes a highly conserved, constitutively active serine/threonine kinase with a short half-life (Saris *et al.* 1991), which phosphorylates substrates involved in e.g. cell cycle progression, apoptosis, proliferation, and mitosis. Proteins phosphorylated by the Pim-1 kinase include transcription factor NFATc, which gets activated by Pim-1 (Rainio *et al.* 2002), transcriptional co-factor protein p100, which in turn interacts with the myeloblastosis oncogene (c-Myb) transcription factor (Leverson *et al.* 1998), phosphatase cell division cycle 25 homolog (Cdc25A), which might link *pim-1* and (v-myc myelocystomatosis viral oncogene homolog (*c-myc)* to each other (Mochizuki *et al.* 1999), nuclear mitosis apparatus (NuMA) protein linking Pim-1 to mitosis (Bhattacharya *et al.* 2002), pro-apoptotic protein Bad, which is inhibited by Pim-1 (Aho *et al.* 2004), retinitis pigmentosa 9 (PAP-1) (Maita *et al.* 2000), Cdc25C associated kinase (C-TAK1) leading to cell cycle progression (Bachmann *et al.* 2004; Bachmann & Moroy, 2005), as well as suppressor of cytokine signaling proteins (SOCS) 1 and SOCS3 resulting in inhibition of transcription factor NFATc1 (Peltola *et al.* 2004).

Various cytokines, hormones, and mitogens induce Pim-1 expression. For instance, the transcription factors STAT3 and STAT5 regulate Pim-1 expression by mediating cytokine signaling. Activators of STATs include interleukins, G-CSF, IFN-γ, EGF, and TNF-α. Pim-1 is in turn capable of regulating STAT activity negatively via the SOCS proteins (Bachmann & Moroy, 2005).

Pim-1 is highly expressed in the human fetal spleen and liver and in adults low levels can be detected in circulating granulocytes (Amson *et al.* 1989). Besides the hematopoietic system, Pim-1 expression has been found in germ cells of the rat, mouse and human testis (Sorrentino *et al.* 1988; Stewart & Rice, 1995; Wingett *et al.* 1992), central nervous system (Eichmann *et al.* 2000) and in human keratinocytes, thymus, prostate, ovary, colon, and small intestine (Stewart & Rice, 1995). The Pim-1 protein can be localized both in the nucleus and cytoplasm of the cells.

The level of Pim-1 protein has been shown to vary during cell cycle progression in a chronic myelogenous leukemia cell line. The highest Pim-1 levels were detected in the G1, G1/S boundary, and G2 phases (Liang *et al.* 1996). Pim-1 regulates also apoptosis, functioning either as an apoptosis inhibitor or enhancer (Aho *et al.* 2004; Lilly *et al.* 1999; Mochizuki *et al.* 1997). To better understand the role of Pim-1, mice deficient of the *pim-1* gene have been generated. In mice lacking *pim-1*, erythrocyte microcytosis (Laird *et al.* 1993), impaired IL-3 response in bone marrow derived mast cells (Domen *et al.* 1993b) as well as impaired response to growth factors IL-7 and steel factor in early B lymphoid compartments in the bone marrow (Domen *et al.* 1993a) have been documented. These null mutant mice lack an obvious phenotype (Laird *et al.* 1993). There are three known Pim family members: Pim-1, Pim-2, and Pim-3. Reduced body size and impaired responses to hematopoietic growth factors is seen in mice lacking all three pim kinases (Mikkers *et al.* 2004).

2.2.2 Pim-1 in cancer

Evidence for the oncogenic characteristic of *pim-1* was provided by van Lohuizen *et al.* who showed by using *pim-1* transgenic mice that 5-10% of the mice overexpressing Pim-1 developed T-cell lymphomas. The low incidence and long latency of lymphoma development in these mice indicated that *pim-1* alone is a weak cell transformation inducer (van Lohuizen *et al.* 1989). Indeed, *pim-1* has been shown to co-operate with other oncogenes like *c-myc, n-myc* (van Lohuizen *et al.* 1989), *gfi-1* (Schmidt *et al.* 1998), *frat-1* (Jonkers *et al.* 1997), and *runx-2* (Blyth *et al.* 2001) in lymphomagenesis. Chemical carcinogens, irradiation, and slow transforming viruses, like murine leukemia virus are capable of accelerating and enhancing lymphomagenesis in Pim-1 overexpressing transgenic mice (Breuer *et al.* 1991; van der Houven van Oordt *et al.* 1998; van Lohuizen *et al.* 1989).

Overexpression of Pim-1 has been detected both in haematological (Wang *et al.* 2001) and non-hematological malignancies like carcinomas of the head and neck (Beier *et al.* 2007; Chiang *et al.* 2006; Peltola *et al.* 2009), pancreas (Reiser-Erkan *et al.* 2008), prostate (Dhanasekaran *et al.* 2001; Rhodes *et al.* 2003; Valdman *et al.* 2004; Xu *et al.* 2005), and bladder (Guo *et al.* 2010). The prognostic role of Pim-1 has been investigated in several non-hematological malignancies and according to the published results Pim-1 may affect cancer prognosis either in a favorable or unfavorable fashion depending on the tumor type. An elevated Pim-1 protein expression has been linked to poor prognosis and radiotherapy resistance in SCC of the head and neck (Peltola *et al.* 2009), whereas another study reported lack of a prognostic role for Pim-1 in oral SCC (Chiang *et al.* 2006). Downregulation of Pim-1 *in vitro* inhibited bladder cancer cell (Warnecke-Eberz *et al.* 2008) growth and sensitized the cells to docetaxel and doxorubicin treatment (Guo *et al.* 2010) indicating a high Pim-1 level being related to poor prognosis.

In contrast, downregulated Pim-1 mRNA expression in non-small cell lung cancer tissue correlated with increased lymph node metastasis (Warnecke-Eberz *et al.* 2008). Similarly, in pancreatic ductal adenocarcinoma a high Pim-1 expression assessed by IHC was related to improved survival (Reiser-Erkan *et al.* 2008). In prostate cancer, a low Pim-1 expression was linked to a shorter prostate specific antigen (PSA) failure free survival in a gene expression profile - protein expression study (Dhanasekaran *et al.* 2001). This finding was confirmed in another independent study later (Rhodes *et al.* 2003). On the contrary, a high Pim-1 level in prostate cancer seems to correlate with poor tumor differentiation according to some reports (Valdman *et al.* 2004; Xu *et al.* 2005). To further complicate this issue, some studies have found no association between Pim-1 expression and clinicopathological variables (Cibull *et al.* 2006) or survival (Valdman *et al.* 2004) in prostate cancer.

Hypoxia which is commonly present in malignant tumors causes chemoresistance and induces Pim-1 kinase expression, thereby linking Pim-1 to chemoresistance. Inhibition of Pim-1 was shown to improve drug sensitivity under hypoxic conditions by Chen and colleagues (Chen *et al.* 2009). Targeting the Pim-1 kinase with a small molecule inhibitor (SGI-1776) *in vivo* enhanced the antitumoral effects of sunitinib in the treatment of renal cell carcinoma (Mahalingam *et al.* 2011). Similarly, SGI-1776

treatment was able to resensitize prostate cancer cells to taxane treatment *in vitro* (Mumenthaler *et al.* 2009). The *KRAS* oncogene participates also in the regulation of Pim-1. *KRAS* mutations cause radioresistance in pancreatic cancer and the inhibition of Pim-1 in pancreatic adenocarcinoma reduced invasion, growth transformation, and radioresistance in a study by Xu *et al.* (Xu *et al.* 2011). This finding might be clinically important since >90% of pancreatic adenocarcinomas are *KRAS* mutated and the lack of anti-*KRAS* drugs warrants the search for other therapeutic target candidates. Another interesting regulator of Pim-1 is EGFR via STAT3/STAT5, which causes nuclear translocation of Pim-1 *in vitro*. One study demonstrated that EGFR-inhibitors (cetuximab and gefinitib) are able to prevent the subcellular translocation of Pim-1 caused by irradiation and the related radioresistance in head and neck SCC (Peltola *et al.* 2009). Pim kinases contribute also to the regulation of lymphocyte growth and proliferation. The immunosuppressive drug rapamycin has modest effect on peripheral T cell survival *in vivo* and $CD8^+$ T cell function is largely rapamycin resistant under normal conditions. Pim-1 and Pim-2 seem to play a role in maintaining the survival and growth of T cells during rapamycin treatment since in Pim-$1^{-/-}$Pim-$2^{-/-}$ mice the immunosuppressive effects of rapamycin are enhanced (Fox *et al.* 2005; Li *et al.* 2011a). An anti-Pim-1 monoclonal antibody (mAb) was shown to selectively inhibit activated lymphocytes suggesting that anti-Pim-1 antibody therapy could possibly be used in the future as an immunosuppressive drug (Li *et al.* 2011a). Pim-1 is upregulated by VEGF-A in endothelial cells during angiogenesis and seems to be necessary for the differentiation of embryonic stem cells to endothelial cells and smooth muscle cells. In human umbilical vascular endothelial cells Pim-1 is needed for VEGF-A dependent proliferation and migration (Zippo *et al.* 2004).

2.3 CD73

2.3.1 Expression and enzymatic role of CD73

CD73 is a cell surface associated glycosyl-phosphatidylinositol anchored protein with ectoenzyme activity (ecto-5′-nucleotidase) widely expressed in the human body (Zimmermann, 1992). CD73 is present both on epithelial cells of various origins as well as on vascular endothelial cells and subsets of peripheral blood B and T lymphocytes (Airas *et al.* 1993; Thomson *et al.* 1990). About 20% of human peripheral blood lymphocytes express CD73 in contrast to granulocytes and monocytes, which are CD73 negative. The majority (70%) of peripheral blood B lymphocytes express CD73. T cells on the other hand express CD73 to a lesser extent: about half of $CD8^+$ T cells and only about 10% of $CD4^+$ T cells are $CD73^+$ (Thomson *et al.* 1990). In mice ecto-5′-nucleotidase activity is higher in T than B lymphocytes isolated from the spleen and lymph nodes (Yegutkin *et al.* 2011).

CD73 functions as a maturation marker on B (Bastian *et al.* 1984; Thompson *et al.* 1986) and T lymphocytes (Edwards *et al.* 1979) and is involved in the activation and proliferation of human peripheral T lymphocytes (Thompson *et al.* 1989). $CD4^+$/$CD25^+$/$FOXP3^+$ T regulatory cells in mice co-express CD73/ecto-5′-nucleotidase and CD39/nucleoside triphosphate diphosphohydrolase (NTPDase) 1.

CD73/CD39 on Treg cells produce adenosine which binds to A_{2A} receptors on activated CD4 effector T cells decreasing their proliferation and cytokine production hence mediating immunsuppressive effects (Deaglio *et al.* 2007; Kobie *et al.* 2006). B- and T-lymphocyte ecto-5´-nucleotidase deficiency is observed in primary immunodeficiency diseases like common variable immunodeficiency (Thompson *et al.* 1986; Webster *et al.* 1978), congenital X-linked agammaglobulinemia (Edwards *et al.* 1979; Edwards *et al.* 1978), and severe combined immunodeficiency (Thompson *et al.* 1984).

The major physiological role of CD73 seems to be its regulatory function of the purinergic signaling cascade, where it dephosphorylates extracellular nucleoside monophosphates into bioactive nucleoside intermediates. This extracellular purinergic pathway consists of both a nucleotide-inactivating cascade and an ATP-regenerating pathway (**Figure 3**). Extracellular purines have been shown to mediate both inflammatory [ATP, adenosine diphosphate (ADP)] and anti-inflammatory (adenosine) effects in human tissues and these can be modulated by CD73 via its enzymatic capability to hydrolyze 5`- adenosine monophosphate (AMP) to adenosine (Yegutkin *et al.* 2002). Adenosine, which is particularly generated at sites of inflammation, hypoxia, organ injury, and traumatic shock signals through four G-coupled adenosine receptors: A_1, A_{2A}, A_{2B}, or A_3. The adenosine receptor-mediated effects depend on which receptor subtype adenosine binds to and on the adenosine tissue concentration. A_1 and A_3 adenosine receptors decrease the intracellular levels of cAMP by inhibiting

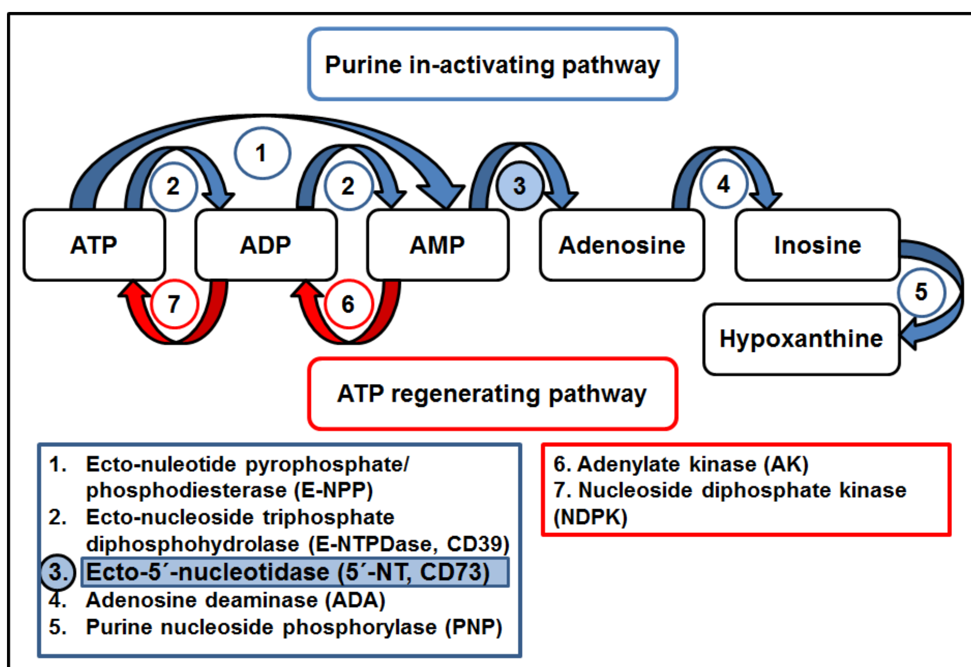


Figure 3 Enzymes involved in the extracellular purine inactivating pathway and the opposing ATP regenerating/adenosine eliminating pathway. CD73 hydrolyzes AMP to adenosine and thereby serves as an important regulator between two counteracting purinergic pathways.

adenylyl cyclase and protein kinase A. In contrast, A_{2A} and A_{2B} adenosine receptors gain immunosuppressive properties by increasing the amount of intracellular cAMP by activating adenylyl cyclase and protein kinase A (Stagg & Smyth, 2010). In neutrophils, which express all four adenosine receptor subtypes, A_1 receptor activation leads to proinflammatory effects (promotes neutrophil adherence to vascular endothelium and chemotaxis), whereas in contrast, A_{2A} and A_{2B} receptor activation result in anti-inflammatory responses (inhibits neutrophil adherence to vascular endothelium and production of toxic oxygen metabolites) (Cronstein *et al.* 1990; Cronstein *et al.* 1992). Adenosine has protective effects against tissue injury caused by hypoxia. One study using CD73^{-}/$^{-}$, A_{2B} adenosine receptor^{-}/$^{-}$, and HIF-1α^{-}/$^{-}$ mice demonstrated that as a response to intestinal ischemia/reperfusion injury HIF-1α induces CD73 and A_{2B} adenosine receptors in intestinal epithelial cells, thereby diminishing the extent of tissue injury (Hart *et al.* 2011).

2.3.2 CD73 in lymphocyte adhesion and transmigration

In addition to its enzymatic activity CD73 functions as a lymphocyte adhesion molecule on blood vessel endothelium (Airas *et al.* 1993). Despite almost identical structures of CD73 on lymphocytes and vascular endothelial cells (Airas *et al.* 1997), CD73 on lymphocytes in contrast to CD73 on endothelial cells, increases lymphocyte binding to endothelial cells (human endothelial cells or human umbilical vascular endothelial cells) via leukocyte integrin LFA-1 clustering (Airas *et al.* 2000). The binding of leukocytes to vascular endothelium inhibits the CD73 mediated adenosine production and the remaining adenosine is deaminated by adenosine deaminase. As a consequence, the vascular barrier function is impaired and leukocyte transmigration increased (Henttinen *et al.* 2003).

Mice lacking CD73 show increased lymphocyte migration rates and enlargement of draining lymph nodes as a response to an inflammatory stimulus (e.g. LPS). CD73 generated adenosine on HEV rather than on lymphocytes, has been shown responsible for regulating lymphocyte transmigration across HEVs into peripheral lymph nodes, through AR_{A2B} signaling (Takedachi *et al.* 2008). The function of CD73 in lymphatic vessels is not known.

2.3.3 CD73 in vasculature

High activities of NTPDase and ecto-5´-nucleotidase and as a consequence a rapid inactivation of ADP/ATP to AMP and further to adenosine are observed in the vasculature (Yegutkin, 2008). The production of adenosine by CD73 has anti-thrombotic effects and reduces leukocyte adhesion acting thereby in a vasoprotective, anti-inflammatory fashion in the vasculature (Koszalka *et al.* 2004). Further evidence for the anti-inflammatory role of CD73 on vasculature has been demonstrated in a study by Zernecke and colleagues who showed that mice deficient of CD73 had an increased expression of vascular cell adhesion molecule (VCAM) 1 as well as sVCAM-1, increased VLA-4/VCAM-1 mediated monocyte recruitment, and enhanced neointimal plaque formation (Zernecke *et al.* 2006). In addition, the generation of CD73 knock-

out mice have demonstrated the important role of CD73 in the maintenance of vascular endothelial barrier function under hypoxic conditions. The extent of vascular leakage increase in different tissues of $CD73^{-/-}$ mice varied; the leakage increase was most prominent in the lungs, moderate in the colon, and absent in the brain (Thompson *et al.* 2004). Hypoxia has been shown to induce CD39 and CD73 on vascular endothelial cells thereby increasing the metabolism of ATP, released from e.g. activated polymorphonuclear granulocytes, to adenosine. Adenosine binds to the A_{2A} and A_{2B} receptors on neutrophils and decreases the neutrophil adhesion to vascular endothelium and transmigration (Eltzschig *et al.* 2004).

CD39 and CD73 dependent adenosine production has a protective role in the lungs during mechanical ventilation induced acute lung injury (ALI) by reducing pulmonary edema and inflammation. This protective function in the capillary-alveolar pulmonary barrier seems to be mediated through the A_{2B} adenosine receptors (Eckle *et al.* 2007). Furthermore, as a response to LPS-induced ALI, CD39 and CD73 attenuate the neutrophil accumulation into the lungs and maintain the capillary-alveolar barrier function (Reutershan *et al.* 2009).

Interferon-β and Interferon-α have been shown to increase CD73 expression on vascular endothelium, thereby maintaining endothelial barrier-function. This leads to a reduction of lymphocyte transmigration and works in an anti-inflammatory fashion by diminishing leukocyte infiltration to sites of inflammation. (Niemelä *et al.* 2004; Niemelä *et al.* 2008). Interferon-β has also been shown to induce CD73 in indirect ALI (caused by intestinal ischemia-reperfusion), thereby promoting the endothelial barrier function and reducing vascular leakage in the lungs (Kiss *et al.* 2007). Taken together, CD73 via an adenosine-mediated mechanism controls endothelial permeability under normal conditions, hypoxia, and lung injury.

CD73 knock-out mice show impaired tubuloglomerular feedback-dependent vasomotor responses (construction of arterioles in response to an increase in the luminal NaCl concentration) in the kidneys. These $CD73^{-/-}$ mice show no gross anatomical abnormalities and their behavior as well as fertility remains normal (Castrop *et al.* 2004).

2.3.4 CD73 in cancer

As a response to dangers, like pathogens, injury, or cell death, a functioning immune system is essential for survival. However, the immune response needs to be precisely regulated to prevent damage to the surrounding normal tissues caused by overactive immune cells. Hypoxia induced adenosine accumulation and its signaling via A_{2A} adenosine receptors is an important downregulator of inflammation and as a consequence protects tissues from inflammatory damage (Ohta & Sitkovsky, 2001; Sitkovsky & Ohta, 2005). Extracellular adenosine is capable of promoting type 2 macrophage activation via A_{2A} and A_{2B} receptors, thus further contributing to immunosuppression (Csóka *et al.* 2012).

Hypoxia is often present in malignant tissues as well and as a consequence increased concentrations of extracellular adenosine. Ohta *et al.* have demonstrated in mice, that A_{2A} adenosine receptors on $C8^+$ T cells inhibit their antitumoral effects and that the antitumoral responses of $CD8^+$ T cells can be enhanced by genetic deletion of adenosine receptors A_{2A} or the use of adenosine receptor A_{2A} antagonist (Ohta *et al.* 2006).

Various types of cancer cells express CD73 and the conversion of AMP to adenosine by CD73 constitutes one of the major sources of adenosine in tumors (Stagg & Smyth, 2010). In mice, tumor derived CD73 suppresses the adaptive immune response via adenosine signaling through A_{2A} receptors. In addition, adenosine generated by tumor derived CD73 increases breast cancer cell migration and metastasis through the activation of A_{2B} receptors. The administration of anti-CD73 mAb was shown to inhibit breast cancer tumor growth in immunocompetent mice in contrast to immunocompromised mice, indicating that the antitumoral effect of blocking CD73 requires the activation of the host immune response. The inhibition of lung metastasis was in contrast independent of the adaptive immune cells since the reduction of lung metastasis was similar in both immunocompetent and immunocomprised mice (Stagg *et al.* 2010). *In vitro* and *in vivo* studies with human breast cancer cell lines by Zhou *et al.* demonstrated that a CD73 inhibitor (α, β-methylene ADP) reduced cancer cell viability and proliferation index as well as diminshed tumor angiogenesis, lymphangiogenesis, and growth (Zhou *et al.* 2007). In addition, CD73 on breast cancer cells has been reported to increase the invasive, migrating, and adhesive properties of the malignant cells (Wang *et al.* 2008).

Not only CD73 expression on cancer cells, but also host-derived CD73 both on hematopoietic and non-hematopoietic tissues have immunosuppressive effects and thereby act in a protumoral fashion. Host-derived CD73 inhibited $CD8^+$ T cell-mediated immune responses against malignant tumors and contributed to the protumoral effect of Tregs in a study by Stagg *et al.*. In addition CD73 on endothelial cells was demonstrated to promote metastasis of circulating malignant melanoma cells (Stagg *et al.* 2011). The importance of host-derived CD73 has also been studied by Yegutkin *et al.* who reported attenuated melanoma tumor growth and metastasis as well as a reduction in number of intratumoral Tregs and MR^+ macrophages in mice lacking CD73. Since CD73 is present on neoangiogenic vessels the amount of blood and lymphatic vessels were investigated in wt and CD73 deficient mice, but no difference in vessel numbers were observed (Yegutkin *et al.* 2011). Taken together, CD73 both on cancer cells and host derived hematopoietic and non-hematopoietic tissues promotes tumor growth.

2.4 Colorectal cancer

2.4.1 Incidence, risk factors, and staging system

Worldwide CRC is the third most common malignancy with 1.2 million new cases diagnosed and about 608 000 deaths caused by the disease estimated annually. CRC

caused mortality rank number four among all cancer related deaths in the world. The majority of the CRC cases (about 60%) are diagnosed in the developed countries (Ferlay *et al.* 2010). In Finland 2648 new CRC cases were diagnosed in year 2009 (Finnish Cancer Registry).

Advanced age, obesity, diabetes, smoking, dietary factors (consumption of red meat, high fat diet, low fibre intake), excessive use of alcohol, inflammatory bowel disease, colonic polyps, and previous CRC increase the risk for developing sporadic CRC. Hereditary syndromes are responsible for the development of CRC in about 6% of the cases. Lynch syndrome, familial adenomatous polyposis, and mut Y homolog associated polyposis are the most common hereditary syndromes associated with increased CRC risk (Cunningham *et al.* 2010).

CRC is classified according to the TNM system, which is maintained by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). The TNM system classifies CRC by the depth of primary tumor invasion/invasion to adjacent organs (T), the number of involved regional lymph nodes (N), and the presence or absence of distant metastasis (M). A minimum of 12 lymph nodes should be examined for reliable staging. Stage is the most important prognostic factor in CRC. The TNM stages for CRC are presented in **Figure 4** (AJCC, 2010).

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

Abbreviations: T, tumor; N, node; M, metastasis

Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into pericolorectal tissues
T4a	Tumor penetrates to the surface of the visceral peritoneum
T4b	Tumor directly invades or is adherent to other organs or structures

Regional Lymph Nodes (n)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in four or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in seven or more regional lymph nodes

Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site
M1b	Metastasis in more than one organ/site or the peritoneum

Figure 4 TNM staging of CRC according to recommendations of AJCC and UICC.

2.4.2 Molecular features of colorectal cancer development

The DNA mismatch repair (MMR) pathway, which is associated with Lynch syndrome and a minority of the sporadic CRC cases (15%), is characterized by somatic mutations or loss of function due to aberrant promoter methylation (Noffsinger, 2009) in one of the DNA MMR genes (*MLH1*, *MSH2*, *MSH6*, or *PMS2*). These mutations lead to dysfunction of the DNA nucleotide mismatch repair system, which normally corrects errors that occur during DNA replication. The loss of this repair function results in mutations in the microsatellite regions (short repetitive sequences of DNA) of the genome, referred to as microsatellite instability (MSI). About 3% of CRC patients have the hereditary Lynch syndrome, which is characterized by e.g. early onset of CRC, accelerated carcinogenesis, and increased risk of malignancies outside the gastrointestinal tract. Lynch syndrome is screened by IHC and diagnosed by the detection of a germline mutation in one of the DNA MMR genes. Both sporadic and Lynch syndrome associated MSI-H are more common among women than men, have mucinous or signet cell differentiation, are usually localized in the proximal colon, and are infiltrated by lymphocytes. About 40% of the sporadic MSI-H cancers also harbor *APC* or *p53* mutations reflecting the involvement of both MMR and chromosomal instability (CIN) pathways (Cunningham *et al.* 2010; Fearon & Vogelstein, 1990; Noffsinger, 2009; Takayama *et al.* 2006; Vogelstein *et al.* 1988).

The serrated pathway includes CRCs that have arisen from serrated polyps [hyperplastic polyps (not sporadic CRC), traditional serrated adenomas, sessile serrated adenomas]. The pathway can be divided into two subtypes according to the precursor lesions and the molecular characteristics of the malignant tumors. The tumors that seem to originate from sessile serrated adenomas are usually located in the right colon and are characterized by the following features: chromosomally stable, *BRAF* mutated, MSI-H, *MLH1* methylated, and a high level of CpG island methylator phenotype (CIMP). The tumors representing the other subtype most likely originate from left-sided traditional serrated adenomas and are characterized by the following molecular features: high CIMP status, microsatellite instability low (MSI-L) or microsatellite stable (MSS), *MGMT* methylation or partial methylation of *MLH1*, chromosomally stable, and *BRAF* mutated (Noffsinger, 2009).

Important components of the major signaling pathways in CRC carcinogenesis described above include the WNT pathway, which gets activated as a consequence of *APC* gene mutations and leads to β-catenin activation through nuclear translocation; the tumor-suppressor TGF-β pathway involving for instance the in CRC commonly occurring loss of the long arm of chromosome 18 (18qLOH) affecting the genes *SMAD4* and *DCC*; the EGFR/*RAS/RAF*/MAPK pathway including the clinically most relevant oncogene *KRAS*; and the PI3K pathway with *PIK3CA* and *PTEN* mutations as well as loss of PTEN expression observed. *KRAS* mutations occur most commonly in codons 12 or 13 and they are mutually exclusive of *BRAF* mutations V600E. *BRAF* mutations are often seen in tumors arisen via the serrated pathway, particularly CIMP-high tumors and they are hardly ever seen in patients with Lynch syndrome (Pritchard & Grady, 2011).

In contrast to the development of primary CRC tumors, the course of an eventual metastatic event, following the establishment of an invasive tumor, is usually short (Nguyen *et al.* 2009). The complex process of developing metastatic disease involves several steps and gene alterations. Proteinases, like MMPs, degrade extracellular matrix components and are important for the proteolysis phase (Takayama *et al.* 2006). In addition, the downregulation of adhesion molecules like cadherins promotes cell detachment from the primary tumor, whereas the upregulation of certain adhesion molecules, e.g selectins on the endothelial cells, platelets, or leukocytes enhances cancer cell adhesion to vascular endothelium, helps CRC cells evade inflammatory reaction, and may aid in the selection of distant metastasis site. Integrins among others are also important players in CRC metastasis (Paschos *et al.* 2009).

2.4.3 Prognostic factors in colorectal cancer

CRC prognosis after radical (R0) surgery is affected by several factors, including preoperative serum carcinoembryonic antigen level, preoperative obstruction or perforation of the gut, TNM stage, tumor differentiation grade, tumor budding, tumor regression grade as a response to neoadjuvant therapy in rectal cancer, the circumferential resection margin in rectal cancer, lymphatic, venous or perineural invasion, as well as histologic type and grade (AJCC, 2010). The most significant prognostic factor is the stage of the disease at the time of diagnosis. Patients with early stage disease (stage I and II) have a 5-year survival rate of 80-90% after radical surgical treatment. In contrast, patients with local lymph node involvement, belonging to the stage III disease group have an obviously worse prognosis with a 5 year survival rate of 60-70% (Howlader *et al.* 2011). The median survival of patients with metastatic stage IV CRC is about 24 months, with less than 10% of the patients alive five years after diagnosis (AJCC, 2010; Cunningham *et al.* 2010).

Prognostic markers are factors separating patients with a good or bad clinical disease outcome regardless of treatment, thus reflecting the natural course of the disease. Numerous prognostic markers have been investigated in CRC, the following being among the most studied; MSI, CIN, 18qLOH/*SMAD4*, *BRAF* V600E mutations, *KRAS* mutations, and *PIK3CA* mutations.

Cancers arising through the CIN pathway have a worse prognosis than the MSI-H cases, which typically have a long OS (Hewish *et al.* 2010). The evidence for both CIN and MSI-H as prognostic factors in CRC is strong and testing is available for MSI, but it is not widely used yet, but might be in the future. About half of CRCs have 18qLOH/*SMAD4* loss which is a sign of poor prognosis, but might instead of being an independent prognostic factor just be a surrogate marker for CIN/MSS colorectal cancers due to the close association between 18qLOH and CIN/MSS. No testing for clinical use is available (Pritchard & Grady, 2011). *BRAF* V600E mutations are associated with poor prognosis in CRC and *BRAF* testing is available for clinical use, but does not influence the treatment choices of CRC patients at present (Price *et al.* 2011; Van Cutsem *et al.* 2011). The role of *KRAS* mutations as prognostic markers is still controversial, but according to a meta-analysis they appear to affect the prognosis of metas-

tatic CRC negatively (Qiu *et al.* 2010). In contrast e.g. studies with a best supportive care arm found no prognostic value for *KRAS* (Karapetis *et al.* 2008). *PIK3CA* mutations might also be associated with a poor CRC survival, but the evidence is still limited (De Roock *et al.* 2011; Pritchard & Grady, 2011).

2.4.4 Colorectal cancer treatment

2.4.4.1 Adjuvant therapy for stage II and III disease

The standard treatment for stage I-III CRC patients is radical surgery. The majority of the patients (70-80%) undergo surgery with curative intent after diagnosis. Even in limited metastatic stage IV disease radical surgery is performed whenever possible since radical surgery is the only curative treatment option available in CRC today.

For selected patients postoperative adjuvant chemotherapy is given for eradication of microscopic metastasis that might be present, even though invisible, after the removal of the primary tumor. For quite some time it has been evident that adjuvant fluoropyrimidine [5-fluorourasil (5-FU) or capecitabine] based therapy benefits stage III CRC patients. Later, evidence has emerged that the addition of oxaliplatin to fluoropyrimidine improves the survival of the patients and currently the standard adjuvant therapy regimen for stage III CRC patients is fluoropyrimidine plus oxaliplatin (FOLFOX, XELOX, or FLOX) for six months. The results of two phase III trials comparing fluoropyrimidine to oxaliplatin/fluoropyrimidine combinations demonstrated the following results: in the MOSAIC trial the 5-year DFS rates were 73.3% *vs* 67.4% and 6-year OS rates 72.9% *vs* 68.7% in favor of oxaliplatin based adjuvant therapy in the stage III subgroup of patients (André *et al.* 2009). OS data as well as updated DFS data were recently published from the other study (NSABP C-07) reporting 5-year DFS rates of 64.2% *vs* 69.4% in the intent to treat patient population (both stage II and III patients), however, 5-year OS rates did not differ between the two treatment arms (Yothers *et al.* 2011). Fluoropyrimidine monotherapy is an alternative to oxaliplatin based regimens for e.g. elderly, non-fit patients because of its better tolerability and possibly equal efficacy too.

The role of irinotecan in the adjuvant therapy setting has also been investigated in phase III trials. Irinotecan in combination with 5-FU/LV (leucovorin) did not benefit stage III colon cancer patients in regard to DFS (HR 0.9), relapse free survival (HR 0.86), or OS (HR not reported, p=0.09) in the PETACC-3 trial. In the stage II and III combined subgroup analysis a small DFS benefit was observed (HR 0.89, p=0.04), whereas in the subgroup of stage II patients the adding of irinotecan to the treatment did not improve DFS or OS. The authors conclude that the addition of irinotecan to 5-FU/LV adjuvant therapy does not benefit stage III CRC patients (Van Cutsem *et al.* 2009b). A smaller phase III trial (ACCORD-02) reported no benefit of adding irinotecan to 5-FU/LV either in high risk stage III colon cancer (Ychou *et al.* 2009). Interestingly, irinotecan based adjuvant therapy benefitted MSI-H stage III colon cancer patients in a study by Bertagnolli *et al.* in contrast to MSI-L/MSS patients (Bertagnolli *et al.* 2009), but the finding needs further validation.

The addition of biological, targeted monoclonal antibodies (bevacizumab or cetuximab) to the adjuvant treatment regimens have not improved the survival of the patients and can therefore not be recommended for use (Allegra *et al.* 2011; Alberts SR *et al.* 2010)

In the group of stage II patients the benefit of adjuvant chemotherapy is more controversial. The 5-year survival rates among these patients range between 72% - 85% and most patients are cured without adjuvant chemotherapy (Lombardi *et al.* 2010). A large study (QUASAR trial) investigated 5-FU based adjuvant therapy *vs* surgery alone in stage II CRC patients and reported a modest absolute 5-year survival benefit of 3.6% in favor of chemotherapy. Of note, they did not perform subgroup analysis for high *vs* low risk stage II patients (Quasar Collaborative *et al.* 2007). Both the MOSAIC trial and NSABP C-07 showed an 5-year OS benefit of only 0.1% for oxaliplatin based chemotherapy in the cohorts of stage II patients (André *et al.* 2009; Yothers *et al.* 2011). Currently high risk stage II patients are offered adjuvant, mainly single fluoropyrimidine therapy but oxaliplatin can also be considered (Lombardi *et al.* 2010; Sargent & Grothey, 2010). High risk characteristics include primary tumor of T4 category, presence of LVI, perineural invasion, poorly differentiated tumors, inadequate number lymph nodes sampled (< 12), presentation with gut perforation or occlusion (Labianca *et al.* 2010). Growing evidence is suggesting the lack of benefit of single fluoropyrimidine based adjuvant therapy in MSI-H stage II colon cancers. Therefore in the future it might become standard practice to test this group of patients for deficient MMR and if diagnosed maybe consider some other treatment than single fluoropyrimidine (Hewish *et al.* 2010; Ribic *et al.* 2003; Sargent *et al.* 2010; Sinicrope *et al.* 2011). Data from large prospective trials is still missing.

2.4.4.2 Treatment of rectal cancer

The treatment of non-metastatic rectal cancer differs from colon cancer treatment, whereas the treatment of metastatic disease follows the same principles. An important aim of rectal cancer therapy is to prevent local disease recurrences in the pelvis since they are usually challenging to treat and cause difficult symptoms to the patient. Another aim is to preserve the sphincter function, which is an important quality of life aspect.

Rectal cancer treatment decisions are influenced by the degree of local invasion of the primary tumor (T-category), clinical N stage, presence of vascular or nerve invasion, and circumferential margin status as evaluated from preoperative MRI. According to these features rectal cancers can be divided into early favorable, intermediate, or locally advanced subgroups. The surgical techniques used in the treatment of rectal cancer include transanal excision, transabdominal resections like low anterior resection, total mesorectal excision (TME), or abdominoperineal resection. The choice of surgical technique depends on the extent and location of the disease.

According to the ESMO guidelines working group recommendations the most favorable subgroup of patients with well or moderately differentiated early cT1N0 disease without LVI, can be treated with only a radical resection. If more unfavorable features are present (cT2 tumors, submucosal layer invasion into middle third) TME is

recommended or alternatively postoperative chemoradiation. TME surgery alone is an appropriate treatment strategy for cT1-2, some early cT3 tumors with negative circumferential margin, and N0 cases.

Patients belonging to the intermediate risk group with cT3(b)c tumors without circumferential margin involvement, some cT4 tumors, or patients with lymph node involvement are treated with preoperative radiation therapy [5 x 5 Gray (Gy)] followed by TME surgery.

Preoperative chemoradiation (50.4 Gy) with concomitant 5-FU-based chemotherapy, followed by radical surgery after 6-8 weeks is recommended for patients belonging to the most locally advanced group of patients with cT3 circumferential margin positivity or cT4 disease not easily surgically removed. Patients not fit for chemoradiation therapy or old patients can alternatively be treated with short course 5 x 5 Gy radiation therapy followed by surgery after about 8 weeks (Glimelius *et al.* 2010).

Preoperative chemoradiotherapy in comparison to postoperative chemoradiotherapy is associated with less toxicity, improved local control rate, and improved sphincter preservation and is therefore the recommended treatment strategy (Sauer *et al.* 2004). The combination of short-term radiotherapy with TME, which is the standard surgical procedure at present has been studied in a large trial which included stage I - IV rectal cancer patients. Preoperative radiation therapy reduced local recurrences with about 50% as compared to the group of patients who were treated with surgery only. The reduction of local recurrences was more pronounced in the patients with a negative circumferential margin (64%). In addition, an overall survival benefit was observed in stage III rectal cancer patients treated with combination treatment with a negative circumferential margin whereas in patients with a favorable prognosis according to preoperative evaluation the adverse events caused by radiotherapy outweighed the benefits (van Gijn *et al.* 2011). The addition of oxaliplatin to the preoperative 5-FU based chemoradiation regimen increases toxicity and does not improve the treatment results and can thus not be recommended (Aschele *et al.* 2011). Postoperative adjuvant chemotherapy is recommended for patients with high risk stage II or stage III disease and those who have received neoadjuvant chemoradiation, albeit its benefit is less well defined than for colon cancer (Glimelius *et al.* 2010).

2.4.4.3 Treatment of metastatic stage IV disease

About 25% of the patients present with metastatic disease at the time of diagnosis and an additional 25% of the patients will develop metastasis at a later time point. Stage IV CRC is in most cases considered incurable.

Liver is the most common metastatic site in CRC and the majority of stage IV CRC patients have unresectable disease. If the patient presents with a potentially resectable, limited disease burden in the liver or lungs a radical metastectomy is performed whenever feasible. Perioperative combination chemotherapy ± a biological drug is usually recommended in these cases to optimize the probability for a successful resection and to improve survival (Nordlinger *et al.* 2008; Van Cutsem *et al.* 2010).

Fluoropyrimidines (5-FU, capecitabine), oxaliplatin, and irinotecan are used as chemotherapy agents in the treatment of metastatic CRC. In addition, biologic targeted therapies bevacizumab (monoclonal anti-VEGF-A antibody) as well as cetuximab and panitumumab (anti-EGFR monoclonal antibodies) are available in the treatment arsenal for metastatic CRC. The adminstering order of cytotoxic drugs, does not seem to affect the outcome of the disease to a significant extent. The efficacy of the cytotoxic doublet regimens 5-FU/LV/oxaliplatin (FOLFOX) or capecitabine/oxaliplatin (XELOX) and 5-FU/LV/irinotecan (FOLFIRI) are in the same range but their toxicity profiles differ, which might affect the choice of treatment (Ducreux *et al.* 2011; Tournigand *et al.* 2004). Moreover, capecitabine has been shown to be equally effective to intravenous 5-FU/LV (Cassidy *et al.* 2011). The usage of all active drugs at some point during the treatment course improves the OS of the patients (Grothey *et al.* 2004). In the palliative treatment setting not only the prolongation of survival, but also the improvement in quality of life is an important goal. The selection of treatment intensity is based on factors including disease burden, disease related symptoms, need for downsizing of liver or lung metastasis with curable resection in mind, co-morbidities, and patient preference. Upfront combination therapy is indicated if maximal tumor shrinkage is needed and if the patient has an aggressive, rapidly progressing disease. If the patient has no chance for a curable resection, is almost symptom free, and has comorbidities a single drug treatment is often a good option.

The optimal treatment duration is still controversial. Treatment of metastatic CRC with the same regimen can continue until disease progression, can be stopped after a certain period, or alternatively be switched to a less toxic maintenance regimen after achieving stabilization of the disease (Cunningham *et al.* 2010; Van Cutsem *et al.* 2010). For instance the OPTIMOX1, OPTIMOX2, and MRC COIN trials have evaluted the consequences of intermittent treatment in metastatic CRC. They showed that after six cycles of oxaliplatin based therapy oxaliplatin can be stopped continuing treatment solely with fluoropyrimidine without affecting the prognosis of the patient negatively (Tournigand *et al.* 2006), whereas a complete stop of the treatment resulted in worse PFS and disease control, but did not affect OS (Chibaudel *et al.* 2009). The results of the MRC COIN trial suggest that patients with a raised platelet count at baseline do worse in terms of OS and quality of life with an intermittent treatment strategy, possibly due to the fact that thrombocytosis reflects cytokine activation related to a more aggressive disease. In contrast, patients with normal baseline platelet counts were not harmed by chemotherapy-free intervals. These findings need confirmation (Adams *et al.* 2011).

The discovery of biological monoclonal antibodies in the treatment of CRC has increased the treatment options in metastatic CRC. Bevacizumab, a monoclonal antibody targeting VEGF improves PFS, OS, and response rates in the first line therapy of CRC when combined to fluoropyrimidine (Hurwitz *et al.* 2005) or irinotecan (Hurwitz *et al.* 2004) based regimens. The combination of bevacizumab with oxaliplatin based therapy in first line improves PFS, not OS or objective response rates (Saltz *et al.* 2008). In second line, bevacizumab treatment combined with oxaliplatin based therapy (FOLFOX4), however, improves PFS, OS, as well as objective response rates

(Giantonio *et al.* 2007; Welch *et al.* 2010). Single bevacizumab therapy is not recommended due to its lack of clinical benefit. The role of bevacizumab in maintenance is not clear at present. Typical side effects caused by bevacizumab include hypertension, gut perforation, impaired wound-healing, thromboembolism, and bleeding (Cunningham *et al.* 2010).

The monoclonal antibodies cetuximab and panitumumab bind to EGFR and block the tyrosine-kinase activation of the receptor, which prevents the activation of EGFR downstream signaling pathways resulting in e.g. pro-apoptotic and anti-proliferative effects. Cetuximab and panitumumab have shown efficacy in several phase III trials in metastatic CRC both as single drugs or in combination with cytotoxic regimens. Anti-EGFR drugs are efficient both in early stages and chemorefractory phase of the disease. *KRAS* gene status is a strong predictor of responsiveness to anti-EGFR therapies and *KRAS* mutated patients do not appear to benefit from treatment.

In phase III trials panitumumab has demonstrated efficacy as single therapy in the chemorefractory phase of the disease (≥ 3rd line) (Amado *et al.* 2008; Van Cutsem *et al.* 2007). Panitumumab in combination with FOLFIRI as 2nd line therapy improved ORR and PFS as compared to FOLFIRI in a study by Peeters *et al.* (Peeters *et al.* 2010). In first line treatment the addition of panitumumab to FOLFOX4 resulted in superior PFS in comparison to FOLFOX4, but OS and ORR were not statistically significantly improved. Liver resection rates did not differ between the two groups either (Douillard *et al.* 2010).

Similar to panitumumab single cetuximab has proven beneficial in the chemorefractory phase of the disease (Karapetis *et al.* 2008). In 2nd line, cetuximab improves PFS and ORR when combined to irinotecan (Sobrero *et al.* 2008). In first line, in combination with FOLFIRI cetuximab improves ORR, PFS, OS, and R0 liver resection rates (Van Cutsem *et al.* 2011). When combining cetuximab with oxaliplatin in 1st line no benefit was seen in the Nordic VII study (Tveit K *et al.* 2010) or the MRC COIN study (Maughan *et al.* 2011). In contrast, in a phase II trial (OPUS) the combination of cetuximab with FOLFOX4 resulted in improved ORR and PFS. Interestingly, the patients with *KRAS* mutated tumors who had received cetuximab prior to the knowledge of the predictive value of *KRAS* did worse than the ones treated with FOLFOX4 only (Bokemeyer *et al.* 2009). Taken together, the data at present seem to encourage the use of anti-EGFR therapy in combination with irinotecan and since response rates with anti-EGFR therapies are acceptable these might be the drug of choice when downsizing of potentially resectable liver or lung metastasis is the aim (Loupakis *et al.* 2011). Typical side effects caused by cetuximab and panitumumab include an acneiform rash, hypomagnesemia, and allergic reactions (Van Cutsem *et al.* 2010).

Head to head comparison of bevazicumab and anti-EGFR therapies are lacking in the *KRAS* wt patient population. In the *KRAS* mutated patient group bevacizumab is usually chosen as part of the combination therapy when aggressive treatment is needed. A phase III trial (AIO KRK-0306) compared FOLFIRI/bevacizumab to FOLFIRI/cetuximab in first line treatment of *KRAS* mutated CRC and found surprisingly no statistically significant difference between the groups in ORR, PFS, or OS.

The study is ongoing in the *KRAS* wt patient group and the results will provide important knowledge in this field (Stintzing *et al.* 2012). The combination of bevacizumab and cetuximab or panitumumab simultaneously with chemotherapy results in decreased survival and increased toxicity and can not be recommended (Hecht *et al.* 2009; Tol *et al.* 2009).

2.4.5 Predictive markers in colorectal cancer

Factors that associate with response or resistance to a particular therapy are called predictive markers. Numerous predictive biomarker candidates have been tested for prediction of cytotoxic therapy efficacy in CRC, but none of those have been implemented into clinical practice yet.

Fluoropyrimidines are used both in the adjuvant and metastatic setting of CRC either as single drug therapy or in combination with irinotecan or oxaliplatin. 5-FU exerts its effects via metabolites that inhibit thymidylate synthase (TS) or get incorporated into DNA or RNA. Stage II and III colon cancer patients with MSI-H (dMMR) tumors do not appear to benefit from adjuvant single fluoropyrimidine therapy as shown in several studies (Ribic *et al.* 2003; Sargent *et al.* 2010) and in stage II patients fluoropyrimidine therapy has been linked to reduced OS (Sargent *et al.* 2010). A recent large retrospective study (n=2141) by Sinicrope *et al.* reported however a decreased recurrence risk, improved DFS and OS for MSI-H stage III colon cancer patients treated with 5-FU based adjuvant therapy, in contrast to stage II patients who showed no benefit. Interestingly the patients with MSI-H tumors with germline mutations, not the ones with sporadic MSI-H, were the ones who benefitted from the treatment (Sinicrope *et al.* 2011). An ongoing US Intergroup E5202 trial is investigating prospectively the predictive role of MSI-H and 18qLOH in stage II CRC patients stratifying the patients post-operatively to an observation arm or adjuvant therapy arm (FOLFOX ± bevazicumab) depending on a risk score. MSI-H does not seem to predict treatment responses in stage IV disease (Hewish *et al.* 2010). Loss of 18qLOH/S*MAD4* may be associated with poor response to 5-FU based adjuvant therapy in stage III CRC. 18qLOH is being tested prospectively in the E5202 trial together with MSI, as mentioned above (Pritchard & Grady, 2011).

Other investigated predictive markers for fluoropyrimidine therapy include for instance dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), and TS. DPD catabolizes 5-FU and a low DPD expression has in some reports been linked to improved adjuvant fluoropyrimidine based responses, but overall the results have been conflicting. TP converts capecitabine to 5-FU and 5-FU to its active metabolite fluorouridine monophosphate but might also have a role in neovascularization. This possibly dual role of TP might explain the conflicting results regarding its role as a predictive marker in CRC. TS, important for DNA synthesis is the primary target of fluoropyrimidine therapy. Both TS protein expression and mRNA levels have been investigated and a high TS level appears to correlate with worse survival, but no firm conclusions on this issue can yet be drawn (Koopman *et al.* 2009).

Topoisomerase-1 (Topo-1) is the target of irinotecan. The inhibition of Topo-1 leads to replication arrest and apoptosis. A high Topo-1 protein expression was demonstrated in a large, randomized trial to associate with an improved responsiveness to irinotecan, but the findings are still lacking validation (Koopman *et al.* 2009; Pritchard & Grady, 2011).

Excision cross-complementing (*ERCC)* gene 1 studied in lung cancer as a predictor of platinum-based chemotherapy (Hubner *et al.* 2011) has also been studied as a predictive marker for oxaliplatin based regimens in CRC. Low ERCC-1 expression correlates with improved survival in some studies, whereas a phase III study failed to find such an association. The data on ERCC-1 are still limited (Koopman *et al.* 2009).

Despite plenty of studies, we still lack predictive markers for bevacizumab therapy, whereas mutation of *KRAS* is a routinely used predictive marker for anti-EGFR therapy.

2.4.5.1 Predictive markers for anti-EGFR therapy

Activating *KRAS* oncogene point mutations, in codons 12 or 13, account for 85-90% of the *KRAS* gene mutations and are associated with non-responsiveness to anti-EGFR monoclonal antibody treatment (cetuximab and panitumumab) in metastatic CRC in several retrospective and prospective trials. The codon 61 mutations (present in only 5% of the cases) appear to be related to anti-EGFR therapy resistance as well, in contrast to the codon 146 mutations (De Roock *et al.* 2011; Karapetis *et al.* 2008; Qiu *et al.* 2010). *KRAS* G13D mutations might predict a favorable anti-EGFR treatment response, differing thereby from the other codon 13 mutations, but this finding needs further validation (De Roock *et al.* 2011). *KRAS t*esting prior to anti-EGFR therapy in metastatic CRC is the only predictive marker used routinely in the treatment of CRC today. However, anti-EGFR therapy does not benefit all *KRAS* wt patients, since only about 60% of the patients treated with anti-EGFR therapy in first line and 30-40% of the patients treated in second line or more respond to treatment (Bokemeyer *et al.* 2009; Chang *et al.* 2009; Lievre *et al.* 2006; Moroni *et al.* 2005; Van Cutsem *et al.* 2009a). Therefore, the search for additional predictive markers in the *KRAS* wt metastatic CRC patient population is ongoing.

BRAF V600E mutations might predict resistance to anti-EGFR therapy, but the results are still controversial (De Roock *et al.* 2011; Pritchard & Grady, 2011). Other markers that may predict resistance to anti-EGFR therapy, but still has limited evidence include *PIK3CA* mutations and phosphatase and tensin homolog (PTEN) loss. *PIK3CA* mutations are present in only 3-10% of *KRAS* wt CRC patients and the results presented have been conflicting, which might be due to the apparently different results obtained with exon-9 and exon-20 *PIK3CA* mutations (De Roock *et al.* 2011; Pritchard & Grady, 2011; Ross *et al.* 2010). Loss of PTEN expression by mutations, deletions, or promoter methylation of the tumor suppressor gene *PTEN* might associate with anti-EGFR therapy resistance in *KRAS* wt CRC patient. However, only about 5% of the *KRAS* wt tumors have PTEN loss, the published results are conflicting, and there is no standardized method at present available (De Roock *et al.* 2011; Ross *et al.*

2010). The ligands of EGFR amphiregulin and epiregulin may also predict anti-EGFR therapy responses, but the evidence is still insufficient (George & Kopetz, 2011).

EGFR gene copy number

In the search for additional predictive markers for EGFR targeted therapies in the *KRAS* wt CRC patient group, one subject of interest has been the EGFR itself. *EGFR* gene mutations are extremely rare (<1%) in CRC (Barber *et al.* 2004). EGFR protein expression has not proved efficient for predicting anti-EGFR therapy responses (Chung *et al.* 2005; Cunningham *et al.* 2004; Saltz *et al.* 2004). In contrast, several studies have reported a correlation between a high *EGFR* gene copy number (GCN) and favorable anti-EGFR mAb therapy response (Cappuzzo *et al.* 2008; Laurent-Puig *et al.* 2009; Personeni *et al.* 2008; Sartore-Bianchi *et al.* 2007; Scartozzi *et al.* 2009; Scartozzi *et al.* 2011). Selected published studies are presented in **Table 2**.

Table 2. Previously published studies examining the prognostic role of *EGFR* GCN.

Study	n	Technique used	Cut-off value	*KRAS* status	TTP/PFS high *EGFR* GCN *vs* low GCN (months)	P-value	OS high *EGFR* GCN *vs* low GCN (months)	P-value
Sartore-Bianchi *et al.*, JCO 2007	**58**	**FISH**	**2.5**	**wt and mt**	**Not reported**	**0.04**	**Not reported**	**0.02**
Personeni *et al.*, Clin Cancer Res 2008	**87**	**FISH**	**2.76**	**wt**	**6.9 *vs* 4.4**	**NS**	**13.3 *vs* 8.4**	**0.02**
Cappuzzo *et al.*, Annals of Oncol 2008	**85**	**FISH**	**2.92**	**wt and mt**	**6.6 *vs* 3.5**	**0.02**	**11.3 *vs* 8.5**	**NS**
Laurent-Puig *et al.*, JCO 2009	**116**	**FISH/CISH**	**4.0**	**wt**	**7.3 *vs* 7**	**NS**	**16.2 *vs* 11.8**	**0.01**
Scartozzi *et al.*, BMC Cancer 2009	**44**	**FISH/CISH**	**2.6/2.12**	**wt**	**7.7/6.4 *vs* 2.9/3.1**	**0.04/0.02**	**16/10.6 *vs* 9.5/10.3**	**NS/NS**
Scartozzi *et al.*, Annals of Oncol 2011	**90**	**CISH**	**2.12**	**wt**	**6 *vs* 3**	**0.003**	**18 *vs* 10**	**0.007**

Abbreviations: CISH, chromogenic *in situ* hybridization; EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; GCN, gene copy number; mt, mutated; NS, non significant; OS, overall survival; PFS, progression-free survival; TTP, time to progression; wt, wild type.

The most commonly used technique for *EGFR* GCN assessement has been fluorescence *in situ* hybridization (FISH). The usage of FISH is challenging due to interpretation difficulties, lack of standardized methodology in the anti-EGFR mAb response predicting setting, and reproducibility concerns. In addition, cut-off values for *EGFR* GCN have shown considerable variation in the studies reported. These factors probably explain why *EGFR* GCN has not been implemented into the clinical practice as yet.

3 AIMS OF THE STUDY

CRC is one of the most common cancers in the world and despite improvements in the treatment about half of the patients will still eventually die from the disease. CRC prognosis is affected by several factors including patient related characteristics, stage of the disease as well as molecular features of the tumor. Prognostic factors help us to predict the course of the disease and to estimate the aggressiveness of therapy needed in each case. In the era of personalized medicine progress has been made in tailoring the treatment according to individual molecular characteristics of the tumors. Nevertheless, a large portion of CRC patients today will end up receiving therapies and suffering from treatment related side-effects un-necessarily. Growing evidence implies that not only characteristics of the cancer cells, but also the microenvironment surrounding the tumor plays an important role in determining the behavior, treatment responsiveness, and prognosis of cancer. The aim of this study was to elucidate new prognostic and predictive markers, related both to the cancer cells and the tumor microenvironment in CRC to further improve our understanding of CRC prognosis and to provide tools for better patient selection to specific treatments. Lymphatic vessels and leukocyte trafficking in lymphatics were also subjects of special interest in this study.

The specific aims of this study were:

I To investigate the functional characteristics and expression of CD73 in lymphatic vessels in comparison to blood vessels.

II To analyze the prognostic impact of lymphatic vessel location and density in colorectal cancer.

III To investigate the prognostic role of tumor associated macrophages by using a pan-macrophage marker CD68 and a type 2 macrophage marker CLEVER-1 in colorectal cancer.

IV To study the predictive value of *EGFR* gene copy number and Chromosome-7 number assessed by EGFR protein expression guided silver *in situ* hybridization in anti-EGFR treated metastatic or locally advanced colorectal cancer patients.

V To elucidate the expression and prognostic value of Pim-1 in colorectal cancer.

4 MATERIALS AND METHODS

The materials and methods are presented in more detail in the original publications I-IV.

4.1 Patient materials and tissue samples

Table 3. Patient materials used in the studies (II, III, IV).

	TAM and lymphatics in CRC n = 159 (II)	*EGFR* GCN in CRC n = 80 (III)	Pim-1 in colorectal cancer, patient cohort 1 n = 124 (IV)	Pim-1 in colorectal cancer, patient cohort 2 n = 25 (IV)
	n (%)			
Median age of patients	72.8	60	73.5	58
Sex				
Male	61 (38.4)	34 (42)	49 (39.5)	16 (64)
Female	98 (61.6)	46 (58)	75 (60.6)	9 (36)
Site of primary tumor				
Colon	136 (85.5)	51 (63.8)	101 (81.4)	-
Rectum	23 (14.5)	28 (35)	23 (18.6)	25 (100)
Unknown	-	1 (1.2)	-	
Tumor differentiation grade				
G1	47 (29.6)	11 (13.8)	40 (32.3)	3 (12)
G2	93 (58.5)	50 (62.5)	76 (61.3)	16 (64)
G3	18 (11.3)	13 (16.2)	7 (5.6)	4 (16)
Grading unreliable/unknown	1 (0.6)	6 (7.5)	1 (0.8)	2 (8)
Stage at diagnosis				
Stage II	89 (56.0)	14 (17.5)	96 (77.4)	7 (28)
Stage III	38 (23.9)	25 (31.25)	28 (22.6)	11 (44)
Stage IV	32 (20.1)	41 (51.25)	-	7 (28)
KRAS mutational status				
KRAS WT	N/A	54 (67.5)	-	16 (64)
KRAS MT	N/A	24 (30)	-	9 (36)
Unknown	159 (100)	2 (2.5)	124 (100)	-
Anti-EGFR treatment				
Cetuximab	N/A	51 (63.8)	N/A	N/A
Panitumumab	N/A	10 (12.5)	N/A	N/A
Both	N/A	1 (1.2)	N/A	N/A
None	N/A	18 (22.5)	N/A	N/A
Follow-up data of the patients				
Alive and free of disease	48 (30.2)	5 (6.2)	47 (37.9)	2 (8)
Alive with disease	2 (1.3)	16 (20)	1 (0.8)	6 (24)
Died of disease	66 (41.5)	59 (73.8)	39 (31.5)	17 (68)
Died of other cause	21 (13.2)	-	19 (15.3)	-
Unknown cause of death	22 (13.8)	-	18 (14.5)	-

Abbreviations: CRC, colorectal cancer; EGFR, epidermal growth factor receptor; GCN, gene copy number; MT, mutated; N/A, not applicable; TAM, tumor associated macrophage; WT, wild type.

Normal human skin (n=3), lymph node (n=4), and intestinal (n=6) fresh frozen tissue samples were used in study I. The skin specimens were obtained from punch biopsies. The lymph node and intestinal samples were taken during routine surgical procedures.

Patients who were diagnosed with CRC and underwent primary tumor surgery in the Intermunicipal Hospital District of Southwest Finland between years 1996 – 1997 were included in the TAM and lymphatics in CRC (II) and Pim-1 in CRC (IV) studies. Study III included 80 patients treated at the Turku University Hospital with metastatic or locally advanced CRC of whom the majority received anti-EGFR therapy (62/80). Of those the treatment response could be reliably evaluated for 54/62 (87%). Response Evaluation Criteria in Solid Tumors (RECIST) was used when treatment responses were evaluated. Complete response (CR) was defined as disappearance of all target lesions. A minimum decrease of 30% in the sum of diameters of target lesions was required for the achievement of a partial response (PR). A 20% minimum increase in the sum of the diameters of the target lesions as well as the appearance of new metastatic lesions was defined as progressive disease (PD). The patients with responses between PR and PD were categorized as having stable disease (SD) (Eisenhauer *et al.* Eur J Cancer, 2009).

A second independent patient cohort of 25 rectal cancer patients was in addition included in study IV. 12 out of 25 of these patients received preoperative radiation therapy and diagnostic tumor biopsy samples were taken from nine of those prior to radiation therapy, which enabled me to study the effect of radiation therapy on Pim-1 expression.

Formalin-fixed, paraffin embedded tumor samples from primary CRC tumors were used in the studies (II, III, IV). The CRC diagnoses were confirmed by experienced pathologists. Patients lacking sufficient clinical data or tumor specimens, patients who died of postoperative complications, and patients who were lost during the follow up were excluded from the study.

Ten fresh frozen primary tumor CRC specimens were in addition used in study II for the evaluation of CLEVER-1 and MR co-localization.

4.2 Animals

Mice deficient for CD73 were generated and backcrossed for 9 generations to the C57/B16/J strain. One polymerase chain reaction was used for the wt and one for the recombined allele for identification of the CD73 wt B6 background strain and CD73-deficient mice (study I).

4.3 Antibodies and immunohistochemistry kits

Table 4. Antibodies and IHC kits used in the studies.

Antibody	Antigen	Isotype	Conjugate	Source	Study
PRIMARY ANTIBODIES					
174/2	Human PV-1	Mouse IgG1	FITC	Niemelä H et al, Blood 2005	I
2-7	CLEVER-1	Rat IgG		Palani et al, Eur J Immunol 2011	II
3-155	Macrophage mannose receptor	Mouse IgG		Irjala et al, JEM 2001	II
3-372	CLEVER-1	Mouse IgG1		Irjala et al, Eur J Immunol 2003	II
3-372	CLEVER-1	Mouse IgG1	Alexa Fluor 488	Irjala et al, Eur J Immunol 2003	II
4G4	Human CD73	Mouse IgG1		Airas et al, J Immunol 1993	I
CD103	Mouse CD103 (clone M290)	Rat IgG2a	PE	BD Pharmingen	I
CD11c	Mouse CD11c (clone HL3)	Hamster IgG1	PerCP-Cy5.5	BD Pharmingen	I
CD3	CD3	Mouse IgG2a		Acris Antibodies GmbH	II
CD40	Mouse CD40 (clone 3/23)	Rat IgG2a	PE	BD Pharmingen	I
CD68	Human CD68	Mouse IgG1	Alexa Fluor 647	Santa Cruz Biotechnology, Inc	I
CD68	Human and mouse CD68	Mouse IgG1		Abcam	II
EGFR	EGFR (intracellular domain, clone 5B7)	Rabbit IgG		Ventana Medical Systems	III
EGFR	EGFR (extracellular domain, clone 3C6)	Mouse IgG1		Ventana Medical Systems	III
Langerin	Human, mouse, rat, swine CD207	Rat IgG2a	Alexa Fluor 647	Dendritics	I
LYVE-1	Human LYVE-1	Rabbit IgG		RELIAtech GmbH	I
LYVE-1	Mouse LYVE-1	Rabbit IgG		RELIAtech GmbH	I
MHC II	Mouse MHC II alloantigens	Rat IgG2b	PE	BD Pharmingen	I
Pim-1	Human and mouse Pim-1 (clone 19F7)	Mouse IgG2a		M Lilly, Loma Linda, California	IV
Pim-1	Human Pim-1 (clone EP2645Y)	Rabbit IgG		Abcam	IV
Podoplanin	Human Podoplanin	Rabbit IgG		D Kerjaschki, Vienna, Austria	I, II
Podoplanin	Mouse podoplanin	Syrian hamster IgG	Biotinylated	BioLegend	I
TY/23	Mouse CD73	Rat IgG2a		BD Pharmingen	I
NEGATIVE CONTROLS					
3G6	T-cells (Chicken)	Mouse IgG1		Marko and Jalkanen, Science 1992	I, II, IV
AK-1		Mouse IgG1		In Vivo Biotech Services GmbH	I
Mouse IgG1 κ isotype control	Unknown	Mouse IgG1	Alexa Fluor 488	BD Pharmingen	II
NS-1	Unknown	Mouse IgG1	FITC	ATCC	I
Mouse IgG2a Isotype control	Keyhole Limpet Hemocyanin (KLH)	Mouse IgG2A		R&D Systems	II
Normal rabbit serum				Serotec	I, II, IV
Normal rat serum				Serotec	I
SECONDARY ANTIBODIES (IF stainings)					
Alexa Fluor 488 goat anti-rabbit IgG				Invitrogen	I
Alexa Fluor 488 goat anti-rat IgG				Invitrogen	I
Alexa Fluor 546 goat anti-mouse IgG1				Invitrogen	I
Alexa Fluor 546 goat anti-mouse IgG				Invitrogen	I, II
Alexa Fluor 546 goat anti-rat IgG				Invitrogen	I
Streptavidin conjugated Alexa Fluor 546				Invitrogen	I
KITS (IHC stainings)					
DakoCytomationEnvisionTM+System-HRP (DAB)				Dako Cytomation Inc	IV
ULTRAview Universal DAB Detection Kit				Ventana Medical System	III
Mouse Vectastain Elite ABC Kit				Vector Laboratories	II
Rabbit Vectastain Elite ABC Kit				Vector Laboratories	II, IV

4.4 Methodology used in the studies

Table 5. Methodology used in the studies.

Method	Study
Confocal microscopy	I, II
Enzyme assay	I
FITC painting	I
Fluorescence *in situ* hybridization	III
Imaging Research MCID M5+ image anlaysis software	IV
Immunofluorescence staining	I, II
Immunohistochemistry	II, III, IV
KRAS gene mutation analysis	III
Light microscopy	II, III, IV
Lymphocyte homing assay	I
Lymphocyte migration assay	I
Silver *in situ* hybridization	III
Skin explant culture	I
Receiver operating characteristic statistical analysis	III, IV

4.4.1 Immunohistochemistry (paraffin-embedded samples)

For detection of CD68$^+$ and CLEVER-1$^+$ TAMs, CD3$^+$ lymphocytes, podoplanin$^+$ and CLEVER-1$^+$ lymphatic vessel numbers, and PIM-1 protein expression levels standard immunohistochemical procedures were used (studies II, IV). Peroxidase activity caused by e.g. hemoproteins (hemoglobin) was suppressed by hydrogen peroxide (H_20_2). In order to improve immunoreactivity the following antigen retrieval methods were used prior to primary antibody incubations: citrate buffer (pH 6.0) heat treatment (anti-Pim-1, clone EP2645Y; anti-CD3), pepsin (anti-CD68), proteinase-K (anti-CLEVER-1, 3-372; anti- Pim-1, clone 19F7), and trypsin (anti-podoplanin). EGFR protein stainings were performed by using the automatized BenchMark XT System (Ventana Roche). Two distinct antibodies recognizing different domains of the EGFR were used: anti-EGFR (clone 3C6) and anti-EGFR (clone 5B7) recognizing the extra-cellular and intracellular domains of human EGFR, respectively (study III).

4.4.2 Immunofluorescence stainings

Immunofluorescence (IF) stainings were performed on acetone-fixed, fresh frozen, 5 μm thick tissue specimens. The primary antibodies were incubated for 30-60 minutes in room temperature except for the anti-human podoplanin antibody, which was incubated overnight in +4°C. The seconday antibodies were incubated protected from light in room temperature for 30 min. Prolong Gold Antifade Reagent was used for mounting (studies I, II).

4.4.3 Evaluation of immunohistochemical stainings

The evaluation of TAMs, lymphatic vessels, and $CD3^+$ lymphocytes was performed by light microscopy. The densities of the variables in question were separately evaluated both in the peri- and intratumoral areas and semi-quantitatively graded (study II).

The EGFR protein expression was analyzed by light microscope and classified into four staining intensity categories. The highest intensity observed (representing a minimum of 10% of the tumor area), the most common staining intensity, and the localization of the staining (membranous, cytoplasmic, or both) were evaluated (study III).

The Pim-1 protein expression intensity was analyzed both by light microscope and by using the Imaging Research MCID M5+ image analysis software (St Catharines, Canada). The highest Pim-1 protein expression area/tumor was analyzed by light microscope and classified into four categories: none (-), weak (+), moderate (++), and strong (+++). The subcellular staining pattern was also evaluated. Areas representing different staining intensities were photographed and the staining intensities from those areas were analyzed by the image analysis software. The Pim-1 expression from the rectal tumors representing the other independent patient material was analyzed by light microscope and graded into four categories: - (none), + (weak), ++ (moderate), and +++ (strong). The mean and the highest expression intensities and subcellular staining pattern were recorded (study IV).

Immunofluorescence stainings were analyzed by using Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss). Pictures were taken with Plan-Neofluar x20/0.5, x40/0.75, and x40/1.3 oil objectives. Zeiss LSM software was used as aquisition software and the images were processed with Zeiss LSM Image Browser (Carl Zeiss MicroImaging). Double positive vessels (study I) and macrophages (study II) were counted from the pictures.

4.4.4 Silver and fluorescence *in situ* hybridization

EGFR DNA probe (Ventana/Roche) and Chr-7 oligonucleotide probe (Ventana/Roche) were used for the detection of *EGFR* gene and Chr-7 from formalin-fixed, paraffin-embedded sections, respectively (study III). The BenchMark XT using ultraVIEW SISH Detection Kit (Ventana/Roche) was used for silver *in situ* hybridization (SISH) analysis. From the areas with the highest EGFR IHC intensity the *EGFR* GCN (number of copies of gene/cell) and Chr-7 number (number of copies of chromosome/cell) were counted from 40 cancer cells/slide, see **Figure 5**. *EGFR* GCN and Chr-7 number from nine selected CRC samples were in addition evaluated by fluorescence *in situ* hybridization using Vysis *EGFR*/CEP 7 FISH Probe Kit (Abbott Molecular Inc., USA).

4.4.5 Enzyme assays

Endothelial cells were cultured in 96-well plates. The enzymatic activities of ATPase, ADPase, ecto-5´-nucleotidase, and adenylate kinase were measured. The radiolabeled

nucleotides and nucleosides were separated and quantified by scintillation β-counting (study I).

4.4.6 Lymphocyte migration assays

Lymphocytes were collected and labeled with CFSE (a fluorescent dye) from lymph nodes and spleen of CD73 deficient and CD73 wt mice. The $CD73^{+/+}$ and $CD73^{-/-}$ lymphocytes were injected subcutaneously into the footpads of both CD73 deficient and wt mice. After 12 hours the popliteal lymph nodes were harvested, passed through a wire mess to obtain a single-cell suspension, and finally analyzed by flow cytometry. The lymphocyte migration was in a similar fashion also investigated after LPS (endotoxin, immune response inducer) injection as well as after incubating the lymphocytes with anti-LFA-1 antibody. LFA-1 is an adhesion molecule belonging to the integrin family (study I).

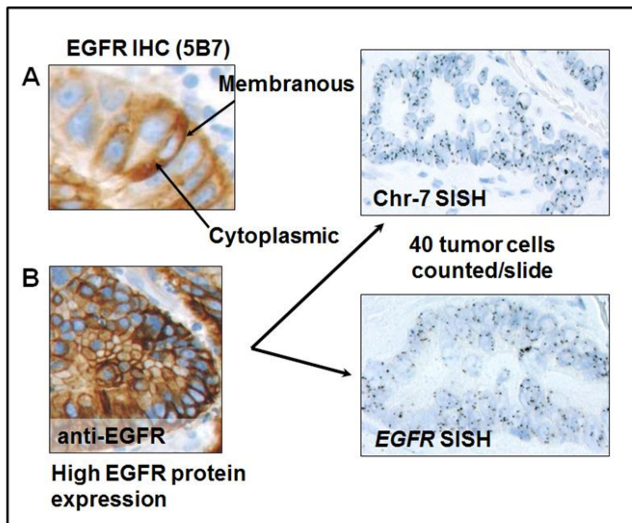


Figure 5 An algorithm combining EGFR protein expression (IHC) and *EGFR* GCN assessed by silver *in situ* hybridization was used for *EGFR* GCN evaluation. (A) EGFR protein expression is observed both in the cytoplasm and membrane of the colorectal cancer cells. (B) *EGFR* GCN analysis was performed from areas with highest EGFR protein expression. *EGFR* gene copy and chromosome 7 numbers were calculated from 40 tumor cells/sample from parallel slides.

4.4.7 Intravenous lymphocyte homing assay

Lymphocytes were collected from CD73 wt and CD73 deficient mice in a similar fashion as when performing the lymphocyte migration assays. The $CD73^{+/+}$ lymphocytes were labeled with CFSE and $CD73^{-/-}$ lymphocytes with TRITC. A pool consisting of both $CD73^{+/+}$ and $CD73^{-/-}$ lymphocytes were injected intravenously into the tail of both CD73 deficient and wt mice. After 4 hours both peripheral and mesenteric lymph nodes as well as spleens were collected, homogenized, and finally lymphocyte suspen-

sions analyzed by flow cytometry. A homing index was calculated for the lymphocytes that had entered the lymph nodes via the HEVs (study I).

4.4.8 FITC painting

Ears of both CD73 wt and deficient mice were painted with FITC on the dorsal side. After 48 hours lymph nodes draining auricular skin were collected, digested, and the tissue pressed through metal strainers to obtain single cell suspension. The cells were stained with dendritic cell markers and analyzed by flow cytometry (study I).

4.4.9 Skin explant culture

The dorsal side of ears of CD73-deficient and wt mice were cultured on 24-well plates for 48 hours. The emigrated dendritic cells were collected and counted and stained for CD73 (study I).

4.4.10 Statistical methods

Statistical analyses were performed with the SAS 9.2 and Enterprise Guide 4.2 computer programs for personal computers (SAS Institute Inc., Cary, NC). Frequency table data were analyzed with the Chi-Square test or Fisher´s exact test. Spearman correlation coefficients were calculated when correlations were analyzed. Statistical analyses comparing findings in CD73 wt and CD73 deficient mice as well as enzymatic activities on vascular and lymphatic endothelial cells were performed with Student´s t-test in study I. Differences in Pim-1 expression intensity, macrophage, and lymphatic vessel numbers between patient subgroups were analyzed with the Mann-Whitney U test. The differences observed in the tested variables within an individual tumor were analyzed with Wilcoxon Signed Rank Test. The cut-off values for *EGFR* GCN, Chr-7 number, and Pim-1 expression intensity were defined with the receiver operating characteristics (ROC) analysis. Univariate survival analyses were performed with Kaplan Meier and Log-rank test as well as Cox proportional hazards regression model. The Cox's proportional hazards regression model was also used when multivariate survival analyses were carried out. The PFS time was calculated from the initiation of anti-EGFR therapy until documented disease progression and the overall survival time from the onset of anti-EGFR therapy until death in study III. In study II and IV the DSS time was calculated from the time of CRC diagnosis until death of CRC. When calculating OS, death of any cause was the end point. P-values < 0.05 were regarded statistically significant.

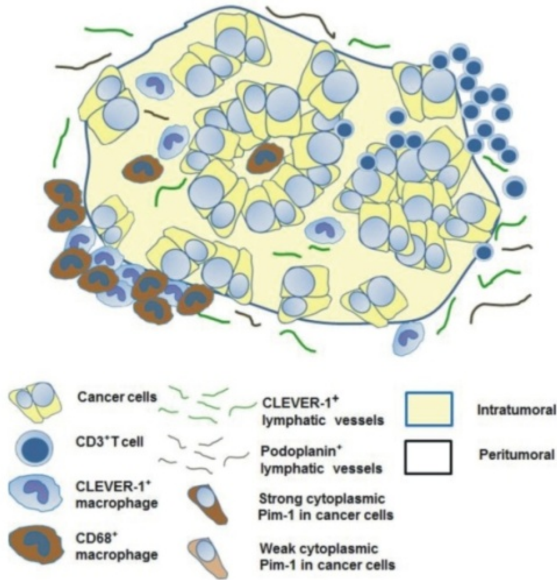
4.5 Ethics

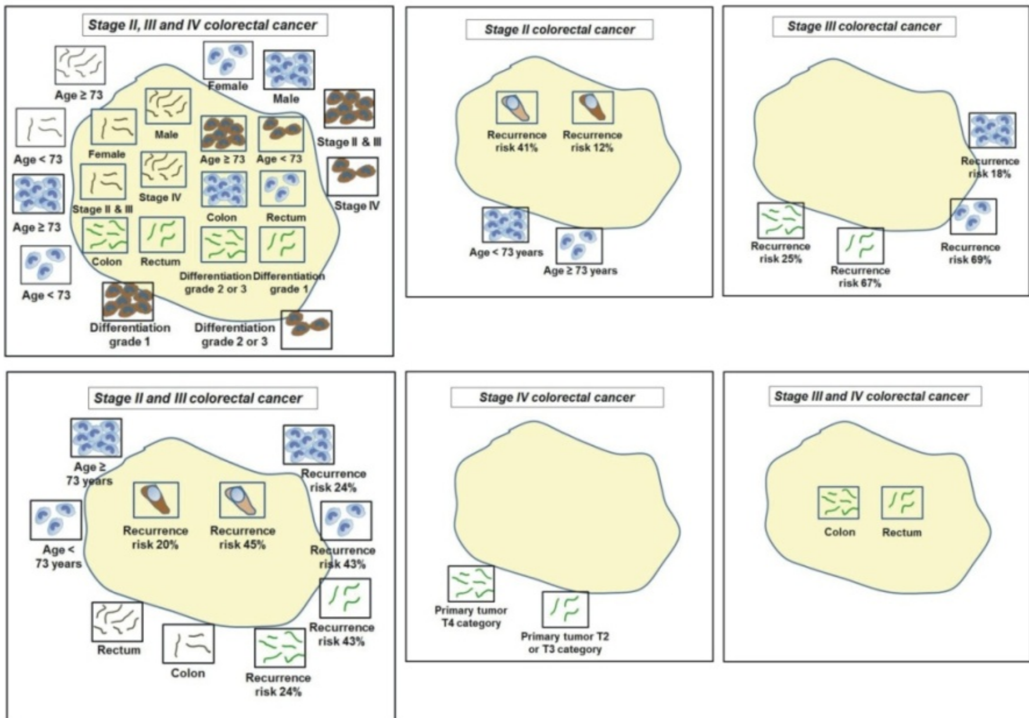
The study was conducted in accordance with the Declaration of Helsinki. The local ethical committee approved the collection of clinical data and usage of CRC tumor samples used in study I. In addition a written informed consent was obtained from the patients. In contrast, a written informed consent was not obtained from the patients participating in studies II-IV since the majority of the patients had already died when

the studies were initiated. Clinical data was collected and histological samples were used with the approval of the National Authority of Medico-Legal Affairs in these studies. The animal studies were approved by the ethical committee of University of Turku.

5 RESULTS

The statistically significant findings (frequency table data) of the variables evaluated in studies II and IV are presented in **Figure 6.**





5.1 CD73 in lymphatics (I)

5.1.1 CD73 expression on lymphatics is heterogenous

CD73 expression on lymphatic vessels of human gut (n=6), peripheral lymph nodes (n=3), and skin (n=3) was analyzed by using double immunofluorescence stainings with lymphatic markers podoplanin and LYVE-1. The majority of the podoplanin$^+$ (74%) and LYVE-1$^+$ (54%) lymphatic vessels in the skin were also CD73$^+$. Most lymphatic vessels in normal gut tissue were in a similar fashion CD73$^+$: podoplanin$^+$/CD73$^+$ and LYVE-1$^+$/CD73$^+$ lymphatic vessels in 61% and 57% of the cases, respectively. Thus, podoplanin$^+$ lymphatic vessels were slightly more commonly co-expressing CD73 than the LYVE-1$^+$ vessels.

CD73 expression on blood vessels was tested by using double IF stainings with PV-1 as a blood vessel marker. A marked variation was seen in the number of CD73 expressing blood vessels in the different tissues tested. The blood vessels of the normal lymph nodes were most often CD73$^+$ (82%), whereas a minority of blood vessels in the gut expressed CD73 (46%). Sixty-eight percent of the skin blood vessels were CD73$^+$.

The CD73 expression studies on lymphatic vessels of wt BALB/C mice foot pad skin samples by double IF stainings revealed that only a minority of the lymphatic vessels of mouse skin are CD73$^+$: 31% and 12% of the podoplanin$^+$ and LYVE-1$^+$ vessels co-expressed CD73, respectively. Similarly to human skin samples the podoplanin$^+$ vessels were more commonly CD73$^+$.

The distribution of CD73 in both human and mice lymph nodes were investigated and regardless of the species the afferent lymphatic vessels and subcapsular sinus were found to express CD73 in contrast to the lymphatic sinuses which were devoid of CD73.

5.1.2 Nucleotide metabolism differs between lymphatic and vascular endothelium

Cultured human microvascular endothelial cells were separated into lymphatic and vascular endothelial cell subgroups and examined for ATPase, ADPase, 5´-nucleotidase, and adenylate kinase activity. Ecto-5´-nucleotidase activity was higher and NTPDase (ATPase, ADPase) activity lower on lymphatic than blood vessel endothelium.

5.1.3 CD73 on lymphocytes affects their migration

CFSE-labeled lymphocytes from wt and CD73 knock-out mice were used for investigating lymphocyte migration via the afferent lymphatics to the draining lymph nodes

Figure 6 Factors examined in studies II and IV and their association with clinicopathological variables of the colorectal cancer patients. Only statistically significant differences observed between the groups analyzed are presented. The investigated factors, located either intra- or peritumorally, were divided into two groups [high *vs* low number (CD3$^+$ T cells, CD68$^+$ and CLEVER-1$^+$ macrophages, podoplanin$^+$ and CLEVER-1$^+$ lymphatic vessels) or high *vs* low expression (Pim-1)]. **(A)** The factors investigated in studies II and IV. **(B)** The statistically significant results are presented separately for different colorectal cancer stage subgroups.

both in wt and CD73-deficient mice. At a 12 hour timepoint the number of migrated lymphocytes was counted in the lymph nodes. $CD73^+$ lymphocytes migrated in a similar fashion regardless of whether the lymphatic endothelial cells expressed CD73 or not. In contrast, the migration of CD73 deficient lymphocytes was statistically significantly attenuated both in wt and CD73 knock-out mice. LPS challenge did not change the $CD73^+$ lymphocyte migration results or the number of LYVE-1^+/$CD73^+$ positive lymphatic vessels in the skin.

5.1.4 LFA-1 is not involved in lymphocyte migration via afferent lymphatics

The involvement of LFA-1 in lymphocyte migration via the afferent lymphatics to the draining lymph nodes was studied by using a function-blocking LFA-1 antibody. The migration of the $CD73^{+/+}$ or the $CD73^{-/-}$ lymphocytes in CD73 wt or CD73 deficient mice was unaltered as a consequence to the anti-LFA-1 antibody treatment.

5.1.5 CD73 does not affect the homing of lymphocytes under normal conditions

The homing via HEVs to lymphoid organs of $CD73^{+/+}$ and $CD73^{-/-}$ lymphocytes were tested both in CD73 wt and CD73-deficient mice. Neither lymphocyte nor endothelial CD73 expression status impacted the homing of the lymphocytes via HEVs.

5.1.6 Dendritic cell trafficking in the afferent lymphatics does not involve CD73

To study whether CD73 takes part in the migration of dendritic cells via afferent lymphatics to the auricular draining lymph nodes both CD73 wt and CD73 deficient mice were used. FITC painting revealed no difference in migrated dendritic cells between CD73 wt and knock-out mice.

5.2 Lymphatics, macrophages, and $CD3^+$ T lymphocytes in colorectal cancer (II)

5.2.1 Lymphatic vessels are heterogenous

Lymphatic vessels in CRC were investigated by using two distinct lymphatic endothelial markers, CLEVER-1 and podoplanin. The appearance of the lymphatic vessels differed between the normal gut tissue and malignant areas. The vessels peri- and intratumorally were smaller in size and had flattened lumens more often than the vessels in the healthy tissue area. Moreover, the density of lymphatic vessels was higher in the peritumoral area than intratumorally. Less than 20% of the $podoplanin^+$ lymphatic vessels co-expressed CLEVER-1.

5.2.2 Intratumoral lymphatics - a sign of poor prognosis

CLEVER-1^+ vessels were more abundant in poorly differentiated tumors than in well differentiated ones and a higher number of intratumoral $podoplanin^+$ lymphatic vessels were observed in stage IV than in stage II and III CRC. Furthermore, a high podo-

planin$^+$ LVD intratumorally associated with a shorter DSS. The number of intratumoral CLEVER-1$^+$ vessels, however, did not associate with survival.

5.2.3 Peritumoral lymphatics - a sign of good prognosis

Stage II and III CRC patients with a high peritumoral CLEVER-1$^+$ LVD had a lower risk of developing a disease recurrence during follow up than the ones with a low peritumoral vessel count (24% *vs* 43%). Among patients with lymph node metastasis at the time of diagnosis (Stage III disease) the recurrence risk difference between the two groups was even higher, 42% (25 *vs* 67%). In addition, patients with a low number of peritumoral CLEVER-1$^+$ LVD had more often a primary tumor representing an advanced T category (T4) than the ones with a high lymphatic vessel count.

5.2.4 CLEVER-1$^+$ vessel and macrophage numbers correlate with each other

CLEVER-1$^+$ vessel density correlated positively with the CLEVER-1$^+$ macrophage density both peri- and intratumorally. In contrast no association was found between the podoplanin$^+$ LVD and macrophage numbers in the peri- or intratumoral areas. The CD68$^+$ macrophage density did not correlate with LVD either.

5.2.5 CLEVER-1 is a marker for type 2 macrophages

To confirm that the macrophages recognized by the CLEVER-1 antibody are indeed type 2 macrophages double IF stainings using both anti-CLEVER-1 and anti-MR antibodies on CRC tissue cryo sections were performed. Almost all MR$^+$ macrophages (95%) were found to co-express CLEVER-1.

5.2.6 The location of macrophages is crucial for CRC outcome

A high number of peritumoral CD68$^+$ macrophages was more often seen in well-differentiated tumors and in early stage disease (stage II and III) than in advanced disease (stage IV). Furthermore, a high CD68$^+$ peritumoral macrophage number associated positively with DSS. When investigating the subpopulation of CLEVER-1$^+$ type 2 macrophages an association between a high number of macrophages in the peritumoral area and a reduced disease recurrence risk was observed. The median DFS time among patients with a high peritumoral CLEVER-1$^+$ macrophage density compared to those with a low number was 103 *vs* 63 months, respectively. In contrast, no statistically significant difference in intratumoral CLEVER-1$^+$ macrophage number between the patients who stayed disease free or those who developed a disease recurrence was found. In early stage disease (stage II and III) a high density of peritumoral CLEVER-1$^+$ macrophages was associated with a longer DSS. In metastatic stage IV disease a high peritumoral CLEVER-1$^+$ macrophage number, in contrast, was associated with a shorter DSS. Stage IV CRC patients with a high number of intratumoral CLEVER-1$^+$ macrophages had a shorter DSS than patients with a low number of these macrophages intratumorally.

5.2.7 Subtype of macrophages influences recurrence site

The balance between the $CD68^+$ and CLEVER-1^+ macrophage numbers were investigated by comparing the tumors with a high number of $CD68^+$ macrophages and a low number of CLEVER-1^+ macrophages (type 1 dominant macrophage score) to all other types of tumors. Only 9% of the patients who developed distant metastasis during follow up had an intratumoral type 1 dominant macrophage score in their primary tumor, whereas a majority (57%) of the patients with only a local disease recurrence had this macrophage score in their primary tumors.

5.2.8 $CD3^+$ lymphocytes in Stage III colorectal cancer

The $CD3^+$ T lymphocyte density both peri- and intratumorally in stage III CRC did not associate with the number of macrophages, recurrence risk, or survival. The number of $CD3^+$ T lymphocytes did not correlate with $podoplanin^+$ or CLEVER-1^+ vessel numbers either.

5.2.9 A new prognostic score is associated with CRC survival

A combined score hypothesized to associate with a good CRC prognosis was created on basis of the results. The score included: a high number of peritumoral $CD68^+$ macrophages, a high number of peritumoral (PT) CLEVER-1^+ macrophages and a low intratumoral $podoplanin^+$ lymphatic vessel number. This $CD68^{+high}PT/Clev^{+high}PT/podo^{+low}IT$ score was observed in 22/159 (14%) of the CRC primary tumors included in this study. Eighteen of these patients had Stage II CRC, three patients had Stage III disease, and only one patient metastatic Stage IV CRC. The CRC patients with the $CD68^{+high}PT/Clev^{+high}PT/podo^{+low}IT$ score in their primary tumors had a longer DSS. Furthermore, the $CD68^{+high}PT/Clev^{+high}PT/podo^{+low}IT$ score was associated with fewer disease recurrences (19% vs 37%) among the Stage II and III CRC patients, but this 18% difference did not reach statistical significance. The median disease-free survival in the subgroup of $CD68^{+high}PT/Clev^{+high}PT/podo^{+low}IT$ stage II CRC patients was notably pro-longed; 11 vs 5.8 years.

5.3 *EGFR* gene copy number as a predictive marker for anti-EGFR treatment in metastatic colorectal cancer (III)

5.3.1 EGFR protein expression correlates with *EGFR* GCN

EGFR protein expression was evaluated with two different monoclonal antibodies against EGFR; clone 5B7, which recognizes the intracellular domain of EGFR and clone 3C6, which recognizes the extracellular domain of EGFR. The EGFR protein expression was heterogenous within the tumors. The most commonly observed EGFR staining intensity was low (+) and the highest staining intensity moderate (++) in the majority of the tumors. Strong (+++) EGFR expression was only seen in one tenth of the tumors. The EGFR expression results obtained with the two different antibodies correlated statistically significantly with each other. Because of the intratumoral het-

erogeneity of EGFR protein expression *EGFR* GCN and Chr-7 number were chosen to be evaluated from the regions with the highest EGFR protein expression.

ROC-curve analysis was used for determination of the optimal cut-off value for *EGFR* GCN and Chr-7 number and turned out to be 4.0 and 4.5, respectively. A high *EGFR* GCN (≥ 4.0) was observed in 64% (51/80) and a high Chr-7 number (≥ 4.5) in 60% (48/80) of the CRC tumors. The EGFR protein expression evaluated with the intracellular domain antibody (clone 5B7) correlated positively with the *EGFR* GCN and Chr-7 numbers. In contrast, the EGFR protein expression results obtained with the extracellular domain antibody (3C6) showed no correlation with *EGFR* GCN despite a positive correlation with Chr-7 number.

5.3.2 *EGFR* GCN predicts responsiveness to anti-EGFR treatment

Metastatic CRC patients with a high *EGFR* GCN (≥ 4.0) in their primary tumors treated with anti-EGFR therapy had a clinical benefit response (CR + PR + SD) in 73% of the cases compared to only 20% for those with a low *EGFR* GCN (< 4.0). When analyzing the predictive value of the marker used routinely today, *KRAS*, a clinical benefit response rate of 59% was observed among the *KRAS* wt patients. A combination of *KRAS* gene status and *EGFR* GCN improved the results: the clinical benefit rate increased to 82% for the *KRAS* wt patients with a high *EGFR* GCN (≥ 4.0). The responses to anti-EGFR therapy are shown more in detail in **Figure 7**.

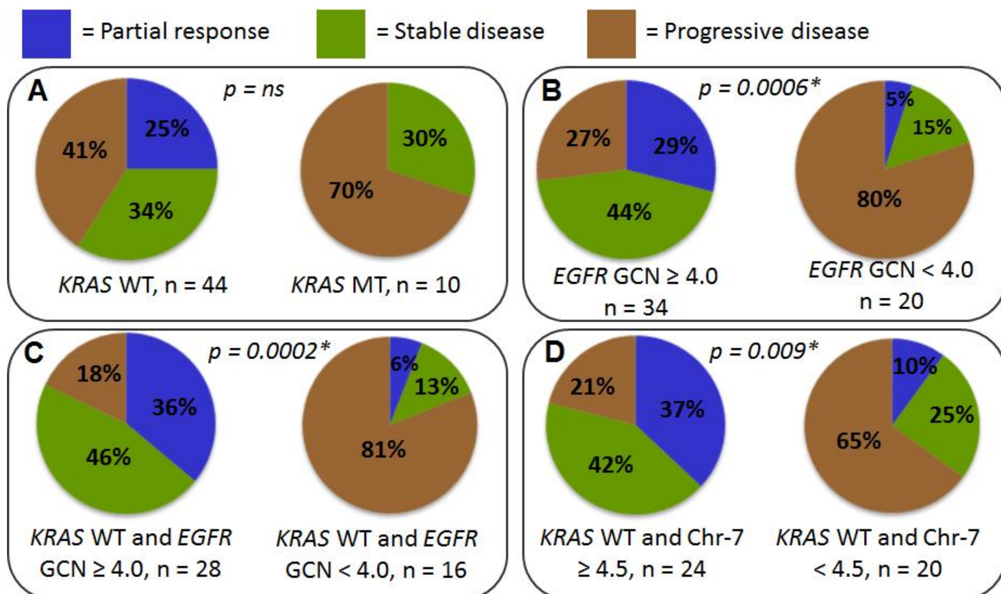


Figure 7 Anti-EGFR treatment responses in metastatic colorectal cancer according to **(A)** *KRAS* gene status and **(B)** *EGFR* gene copy number. The treatment responses in the *KRAS* wild type subgroup of patients according to **(C)** *EGFR* gene copy number and **(D)** Chromosome 7 number. Adapted from Algars *et al.* Br J Cancer 2011 (study III).

Subgroup analyses were performed to investigate the predictive value of *EGFR* GCN also in *KRAS* wt metastatic CRC patients treated with anti-EGFR therapy in first line (n = 5), second line or more (n = 39), and in the chemorefractory phase of the disease (≥ third line, n = 25). From the chemorefractory subgroup of patients the ones with a prolonged SD (≥ 24 weeks) were also separately analyzed. *KRAS* wt patients with a high *EGFR* GCN responded statistically significantly better to anti-EGFR treatment than the ones with a low *EGFR* GCN in all subgroups analyzed. The best clinical benefit rate (84%) was recorded in the chemorefractory CRC patient group.

5.3.3 A high *EGFR* GCN is associated with improved survival

PFS was statistically significantly longer in anti-EGFR treated metastatic CRC patients with *KRAS* wt tumors as compared to *KRAS* mutated ones (22 *vs* 12 weeks) and in patients with a high *EGFR* GCN in their primary tumors in comparison to a low *EGFR* GCN (32 *vs* 12 weeks). The combination of these two markers improved the results: patients with *KRAS* wt tumors with a high *EGFR* GCN had an almost three times longer PFS than the ones with a low *EGFR* GCN (35 *vs* 12 weeks). Subgroup PFS survival analyses were performed by including the *KRAS* wt patients that had been treated with anti-EGFR therapy in ≥ second line or the ones treated at a chemorefractory phase of the disease. From the chemorefractory patient material a further subgroup analysis was carried out by excluding the patients with a short SD duration (< 24 weeks). In all patient subgroups analyzed a high *EGFR* GCN associated with a prolongation of PFS. *EGFR* GCN proved to be an independent predictor of PFS in the multivariate survival analysis also.

OS was in a similar fashion improved in metastatic CRC patients treated with anti-EGFR therapy with a high *EGFR* GCN in their primary tumors as compared to a low *EGFR* GCN (15.9 *vs* 4.4 months). OS for patients with *KRAS* wt tumors was longer than for patients with *KRAS* mutated tumors (11.6 *vs* 8.2 months), but the difference did not reach statistical significance. *KRAS* wt/*EGFR* GCN ≥ 4.0 patients had a more than four times longer OS than the *KRAS* wt/ *EGFR* GCN < 4.0 patients (19.7 *vs* 4.4 months). OS remained longer in the *KRAS* wt/*EGFR* GCN ≥ 4.0 patients in the subgroup analyses including patients treated with anti-EGFR therapy in ≥ second line or patients treated in the chemorefractory phase of the disease. *EGFR* GCN independently predicted OS also in multivariate survival analysis.

5.4 Pim-1 in colorectal cancer (IV)

5.4.1 Pim-1 protein expression is heterogenous in CRC

Pim-1 protein expression in primary stage II and III CRC tumors (n = 124) was evaluated with a monoclonal mouse Pim-1 antibody (clone 19F7). The expression profile was exclusively cytoplasmic and showed heterogeneity within the tumors. Despite testing different antigen retrieval methods no Pim-1 expression in the nuclei of the CRC cells was seen. Strong staining (+++) was seen in 37% (46/124) of the tumors. Only 4% (5/124) of the tumors were completely devoid of Pim-1 expression.

The Pim-1 expression categories obtained by visual evaluation correlated statistically significantly with the intensity values of Pim-1 expression provided by the image analysis computer program. The Pim-1 expression values provided by the image analysis computer program were used in statistical analyses. The cut-off value for Pim-1 expression was determined by ROC analysis generated on disease outcome (DSS, OS, disease recurrence) and set at 60 percentile (image analysis value 0.1185).

A second independent patient cohort consisting of rectal cancer patients (n=25) was evaluated for Pim-1 expression by using a commercially available monoclonal Pim-1 antibody (clone EP2645Y). The staining pattern was heterogenous and in contrast to the 19F7 antibody results nuclear Pim-1 staining was seen in a majority of the tumors. Seventy-two percent of the tumors had Pim-1 expression present in the cytoplasm of the cancer cells, whereas nuclear staining was seen in almost all (92%) cases.

To further investigate the clearly different staining pattern observed with the two distinct Pim-1 antibodies, ten CRC tumor specimens of the stage II and III patient material were stained with the EP2645Y antibody, in addition to the 19F7 antibody. An unquestionable difference in the nuclear staining capability was observed as all specimens had Pim-1 in the nucleus, when the clone EP2645Y antibody was used in contrast to no nuclear Pim-1 staining with the 19F7 antibody.

5.4.2 Radiation therapy alters subcellular Pim-1 expression levels

From nine rectal cancer patients, both a pre- and post-radiation tumor sample was available, which made it possible to study the effect of radiation therapy on Pim-1 localization and expression intensity. The cytoplasmic Pim-1 expression was increased in 33% (mean expression) or 44% (highest expression) of the cases or alternatively unaltered. No reduction of Pim-1 expression was observed. In contrast, a decrease in nuclear Pim-1 expression was noticed in 22% (highest expression) or 44% (mean expression) of the cases.

5.4.3 High Pim-1 expression is associated with a favorable CRC outcome

Stage II and III CRC patients with a high Pim-1 expression (above the 60 percentile) in their primary CRC tumors experienced fewer disease recurrences than the ones with low Pim-1 expressing tumors (20% *vs* 45%). In addition, both DSS and OS were longer for patients with high Pim-1 expressing tumors than for those with low levels of Pim-1. A 7% difference in the 2-year survival rate was observed in benefit of the patients with high Pim-1 expressing tumors (88% *vs* 81%). The 5-year survival difference was 13% in favor of the patients with elevated Pim-1 expression (70% *vs* 57%). A high Pim-1 expression associated positively with DSS also in multivariate survival analysis, whereas the association with OS was only borderline significant ($p = 0.05$).

In the independent rectal cancer patient cohort patients with negative nuclear Pim-1 expression as the mean expression category (most commonly observed nuclear staining pattern) had a shorter DSS than the ones with Pim-1 present in the cancer cell nuclei.

The DSS advantage for those patients with more Pim-1 in the nucleus remained significant when the patients with stage IV disease at the time of diagnosis were excluded from the analysis. Kaplan Meier survival curves and IHC figures of selected markers investigated in this study are shown in **Figure 8**.

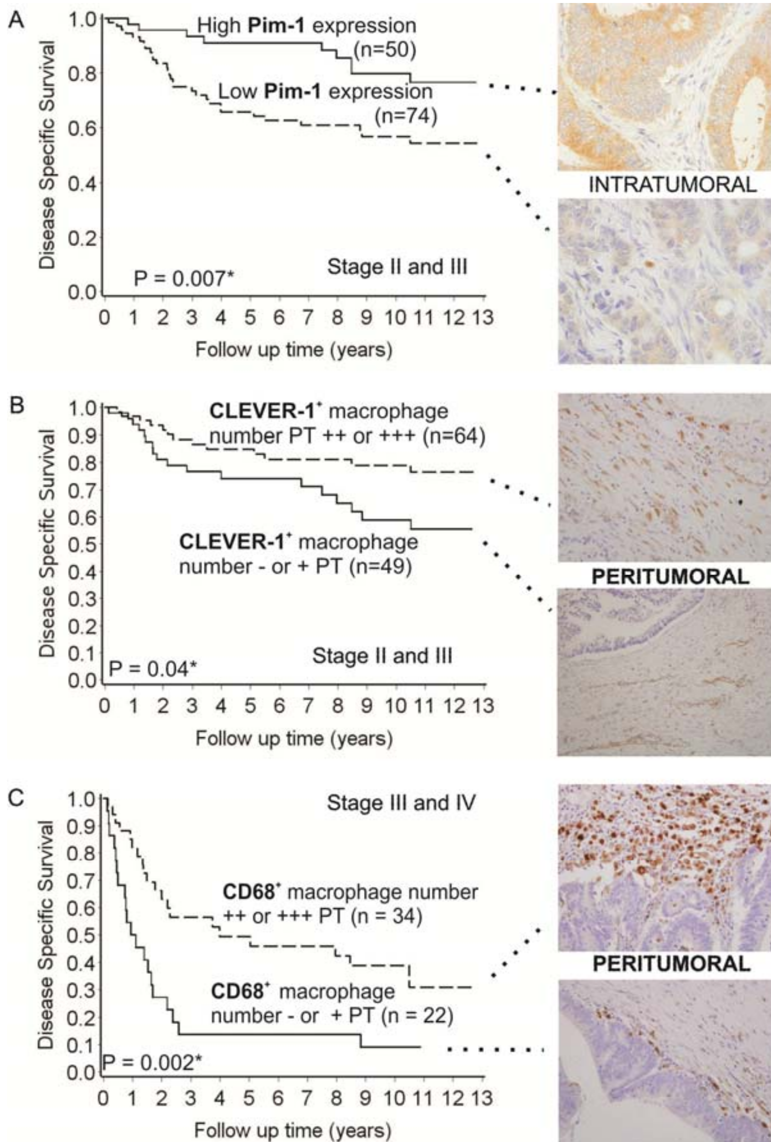


Figure 8 Kaplan Meier survival curves and immunohistochemical stainings for some of the markers investigated in this study. **(A)** High Pim-1 expression and **(B)** a high number of CLEVER-1$^+$ type 2 macrophages in the peritumoral (PT) area associate with improved disease-specific survival in stage II and III colorectal cancer patients. **(C)** A high density of CD68$^+$ macrophages in the peritumoral area of stage III and IV colorectal cancer patients is associated with longer disease-specific survival.

6 DISCUSSION

6.1 CD73 in lymphatics

This study demonstrates that CD73 is a molecule involved in lymphocyte trafficking via the afferent lymphatics to the draining lymph nodes and thus adds CD73 to the repertoire of just a few molecules known at present to take part in this event. The molecules involved in leukocyte trafficking in blood vessels are in contrast well characterized (Ley *et al.* 2007). CD73 on lymphocytes, not lymphatic endothelial cells, participates in lymphocyte migration and trafficking via the afferent lymphatics according to the results of this study. By contrast, the binding of lymphocytes to vascular endothelium has been demonstrated to inhibit the enzymatic activity of endothelial CD73 and thereby decrease the amount of adenosine. This enhances leukocyte transmigration as a consequence of impaired vascular barrier function (Henttinen *et al.* 2003). The leukocyte trafficking in the afferent lymphatics *in vivo* occurred independent from integrin LFA-1 in study I opposite to the blood vessels, where *in vitro* data show that CD73 on lymphocytes increases lymphocyte binding to endothelial cells via LFA-1 clustering (Airas *et al.* 2000).

This study investigated also the homing of lymphocytes via blood vasculature and demonstrated that neither on lymphocytes nor on endothelial cells did CD73 affect the homing of lymphocytes via HEVs to the spleen, blood, peripheral or mesenteric lymph nodes under normal, noninflamed conditions, which is in line with the findings of Takedachi *et al.* Takedachi and colleagues investigated also the migration of T and B lymphocytes across HEVs after LPS challenge and showed that adenosine generated by CD73 on HEVs, not lymphocytes, decreases lymphocyte migration via adenosine receptor A_{2B} signaling (Takedachi *et al.* 2008). Lymphocyte migration through HEVs during LPS challenge was not investigated in study I. Instead, the effect of LPS on lymphocyte migration in afferent lymphatic vessels was examined and it did not affect the migration. Taken together, the role of CD73 as a leukocyte trafficking molecule differs between the blood and lymphatic vasculature.

The expression analyses of CD73 in lymphatic and blood vessels of normal human and mouse skin, lymph node, and gut tissues revealed that both lymphatic and blood vessels are heterogenous. Interestingly, more than half of the lymphatic vessels in human skin samples expressed CD73, whereas only a minority of the lymphatics in mouse skin was CD73 positive. Both in human and mouse the podoplanin$^+$ lymphatic vessels were more often CD73$^+$ than the LYVE-1$^+$ lymphatics. The finding that CD73 is present only on afferent lymphatics is extraordinary, since all known lymphatic markers known today are present both on afferent and efferent lymphatics. Higher activities of ATPase and ADPase were seen on vascular endothelial cells in comparison to lymphatic endothelium. In contrast, ecto-5´-nucleotidase activity was higher on lymphatic than vascular endothelium further emphasizing the differences between the two vascular beds.

Previously only one molecule expressed on lymphocytes, CCR7 has been shown to take part in leukocyte trafficking in the afferent lymphatics (Debes *et al.* 2005). Therefore the discovery of a new molecule participating in leukocyte trafficking through the

lymphatics is important and possibly opens the door for future research in the fields of for instance inflammation and cancer. Tumor cells utilize the lymphatic vessel conduits for metastatic spread. Cancer cells expressing CD73 could potentially use the same mechanism for disseminating to the lymph nodes as the lymphocytes in this study. At present we do not know the mechanism of how CD73 on the lymphocytes facilitates the trafficking in the afferent lymphatics.

Several protumoral effects have been linked to CD73 or adenosine generated by CD73, e.g. the ability to increase tumor cell chemotaxis, increased tumor cell migration, suppression of antitumoral immunity, and anti-apoptotic effects. Targeting of CD73 would therefore be appealing. In cancer, potential treatment target candidates include the blocking of A_{2A} adenosine receptors or CD73 by anti-CD73 antibodies or small molecular inhibitors of CD73 (Salmi & Jalkanen, 2011; Stagg & Smyth, 2010). By contrast, in inflammatory diseases the upregulation of adenosine or CD73 is the goal, which can be achieved by e.g. interferon-α or -β therapy, which both increase CD73 on vasculature without affecting the CD73 levels on cancer or inflammatory cells. Statins are also known inducers of CD73 (Salmi & Jalkanen, 2011).

CD73 is a molecule involved both in inflammation and lymphatics, which are both factors capable of affecting the course of CRC. Therefore the role of CD73 in human CRC would be interesting to study in the future. Finally, when interpreting data of *in vitro* and *in vivo* murine studies one should keep in mind that the results obtained from mice experiments might not directly translate to humans.

6.2 Lymphatics in colorectal cancer

The results in study II by using two distinct lymphatic endothelial markers, podoplanin and CLEVER-1, showed that the location and number of lymphatic vessels both intra- and peritumorally may influence the outcome of CRC. Quite interestingly the LVD seemed to impact the disease prognosis inversely depending on the location of the lymphatic vessels. A high LVD intratumorally was associated with a decreased survival of the patients, whereas, in contrast, a high LVD peritumorally was associated with fewer disease recurrences.

To my knowledge only a few studies have investigated the association between LVD and patient survival in CRC previously. Three studies reported, similar to these results, an association between a high intratumoral LVD and decreased survival (Li *et al.* 2011b; Matsumoto *et al.* 2007; Yan *et al.* 2008), whereas a study by Omachi *et al.* found no such association (Omachi *et al.* 2007). Only one of the studies mentioned above investigated the peritumoral LVD separately and found no association between peritumoral LVD and survival (Matsumoto *et al.* 2007). The findings regarding the relationship between a high peritumoral LVD and good prognosis demonstrated in my study are not unique, since similar findings have been published in other malignancies like SCC of the head and neck (Maula *et al.* 2003) and melanoma (Straume *et al.* 2003). One possible mechanism for this phenomenon could be an enhanced immune

response towards the cancer cells provided by the peritumoral lymphatics serving as a conduit for antigen presenting cells

In study II the LVD was higher peritumorally than intratumorally but nevertheless lymphatic vessels were observed intratumorally in a majority of the tumors (84%). In some studies intratumoral lymphatics have been extremely rare (Ishikawa *et al.* 2008; Liang *et al.* 2006; Liang *et al.* 2007), whereas other studies have detected intratumoral lymphatics similar to these findings (Longatto-Filho *et al.* 2008; Matsumoto *et al.* 2007; Saad *et al.* 2006). No proliferative marker to examine the functionality of the intratumoral lymphatic vessels was used in this study, but patients with distant metastasis had more vessels intratumorally than patients with stage II and III disease suggesting that these vessels may be involved in the metastatic spread of CRC. Proliferative markers (Ki-67) have been used in addition to lymphatic markers in only a few of the studies investigating LVD in CRC (Liang *et al.* 2006; Omachi *et al.* 2007). Of those, proliferative lymphatic vessels were detected in only one of the studies (Omachi *et al.* 2007).

The retrospective nature and heterogenous patient material are two unquestionable weaknesses of this study. Furthermore, rather unexpectedly no difference in LVD between stage II (N0) and III (N+) disease was observed. This could be explained by possible under-staging due to inadequate numbers of lymph nodes examined at the time of diagnosis, resulting in lymph node positive patients being diagnosed falsely into lymph node negative ones. At the time period 1996 - 1997, when the patient population of this study underwent CRC primary tumor surgery an adequate number of lymph nodes (≥12) in accordance with today`s standards were seldom investigated. According to recent knowledge not only the increase in number of lymphatic vessels but also qualitative changes in lymphatic vessels are capable of enhancing the spread of malignant cells to the regional lymph nodes. However, in this study the morphological changes in the lymphatic vessels were not studied.

The strengths of this study include the usage of two different lymphatic markers, even though one should remember, when interpreting the results regarding CLEVER-1^{+} vessels that CLEVER-1 can in addition to lymphatic endothelium be expressed on neovasculature in malignancies. Podoplanin on the other hand is probably one of the most reliable lymphatic vessel markers available today. Other strengths of this study include the separate analysis of both the intra- and peritumoral regions of the tumor samples as well as the inclusion of survival data of the patients.

The role of angiogenesis in tumor development has been widely studied resulting in anti-angiogenic drugs being incorporated into routine cancer care. Bevacizumab is an anti-VEGF-A monoclonal antibody used in the treatment of CRC, lung cancer, and renal cell carcinoma. Sunitinib (Gan *et al.* 2009), sorafenib (Mulder *et al.* 2010), and pazopanib (Schutz *et al.* 2011) are examples of other cancer drugs available with anti-angiogenic effects (Holopainen *et al.* 2011). The discovery of specific lymphatic endothelial markers has increased the knowledge also in the field of tumor-associated lymphangiogenesis. Lymphatic vessels are thought to provide a conduit for cancer cells to utilize for metastasizing to the regional lymph nodes and LVI is regarded a strong negative prognostic factor in most malignancies. As a consequence, numerous studies have attempted to find factors

involved in this crucial step of tumorigenesis in order to find means to prevent this harmful event. Focus has lied on growth factors known to be involved in lymphangiogenesis, their receptors, LVD and LVI. Chemotactic factors have also been investigated, among others. For instance VEGF-A, VEGF-C, VEGF-D, VEGFR-2, and VEGFR-3 are possible anti-lymphangiogenic treatment targets. They can be inhibited by neutralizing antibodies directed against the ligands or receptors or alternatively soluble versions of the receptors (e.g. VEGF-Trap aflibercept). Small molecule tyrosine kinase inhibitors of VEGFR-2 and VEGFR-3 are possible drug candidates and an on-going phase II trial is investigating mFOLFOX6 cytotoxic therapy in combination with either bevacizumab (anti-VEGF-A antibody) or tivozanib (an inhibitor of VEGFR-1, -2, and -3) in metastatic colorectal cancer. One potential indication for anti-lymphangiogenic therapy could be chemoprevention. Interestingly celecoxib, a COX-2 inhibitor, which is approved for chemoprevention of CRC, inhibits both angiogenesis and lymphangiogenesis (Nagahashi *et al.* 2010). An additional possible indication could be targeting tumor associated lymphatic vessels to restrict metastatic spread of the CRC cells after non-radical removal of the primary tumor or in those cases, where the primary tumor is inoperable. Anti-lymphangiogenic therapy might also work in the post-operative adjuvant setting despite the disappointing results of two large phase III trials that failed to show any benefit of adding the anti-angiogenic drug bevacizumab to chemotherapy after complete surgical removal of the primary tumor. In contrast bevacizumab shows benefit in patients with metastatic CRC (Allegra *et al.* 2011). Importantly, according to the results presented here the timing of lymphatic vessel targeting should be right, since at certain stages of disease the lymphatic vessels in the peritumoral area seem to be beneficial for patient outcome. Destroying the vessels at the wrong phase of the disease might in the worst scenarios be detrimental for the patient.

If LVD in the future proves to be a prognostic factor in CRC it could potentially be used for selecting stage II patients benefitting from post-operative adjuvant therapy as well as for selecting the intensity of the adjuvant therapy needed. Studies investigating the potential role of LVD as predictive marker for CRC treatments would also be interesting to carry out.

Future prospective studies with a more homogenous patient material would be recommended to confirm these results. Since apparent discrepancies exists in the results reported by different groups, even in studies where the lymphatic marker used and the cancer type investigated have been the same, an international consensus report has been published for standardization of lymphangiogenesis assessment. The recommended methodology includes the usage of double immunostains with both lymphatic marker D2-40 and proliferation marker Ki-67, the detection of vascular hot-spots by light-microscope, and the quantification of lymphatic vessels with the Chalkley point gratitude method, which is a measure of the lymphatic vasculature area rather than lymphatic vessel number. The consensus proposal recommends counting of both proliferative and non-proliferating LECs and that the analysis should be sequentially performed by two investigators (Van der Auwera *et al.* 2006).

6.3 Tumor associated macrophages in colorectal cancer

In this study a high density of peritumoral $CD68^+$ macrophages was a sign of good prognosis, whereas the impact of type 2 CLEVER-1^+ macrophages on CRC outcome varied according to the stage of the disease and the location of the macrophages.

The results regarding an improved survival of the patients with a high peritumoral $CD68^+$ number is supported by other findings in this field (Forssell *et al.* 2007; Lackner *et al.* 2004; Zhou *et al.* 2010). The association between a high number of type 2 CLEVER-1^+ macrophages peritumorally with a good prognosis in stage II and III CRC was surprising taking into account the expected protumoral function of the type 2 macrophages. To my knowledge only one study has investigated the prognostic impact of type 2 macrophages in CRC before (Nagorsen *et al.* 2007). They used CD163 as a type 2 macrophage marker and reported in support of my findings, that a high $CD163^+$ macrophage number in the tumor stroma was related to improved survival. They included CRC patients of all stages (stage I-IV) and probably due to the small patient number (n=40) did not perform stage specific subgroup analyses. Type 2 macrophages are known to induce immunosuppressive T regulatory cells (Savage *et al.* 2008) and therefore the finding by Salama *et al.* that a high $FOXP3^+$ Treg density in a big stage II and III CRC patient material (n=967) was associated with improved survival, further supports these results (Salama *et al.* 2009). One recent study examining immune cells at the tumor budding regions in CRC reported a similar relationship between a high $FOXP3^+$ Treg density and a favorable disease outcome (Zlobec *et al.* 2011). Indirectly supporting the findings in study II is one study by Erdman *et al.* who examined $CD45^+CD25^+$ regulatory T lymphocytes in a mouse model of human intestinal cancer and showed that Tregs inhibited the development of adenomas in an IL-10 dependent manner and induced regression of intestinal adenomas, enhanced apoptosis, and down-regulated COX-2 within tumors (Erdman *et al.* 2005). Due to the known connection between T lymphocytes and macrophages the $CD3^+$ T lymphocytes in the stage III subpopulation was investigated in my study, but no correlation between numbers of type 2 macrophages and $CD3^+$ lymphocytes was found. The usage of a T cell subpopulation marker could have yielded different results.

In metastatic stage IV disease, a high number of CLEVER-1^+ macrophages both peri- and intratumorally associated with worse survival in contrast to the findings in earlier stages of CRC. The findings in stage IV CRC disease are easier to comprehend, taking into account the expected protumoral functions of type 2 macrophages. It appears, that the function and role of the macrophages change during cancer progression and that the location of the macrophages is crucial, as well. In support of this theory a mouse tumor model study by Movahedi *et al.* showed evidence for the fact that different subset of TAMs are present in different tumor regions and increased in number during tumor progression (Movahedi *et al.* 2010). Moreover, Erreni *et al.* speculated that, the macrophages in the peritumoral region might be exposed to less tumor-derived cytokines and be located in a tissue better oxygenated than the intratumoral region, both factors, which might bring about an antitumoral macrophage phenotype (Erreni *et al.* 2011).

The influence of the type 1 and 2 macrophage balance on CRC recurrence patterns was also investigated. A type 1 dominant ($CD68^{high}$ and $CLEVER\text{-}1^{neg\ or\ low}$) intratumoral macrophage score associated more often with local recurrences than distant metastasis and only a minority of the patients with liver metastasis had this score in their primary tumor. Two other studies reported similar to these findings, that a high intratumoral $CD68^{+}$ macrophage number was protective against distant metastasis (Nagtegaal *et al.* 2001) and liver metastasis (Zhou *et al.* 2010).

No association between $CD3^{+}$ lymphocyte counts and prognosis in the stage III CRC patient subpopulation was found in this study, which is in line with the findings of Laghi *et al.* (Laghi *et al.* 2009) as well as Nosho *et al.* (Nosho *et al.* 2010). Most other studies though have found opposite to these results an association between a high $CD3^{+}$ T cell number and favorable CRC prognosis. In this study some tumors analyzed for $CD3^{+}$ density were done on TMA samples and the analysis was only performed from the intratumoral regions. Nosho *et al.* used TMA samples similar to this study. Laghi *et al.* on the other hand, analyzed only the peritumoral part of the tissue specimen. The usage of TMA tissue samples prevents taking into account the heterogeneity often seen within CRC tumors, therefore the results might have differed, if whole tissue specimens would have been used.

A positive correlation was observed between the numbers of type 2 $CLEVER\text{-}1^{+}$ macrophages and $CLEVER\text{-}1^{+}$ vessels in this study, whereas the density of $CD68^{+}$ macrophages did not correlate with $podoplanin^{+}$ or $CLEVER\text{-}1^{+}$ vessel numbers. A positive correlation between numbers of macrophages and lymphatic or blood vessels would have been expected due to the proangiogenic and prolymphangiogenic factors secreted by macrophages. Ammar *et al.* found a positive correlation between the numbers of $CLEVER\text{-}1^{+}$ macrophages and $CLEVER\text{-}1^{+}$ blood as well as lymphatic vessel numbers in breast cancer, in support of these results (Ammar *et al.* 2011). In line with my findings Hasita *et al.* reported a closer association between numbers of type 2 $CD163^{+}$ macrophages and vessels than between $CD68^{+}$ macrophages and vessels (Hasita *et al.* 2010).

The strengths of this study include the usage of both a pan-macrophage marker and type 2 macrophage marker as well as the investigation of both the intratumoral and peritumoral areas. The role of CLEVER-1 as a type 2 macrophage marker was validated by performing double stainings with an established type 2 macrophage marker, MR. The results demonstrated that CLEVER-1 indeed recognizes type 2 macrophages in CRC tissue. The prognostic significance of type 2 $CLEVER\text{-}1^{+}$ macrophages in CRC is not known and therefore, this study brings new knowledge to this field. The limitations of this study comprise the retrospective nature of this study material and the lack of a validation patient cohort.

Tilting the balance of pro- and antitumoral immune responses towards an antitumoral, adaptive immunity favoring one and to find means for selective depletion of protumoral inflammatory cells are two interesting therapeutic possibilities in addition to the immunotherapeutic agents already available on the market. Thus reduction of TAMs, their depletion, or re-education could be possible future treatment strategies.

IFN-γ, an activator of innate immunity cells has the ability to re-educate TAMs towards antitumoral activity. In an interesting study by Kodumudi *et al.* docetaxel treatment in a breast cancer mouse model decreased MDSC number and polarized MDSCs toward a M1 like phenotype (Kodumudi *et al.* 2010). Blocking CCL-2, a chemokine that attracts monocytes and Tregs to the tumor microenvironment might also be beneficial in CRC (Popivanova *et al.* 2009). It would be crucial to clarify which particular immune cell subtype is prognostic for which type of cancer at which disease stage. We are still lacking robust data in this regard, which is an obstacle for drug development in this field.

Despite numerous studies addressing the prognostic role of specific immune and inflammatory cells in CRC, they cannot be implemented into clinical practice as yet. The studies reported show considerable variation in e.g. sample size, tumor specimens (TMA *vs* whole tissue samples), region of interest (peri- *vs* intratumoral), immunohistochemical markers, methodology, and analyzing techniques used. Standardized methodology for evaluation of immune-responses is needed and the REporting recommendations for tumor MARKer prognostic studies (REMARK) guidelines should be followed, when carrying out prognostic biomarker studies (McShane *et al.* 2005; Ogino *et al.* 2011).

6.4 *EGFR* GCN in metastatic colorectal cancer

EGFR GCN assessed by automated SISH guided by EGFR protein expression analysis was shown to predict responsiveness to anti-EGFR treatment, PFS, and OS in metastatic CRC even better than the recommended, routinely used *KRAS* test. The predictive value was further improved by combining the *KRAS* and *EGFR* GCN analyses.

The results of this study seem to have a better predictive value than the results previously reported by other groups (see **Table 2** in the review of the literature section on page 56). In addition, the *EGFR* GCN cut-off value was higher than in most previous publications (Cappuzzo *et al.* 2008; Personeni *et al.* 2008; Sartore-Bianchi *et al.* 2007; Scartozzi *et al.* 2009; Scartozzi *et al.* 2011). Potential explanations for these differences include the usage of SISH as well as a different *EGFR* probe for *EGFR* GCN evaluation. SISH analysis performed by conventional bright field light microscopy enables improved morphological tissue identification as compared to FISH, which might have affected the results. However, a study investigating the concordance between *HER2* gene amplification analyzed by FISH in comparison to SISH in breast cancer, reported a high concordance rate between the two methods (96%). The discrepancies that occurred were in tumors exhibiting *HER2* gene copy number heterogeneity. CRC primary tumors are heterogenic in nature and that might cause differences when analyzing *EGFR* GCN by SISH or FISH in CRC. But, these arguments do not explain the differences observed, when comparing our results to the ones performed by chromogenic *in situ* hybridization (CISH) (Laurent-Puig *et al.* 2009; Scartozzi *et al.* 2009; Scartozzi *et al.* 2011). Scartozzi *et al.* compared *EGFR* GCN evaluation by FISH and CISH in CRC. The cut-off values showed some variation (see **Table 2** in the review of the literature section) but they both predicted response to anti-EGFR therapy and the authors concluded that both FISH and

CISH are suitable for *EGFR* GCN assessment in CRC (Scartozzi *et al.* 2009). *HER2* FISH and CISH comparison in breast cancer yielded, in a similar manner, a high concordance rate (Tanner *et al.* 2000).

The usage of EGFR protein expression (area with highest intensity) to guide the selection of the area for *EGFR* GCN analysis might explain the superior results and the higher *EGFR* GCN cut-off value observed in this study. A new anti-EGFR antibody, clone 5B7, which recognizes the functionally active intracellular domain of EGFR was used in this study. This might also explain the differences observed, since other commercially available antibodies are directed against the extracellular domain of EGFR. An antibody, clone 3C6 directed against the extracellular domain of EGFR, was tested too, but it did not correlate with *EGFR* GCN in contrast to the results obtained with the clone 5B7. Taken together, the most likely cause for the observed differences in cut-off values and predictive strengths between this study and others is in my opinion the combination of EGFR IHC and *EGFR* GCN assessed by the SISH method.

The *EGFR* GCN/Chr-7 ratio was also assessed and a true *EGFR* amplification (ratio > 2.2) was observed in only 2 out of 78 tumors. Therefore, the observed *EGFR* GCN increase (≥ 4.0) in 65% (51/78) of the CRC tumors was related to Chr-7 polysomy. Nevertheless, at least according to these results, a GCN gain seems to predict responsiveness to anti-EGFR therapy in CRC despite the concurrent presence of Chr-7 polysomy. The importance of the gene/chromosome ratio is being questioned also in HER2 positive breast cancer patients treated with trastuzumab, where a study showed that patients with *HER2* amplified tumors, regardless of Chr-17 number and *HER2*/centromere 17 ratio, benefitted from treatment (Perez *et al.* 2010).

Weaknesses of this study include the inclusion of a relatively small patient number, the retrospective nature of this study as well as the heterogeneity in the treatment regimens. Subgroup analyses were performed in the *KRAS* wt patient population to evaluate the predictive value of *EGFR* GCN in more homogenously treated patients. A high *EGFR* GCN remained as a statistically significant predictor of a favorable anti-EGFR therapy response in all subgroups evaluated.

The strengths of this study include the usage of a fully automated SISH methodology, which minimizes methodological reproducibility concerns. In addition, a low SISH failure rate was observed, 2.5% (2/80), which is clearly less than e.g. in a study by Laurent-Puig *et al.* who reported a drop-out rate of 20% due to technical problems (Laurent-Puig *et al.* 2009). Furthermore, the chromogen of SISH is stable unlike fading fluorochromes of FISH, and does not require special analyzing instruments. In one study by Dietel *et al.* the interobserver variation for *HER2* SISH in breast cancer was low (Dietel *et al.* 2007) similar to CISH (Isola *et al.* 2004), whereas Sartore-Bianchi *et al.* who investigated the inter-laboratory reproducibility of *EGFR* GCN FISH in CRC showed marked variation between different experienced laboratories (Sartore-Bianchi *et al.* 2011).

Lack of standardized methodology, differences in cut-off values used, and absence of robust validation of *EGFR* GCN assessment are most probably the factors responsi-

ble for preventing the incorporation of *EGFR* GCN into the clinical practice. Therefore, the methodology used in this study could offer a robust, reproducible, objective, and easy to perform alternative for *EGFR* GCN analysis. But naturally, prior to that, the results need validation, ideally in a prospective, homogenously treated, larger patient material.

6.5 Pim-1 in colorectal cancer

This study shows that a high Pim-1 expression in CRC is a sign of good disease outcome in two distinct patient materials, using two different anti-Pim-1 antibodies. The findings are in line with findings in lung cancer (Warnecke-Eberz *et al.* 2008), pancreatic ductal adenocarcinoma (Reiser-Erkan *et al.* 2008), and prostate cancer (Dhanasekaran *et al.* 2001; Rhodes *et al.* 2003). Since Pim-1 has been linked to many protumoral functions these results are at least to some extent unexpected. One explanation for high Pim-1 expression being a sign of good cancer prognosis may be the role of Pim-1 in mitosis by interacting with NuMA, HP1β, dynein, and dynactin (Bhattacharya *et al.* 2002). Pim-1 overexpression in prostate epithelial cells was reported to induce genomic instability by disrupting the normal function of the mitotic spindle checkpoint as shown in a study by Roh *et al.* (Roh *et al.* 2003). They speculated that during cancer progression Pim-1 expression is downregulated in order to stabilize the genomic abnormalities in accordance with the so called genetic convergence theory (Heim *et al.* 1988).

Pim-1 has been found both in the cytoplasm and nucleus of cancer cells. The staining patterns in CRC tumor samples were strikingly different when two different anti-Pim-1 antibodies were used in study IV. Both anti-Pim antibodies have been used previously in other studies and according to the positive internal control (leukocytes within the tissue specimen) seemed to work reliably. Because of the surprising lack of nuclear staining observed with the 19F7 Pim-1 antibody, in contrast to the results with the commercial EP2645Y Pim-1 antibody, various antigen retrieval methods were tested, but they did not alter the result. To my knowledge, only one study investigating Pim-1 expression in CRC has been reported earlier. In that study, Pim-1 was found in a subset of CRC cells located in the cytoplasm, when using the same 19F7 anti-Pim-1 antibody (Shah *et al.* 2008). Nga and colleagues used the same antibody too and reported strong vacuolar cytoplasmic staining in myxoid liposarcoma, whereas only weak cytoplasmic Pim-1 expression was observed in other non-adipocytic myxoid tumors. In line with my findings, no nuclear expression was seen (Nga *et al.* 2010). Also in prostate cancer 19F7 showed only cytoplasmic Pim-1 expression (Cibull *et al.* 2006). On the contrary, in SCC of the head and neck (Peltola *et al.* 2009) and bladder cancer (Guo *et al.* 2010) 19F7 Pim-1 antibody exhibited both cytoplasmic and nuclear subcellular staining. Therefore, the expression of Pim-1 seems to vary between different malignancies. The strong nuclear staining seen in my CRC patient material with the EP2645Y antibody, gives rise to at least two possible explanations to this discrepancy. First, the Pim-1 molecule may be truncated and loose the 19F7 epitope in the nucleus in CRC or alternatively become non-accessible in the nucleus for the 19F7 antibody.

As a response to radiation therapy Pim-1 expression was increased in the cytoplasm, decreased in the CRC cell nuclei, or alternatively unchanged. These findings are in striking contrast to SCC of the head and neck where Pim-1 was translocated to the nucleus following irradiation (Peltola *et al.* 2009).

Weaknesses of this study include the lack of a comparable validation patient cohort and the usage of two different antibodies for testing the prognostic value of Pim-1 in CRC. Due to 19F7 antibody availability problems I was forced to change the antibody to a commercial one when expanding the patient material. Two different analyzing techniques (computer based image analysis and semi-quantitative grading by light microscope) were also used. In addition, the retrospective nature of this study is a weakness of note.

At least a couple of the weaknesses listed above can be considered both a weakness and strength. For instance the comparable results with the two clearly differently working anti-Pim-1 antibodies, strengthens the somewhat surprising findings. Furthermore the change of the antibody to a commercially available one without losing the prognostic value of Pim-1 makes it feasible to use an easily available commercial antibody in future validation experiments. The same holds true for the analyzing technique. An image analysis program might be considered more objective and the results easier to reproduce. But, even the usage of an image analysis program requires the subjective selection of the areas of interest within the tumor specimens as well as specific equipment adjustments which might cause interlaboratory variation. Furthermore, light microscopes are available in most laboratories, which might not be the case for specific image analysis software programs.

Pim-1 kinase inhibitors have been developed and at least one of them SGI-1776 has been tested in phase I clinical trials in prostate cancer, non-Hodgkin´s lymphoma, and leukemia. These trials have unfortunately been terminated due to cardiac toxicity. My results do not encourage pharmacological inhibition of Pim-1 as a treatment strategy in CRC at this point. But further studies are naturally needed to confirm these findings. If confirmed, an appealing approach would be to utilize Pim-1 as a prognostic marker in stage II and III CRC in the postoperative setting. Due to the high risk for disease relapse, lymph node positive stage III CRC patients are postoperatively treated with combination adjuvant chemotherapy regardless of other prognostic factors. By contrast, in the stage II patient group the benefit of adjuvant chemotherapy is less clear and robust prognostic and predictive markers would be needed for improved patient selection for adjuvant therapy *vs* no therapy or adjuvant single agent therapy *vs* combination therapy. Today the postoperative adjuvant therapy option choice is influenced by the so called high risk features of stage II disease including depth of primary tumor invasion, differentiation grade of tumor, number of examined regional lymph nodes, presence of LVI, and preoperative occlusion or perforation of the gut. As a final note, the prognostic value of Pim-1 in colorectal cancer has not previously been reported and therefore this study brings new knowledge to this field.

7 SUMMARY AND CONCLUSIONS

This study focused on factors potentially important for colorectal cancer progression, prognosis, and treatment responsiveness. Cancer cell properties as well as certain characteristics of the tumor microenvironment were identified as important effectors of colorectal cancer outcome. Interestingly, several of the variables studied yielded unexpected results reflecting the complexity of cancer biology.

The number of lymphatic vessels was shown to impact the survival of colorectal cancer patients in a negative fashion if located within the tumor, whereas a high number of peritumoral vessels were associated with a favorable survival. The dual role for lymphatic vessels in CRC prognosis might be explained by the fact that lymphatic vessels play an essential role in host defence but may also serve as a route for the spread of malignant cells.

A high total number of tumor associated macrophages in the peritumoral area affected the outcome of the patients positively. A high number of type 2 peritumoral CLEVER-1$^+$ macrophages in early stage disease was also a sign of good prognosis, whereas in advanced stage IV disease the type 2 macrophages associated with poor CRC survival regardless of their location. There is no clear explanation for this somewhat unexpected finding but clearly the composition of immune cell infiltrates and their function vary between different stages of disease as well as different malignancies. The macrophages in the early stages of disease appear to work in an antitumoral fashion, whereas in advanced disease they seem to promote tumor growth in CRC.

The knowledge regarding CD73 in lymphatics has been lacking. The results presented here show that CD73 on lymphocytes is crucial for lymphocyte trafficking via afferent lymphatics to the lymph nodes. In addition, a marked heterogenous phenotype was observed in the lymphatic vessels both in healthy and malignant human tissues as well as normal mice tissues. Of note, CD73 appears to be the only marker known today with the ability to distinguish between afferent and efferent lymphatics. CD73 was not found on efferent lymphatics in human or mouse lymph nodes.

Colorectal cancer, which is one of the most common malignancies worldwide, still has a high mortality rate despite advances in the treatment modalities. The discovery of tools enabling better prognostication of disease outcome as well as means for better prediction of treatment efficacy for individual patients would probably improve the outcome of the disease. *KRAS* testing prior to anti-EGFR therapy is the only molecular predictive marker used in the treatment of CRC in the clinic at present. A high *EGFR* gene copy number assessed by IHC guided SISH in metastatic *KRAS* wt CRC, was demonstrated to efficiently predict responsiveness to anti-EGFR therapy. In addition, a high Pim-1 expression was surprisingly linked to a favorable CRC prognosis.

In summary, the results show that both the location and number of lymphatic vessels; location, subtype, and number of macrophages, and the expression level of Pim-1 appear to affect CRC outcome. All these findings need however further validation,

preferably in a prospective setting with larger patient materials. *EGFR* GCN as analyzed by a reproducible SISH method is a promising predictive marker candidate for anti-EGFR therapy in metastatic CRC. The findings regarding *EGFR* GCN require further validation as well, but the incorporation of *EGFR* GCN evaluation by SISH prior to anti-EGFR therapy in routine clinical work does not seem to be an unrealistic goal in the forseeable future. Targeting the factors tested in this study pharmacologically in the future could be possible in several ways, but prior to that we need to know what exactly to target and be aware of the right moment for the intervention in order to prevent the scenario of doing more harm than good.

The results emphasize the complexity of cancer biology and bring to mind the enormous efforts needed, usually by several groups, before a single prognostic/predictive marker can be recommended for routine use in daily clinical practice. Inevitable some initially promising markers will not prove so in validation studies, leading to much effort, time, and money going to waste. But even one new prognostic/predictive marker making it all the way to implementation into every day clinical work will eventually benefit thousands of patients and bring us again one step closer to the ultimate goal of personalized medicine. Collaboration and standardized methodology are needed in the future.

8 ACKNOWLEDGEMENTS

This work was carried out at the Department of Oncology and Radiotherapy, MediCity Research Laboratory, and Department of Medical Microbiology and Immunoloy, University of Turku. My sincere thanks to the heads of these institutions, Professors Sirpa Jalkanen, Seppo Pyrhönen, and Pentti Huovinen for providing excellent facilities for scientific work and for creating an inspiring atmosphere. I am also grateful to the National Graduate School of Clinical Investigation (CLIGS) for financial and educational support.

I am deeply grateful to my supervisor Professor Sirpa Jalkanen for her support, never-ending patience, extraordinary optimism, and enthusiasm. I admire her excellent skills and knowledge in the field of science and I thank her for guidance when the "red line" has been difficult to find. I express my gratitude for her exceptional ability to find possibilities also in the most surprising and impossible situations and for being such a great role model. I express my warmest thanks to my other supervisor Docent Raija Ristamäki for recruiting me on-board to the world of science and for introducing me to her "old lab" where she knew I would enjoy being. I admire her unbelievable memory which has been helpful on numerous occasions and her great clinical skills. I am deeply grateful for Raija`s support, patience, encouraging words when needed, and skilful guidance during the different phases of this thesis project. I also want to express my deepest gratitude to Professor Seppo Pyrhönen who has supported me all these years. I am indebted to his encouraging attitude towards research work, which has made intermittent full time research work possible. I value Seppo`s scientific and clinical skills and thank him for the interest he has shown towards my thesis project.

I wish to express my sincerest gratitude to the excellent reviewers of this thesis, Professor Ari Ristimäki and Docent Minna Tanner, who despite a tight time schedule provided valuable comments and constructive criticism. Their remarks, without doubt, improved this thesis.

The members of my supervisory committee are thanked for their time, support, and advice.

I express my warmest thanks to all my co-authors. Without their professional contribution this thesis would not exist. Professor Marko Salmi who managed to solve the "3-372 problem", Gennady Yegutkin for robust expertise in the field of CD73, Marika Karikoski for skilled *in vivo* work, Jussi Niemelä and Patrizia Stoitzner for their contribution to the CD73 project, Harry Kujari and Jukka Laine for expert help with tissue samples, Heikki Irjala and Samuli Vaittinen for doing the very exhausting microscope analysis without giving up, Heikki Irjala and Heikki Huhtinen for providing samples to this project, Professor Olli Carpen, Jari Sundström, and Minnamaija Lintunen for their invaluable contribution to the EGFR project, and Päivi Koskinen, Niina Santio, and Jari Sundström for jumping on board our Pim-1 project.

My warmest thanks to the members of the "team EGFR", Professor Olli Carpén, Raija Ristamäki, Jari Sundström, Minnamaija Lintunen, Tuulia Avoranta, Eva-Maria Birkman, and Terhi Jokilehto for an inspiring, enthusiastic team work, where the contribution of each and every one is appreciated. I truly enjoy our regulary meetings! I want to thank Eija Korkeila and Kaisa Lehtiö for inspiring and "therapeutical" conversations about science and life in general during occasional lunch meetings.

The skilled lab personnel, Pirjo Heinilä, Teija Kanasuo, Sari Mäki, Mari Parsama, Maritta Pohjansalo, Laila Reunanen, Riikka Sjöroos, and Etta-Liisa Väänänen are thanked for creating a friendly and helpful atmosphere in the lab. I am especially indebted to Pirjo and Sari who introduced me to the mysteries of lab work. Thank you for your patience and friendliness and for help with IHC. I am deeply grateful to Anne Sovikoski-Georgieva for skilled secretarial help, always provided in a friendly and efficient manner. Sinikka Kollanus is acknowledged for her excellent help with tissue samples, Tero Vahlberg for his expertise and help in the field of statistics, Jouko Sandholm and Markku Saari for teaching me the wonders of confocal microscopy, Elina Wiik for answering my questions about bureaucracy, Ioan "John" Iagar and Antti-Pekka Laine for handy technical help.

Both former and present students and postdocs of our lab that I have had the priviledge to work with: Kristiina Aalto, Laura Airas, Kaisa Auvinen, Minna Boström, Johannes Dunkel, Dominik Eichin, Heli Elovaara, Kati Elima, Heikki Irjala, Sam Kaitaniemi, Marika Karikoski, Imtiaz Iftakhar-E-Khuda, Johannes Keuschnigg, Juha Laurila, Teija Kilpeläinen, Jan Kiss, Sanna-Mari Maula, Fumiko Marttila, Nathalie Mercier, Mari Miiluniemi, Jussi Niemelä, Mikael Maximov, Senthil Palani, Pia Rantakari, Maija Saraste, Outi Sareila, Tibor Veres, Gennady Yegutkin and all the rest are thanked for all hilarious moments that has made the countless hours spent on this thesis project tolerable and enjoyable. It has always felt like coming home after longer periods away from the lab, thank you for making me feel that way. My regular lunch buddies Krisse, Kati, Marika, and Sam deserve special thanks for sharing both good and bad days with me and for continual support and help. Krisse and Sam are thanked for being the best roommates one can imagine.

I have been fortunate to have two great work places to alternate between. I want to thank all my present and former friends and colleagues at the Department of Oncology and Radiotherapy (there´s just too many of you for individual mentioning) for their supportive attitude towards my research work. Long abscences from the clinical work has not prevented you from welcoming me back and I have always felt like coming home when going in this direction too. Thank you all for that! Thank you Pia for being a great room mate.

"Pargas gänget", "Med school friends", "Lohja friends", "S:t Karins friends" and all the rest, I am deeply grateful for having friends like you and cherish the moments I get to spend with you. Thank you for being there, for making me laugh, for charging my batteries, and for making life feel like an adventure.

I am deeply grateful to my dear parents Tuula and Rainer for always unconditionally believing in me and supporting me. The value of your help and love over the years is priceless. No words are enough. THANK YOU for everything! Warmest thanks to my dear sisters Monica and Jessica. I am very fortunate to have sisters like you! Johan, my nephew William, nieces Olivia and Maya, Juha, Terttu, Eino, Jukka, Sari, Janne, Susanna, Sini, Mikko + all your beautiful children are thanked for all the fun moments I have shared with you and the counterbalance to science you have given me.

Last, but not least, I owe my deepest gratitude to my amazing family. I want to thank you from the depths of my heart for your unwavering support and love during this journey. I want to thank you, my beloved husband Jyrki for putting up with me and encouraging me at times of tight deadlines, setback after setback, and for sharing the moments of joy with me, as well. Without you this project would have been impossible to complete. Alex and Therese, thank you for being you, for bringing tons of happiness and laughter to my life, for counterbalancing work related matters, and for making it crystal clear to me what truly matters in life. Your eager awaiting for “BOKEN” to be completed has finally come to an end.

This work was supported financially by the National Graduate School of Clinical Investigation, EVO funding of Turku University Hospital, The Finnish Medical Foundation, the Cancer Society of South-Western Finland, the Finnish Cancer Organizations, the Finnish Cultural Foundation, and the Medical Society of Finland.

S:t Karins, April 2012

9 REFERENCES

Achen MG, McColl BK, Stacker SA (2005) Focus on lymphangiogenesis in tumor metastasis. *Cancer Cell* 7(2): 121-7

Adams RA, Meade AM, Seymour MT, Wilson RH, Madi A, Fisher D, Kenny SL, Kay E, Hodgkinson E, Pope M, Rogers P, Wasan H, Falk S, Gollins S, Hickish T, Bessell EM, Propper D, Kennedy MJ, Kaplan R, Maughan TS, Investigators MCT (2011) Intermittent versus continuous oxaliplatin and fluoropyrimidine combination chemotherapy for first-line treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet Oncol* 12(7): 642-53

Aggarwal BB, Gupta SC, Kim JH (2012) Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. *Blood* 119(3): 651-65

Aho TL, Sandholm J, Peltola KJ, Mankonen HP, Lilly M, Koskinen PJ (2004) Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser112 gatekeeper site. *FEBS Lett* 571(1-3): 43-9

Airas L, Niemelä J, Jalkanen S (2000) CD73 engagement promotes lymphocyte binding to endothelial cells via a lymphocyte function-associated antigen-1-dependent mechanism. *J Immunol* 165(10): 5411-7

Airas L, Niemelä J, Salmi M, Puurunen T, Smith DJ, Jalkanen S (1997) Differential regulation and function of CD73, a glycosyl-phosphatidylinositol-linked 70-kD adhesion molecule, on lymphocytes and endothelial cells. *J Cell Biol* 136(2): 421-31

Airas L, Salmi M, Jalkanen S (1993) Lymphocyte-vascular adhesion protein-2 is a novel 70-kDa molecule involved in lymphocyte adhesion to vascular endothelium. *J Immunol* 151(8): 4228-38

AJCC (2010) Part III Digestive System: Colon and Rectum. In *Cancer Staging Handbook*, Stephen BE BD, Compton CC, Fritz AG, Greene FL, Trotti A (ed), Seventh edition edn, Chapter Part III Digestive System, pp 173-206. New York: Springer

Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y, Sugimachi K (2000) Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer* 83(7): 887-91

Alberts SR, Sargent DJ, Smyrk TC, Shields AF, Chan E, Goldberg RM, Gill S, Kahlenberg MS, Thibodeau SN, Nair S. Adjuvant mFOLFOX6 with or without cetuximab (Cmab) in KRAS wild-type (WT) patients (pts) with resected stage III colon cancer (CC): Results from NCCTG Intergroup Phase III Trial N0147. *Journal of Clinical Oncology, 2010 ASCO Annual Meeting Proceedings (Post meeting Edition), Vol 28, No 18_suppl (June 20 Supplement), 2010: CRA 3507*

Allavena P, Sica A, Garlanda C, Mantovani A (2008) The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunol Rev* 222: 155-61

Allegra CJ, Yothers G, O'Connell MJ, Sharif S, Petrelli NJ, Colangelo LH, Atkins JN, Seay TE, Fehrenbacher L, Goldberg RM, O'Reilly S, Chu L, Azar CA, Lopa S, Wolmark N (2011) Phase III trial assessing bevacizumab in stages II and III carcinoma of the colon: results of NSABP protocol C-08. *J Clin Oncol* 29(1): 11-6

Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD (2008) Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 26(10): 1626-34

Ammar A, Mohammed RA, Salmi M, Pepper M, Paish EC, Ellis IO, Martin SG (2011) Lymphatic expression of CLEVER-1 in breast cancer and its relationship with lymph node metastasis. *Anal Cell Pathol (Amst)* 34(1-2): 67-78

Amson R, Sigaux F, Przedborski S, Flandrin G, Givol D, Telerman A (1989) The human protooncogene product p33pim is expressed during fetal hematopoiesis and in diverse leukemias. *Proc Natl Acad Sci U S A* 86(22): 8857-61

André T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, Bonetti A, Clingan P, Bridgewater J, Rivera F, de Gramont A (2009) Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 27(19): 3109-16

Ara T, Declerck YA (2010) Interleukin-6 in bone metastasis and cancer progression. *Eur J Cancer* 46(7): 1223-31

Aschele C, Cionini L, Lonardi S, Pinto C, Cordio S, Rosati G, Artale S, Tagliagambe A, Ambrosini G, Rosetti P, Bonetti A, Negru ME, Tronconi MC, Luppi G, Silvano G, Corsi DC, Bochicchio AM, Chiaulon G, Gallo M, Boni L (2011) Primary tumor response to preoperative chemoradiation with or without oxaliplatin in locally advanced rectal cancer: pathologic results of the STAR-01 randomized phase III trial. *J Clin Oncol* 29(20): 2773-80

Bachmann M, Hennemann H, Xing PX, Hoffmann I, Moroy T (2004) The oncogenic serine/threonine kinase Pim-1 phosphorylates and inhibits the activity of Cdc25C-associated kinase 1 (C-TAK1): a novel role for Pim-1 at the G2/M cell cycle checkpoint. *J Biol Chem* 279(46): 48319-28

Bachmann M, Moroy T (2005) The serine/threonine kinase Pim-1. *Int J Biochem Cell Biol* 37(4): 726-30

Baeten CI, Castermans K, Hillen HF, Griffioen AW (2006) Proliferating endothelial cells and leukocyte infiltration as prognostic markers in colorectal cancer. *Clin Gastroenterol Hepatol* 4(11): 1351-7

Balkwill F, Mantovani A (2010) Cancer and inflammation: implications for pharmacology and therapeutics. *Clin Pharmacol Ther* 87(4): 401-6

Baluk P, McDonald DM (2008) Markers for microscopic imaging of lymphangiogenesis and angiogenesis. *Ann N Y Acad Sci* 1131: 1-12

Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, Jeltsch M, Petrova TV, Pytowski B, Stacker SA, Ylä-Herttuala S, Jackson DG, Alitalo K, McDonald DM (2005) Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest* 115(2): 247-57

Baluk P, Yao LC, Feng J, Romano T, Jung SS, Schreiter JL, Yan L, Shealy DJ, McDonald DM (2009) TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. *J Clin Invest* 119(10): 2954-64

Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG (1999) LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 144(4): 789-801

Barber TD, Vogelstein B, Kinzler KW, Velculescu VE (2004) Somatic mutations of EGFR in colorectal cancers and glioblastomas. *N Engl J Med* 351(27): 2883

Bass A (2011) Impact of KRAS and BRAF gene mutations on targeted therapies in colorectal cancer. *J Clin Oncol* 29(19): 2728-9

Bastian JF, Ruedi JM, MacPherson GA, Golembesky HE, O'Connor RD, Thompson LF (1984) Lymphocyte ecto-5'-nucleotidase activity in infancy: increasing activity in peripheral blood B cells precedes their ability to synthesize IgG in vitro. *J Immunol* 132(4): 1767-72

Beier UH, Weise JB, Laudien M, Sauerwein H, Gorogh T (2007) Overexpression of Pim-1 in head and neck squamous cell carcinomas. *Int J Oncol* 30(6): 1381-7

Bertagnolli MM, Niedzwiecki D, Compton CC, Hahn HP, Hall M, Damas B, Jewell SD, Mayer RJ, Goldberg RM, Saltz LB, Warren RS, Redston M (2009) Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: Cancer and Leukemia Group B Protocol 89803. *J Clin Oncol* 27(11): 1814-21

Bhattacharya N, Wang Z, Davitt C, McKenzie IF, Xing PX, Magnuson NS (2002) Pim-1 associates with protein complexes necessary for mitosis. *Chromosoma* 111(2): 80-95

Blyth K, Terry A, Mackay N, Vaillant F, Bell M, Cameron ER, Neil JC, Stewart M (2001) Runx2: a novel oncogenic effector revealed by in vivo complementation and retroviral tagging. *Oncogene* 20(3): 295-302

Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, Donea S, Ludwig H, Schuch G, Stroh C, Loos AH, Zubel A, Koralewski P (2009) Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 27(5): 663-71

Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, Kerjaschki D (1997) Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. *Am J Pathol* 151(4): 1141-52

Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, Kerjaschki D (1999) Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol* 154(2): 385-94

Breuer M, Wientjens E, Verbeek S, Slebos R, Berns A (1991) Carcinogen-induced lymphomagenesis in pim-1 transgenic mice: dose dependence and involvement of myc and ras. *Cancer Res* 51(3): 958-63

Britschgi MR, Favre S, Luther SA (2010) CCL21 is sufficient to mediate DC migration, maturation and function in the absence of CCL19. *Eur J Immunol* 40(5): 1266-71

Bronkhorst IH, Ly LV, Jordanova ES, Vrolijk J, Versluis M, Luyten GP, Jager MJ (2011) Detection of M2-macrophages in uveal melanoma and relation with survival. *Invest Ophthalmol Vis Sci* 52(2): 643-50

Burn J, Gerdes AM, Macrae F, Mecklin JP, Moeslein G, Olschwang S, Eccles D, Evans DG, Maher ER, Bertario L, Bisgaard ML, Dunlop MG, Ho JW, Hodgson SV, Lindblom A, Lubinski J, Morrison PJ, Murday V, Ramesar R, Side L, Scott RJ, Thomas HJ, Vasen HF, Barker G, Crawford G, Elliott F, Movahedi M, Pylvanainen K, Wijnen JT, Fodde R, Lynch HT, Mathers JC, Bishop DT, Investigators C (2011) Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet* 378(9809): 2081-7

Böckle BC, Sölder E, Kind S, Romani N, Sepp NT (2008) DC-sign+ CD163+ macrophages expressing hyaluronan receptor LYVE-1 are located within chorion villi of the placenta. *Placenta* 29(2): 187-92

Canioni D, Salles G, Mounier N, Brousse N, Keuppens M, Morchhauser F, Lamy T, Sonet A, Rousselet MC, Foussard C, Xerri L (2008) High numbers of tumor-associated macrophages have an adverse prognostic value that can be circumvented by rituximab in patients with follicular lymphoma enrolled onto the GELA-GOELAMS FL-2000 trial. *J Clin Oncol* 26(3): 440-6

Cappuzzo F, Finocchiaro G, Rossi E, Janne PA, Carnaghi C, Calandri C, Bencardino K, Ligorio C, Ciardiello F, Pressiani T, Destro A, Roncalli M, Crino L, Franklin WA, Santoro A, Varella-Garcia M (2008) EGFR FISH assay predicts for response to cetuximab in chemotherapy refractory colorectal cancer patients. *Ann Oncol* 19(4): 717-23

Cassidy J, Saltz L, Twelves C, Van Cutsem E, Hoff P, Kang Y, Saini JP, Gilberg F, Cunningham D (2011) Efficacy of capecitabine versus 5-fluorouracil in colorectal and gastric cancers: a meta-analysis of individual data from 6171 patients. *Ann Oncol* 22(12): 2604-9

Castrop H, Huang Y, Hashimoto S, Mizel D, Hansen P, Theilig F, Bachmann S, Deng C, Briggs J, Schnermann J (2004) Impairment of tubuloglomerular feedback regulation of GFR in ecto-5'-nucleotidase/CD73-deficient mice. *J Clin Invest* 114(5): 634-42

Chan AT, Giovannucci EL, Meyerhardt JA, Schernhammer ES, Curhan GC, Fuchs CS (2005) Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. *Jama* 294(8): 914-23

Chan AT, Ogino S, Fuchs CS (2009) Aspirin use and survival after diagnosis of colorectal cancer. *Jama* 302(6): 649-58

Chang DZ, Kumar V, Ma Y, Li K, Kopetz S (2009) Individualized therapies in colorectal cancer: KRAS as a marker for response to EGFR-targeted therapy. *J Hematol Oncol* 2: 18

Chen J, Kobayashi M, Darmanin S, Qiao Y, Gully C, Zhao R, Yeung SC, Lee MH (2009) Pim-1 plays a pivotal role in hypoxia-induced chemoresistance. *Oncogene* 28(28): 2581-92

Chiang WF, Yen CY, Lin CN, Liaw GA, Chiu CT, Hsia YJ, Liu SY (2006) Up-regulation of a serine-threonine kinase proto-oncogene Pim-1 in oral squamous cell carcinoma. *Int J Oral Maxillofac Surg* 35(8): 740-5

Chibaudel B, Maindrault-Goebel F, Lledo G, Mineur L, André T, Bennamoun M, Mabro M, Artru P, Carola E, Flesch M, Dupuis O, Colin P, Larsen AK, Afchain P, Tournigand C, Louvet C, de Gramont A (2009) Can chemotherapy be discontinued in unresectable metastatic colorectal cancer? The GERCOR OPTIMOX2 Study. *J Clin Oncol* 27(34): 5727-33

Chien Y, Scuoppo C, Wang X, Fang X, Balgley B, Bolden JE, Premsrirut P, Luo W, Chicas A, Lee CS, Kogan SC, Lowe SW (2011) Control of the senescence-associated secretory phenotype by NF-κB promotes senescence and enhances chemosensitivity. *Genes Dev* 25(20): 2125-36

Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, Kuo WL, Ljung BM, Chew K, Myambo K, Miranda M, Krig S, Garbe J, Stampfer M, Yaswen P, Gray JW, Lockett SJ (2004) In situ analyses of genome instability in breast cancer. *Nat Genet* 36(9): 984-8

Chung KY, Shia J, Kemeny NE, Shah M, Schwartz GK, Tse A, Hamilton A, Pan D, Schrag D, Schwartz L, Klimstra DS, Fridman D, Kelsen DP, Saltz LB (2005) Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 23(9): 1803-10

Cibull TL, Jones TD, Li L, Eble JN, Ann Baldridge L, Malott SR, Luo Y, Cheng L (2006) Overexpression of Pim-1 during progression of prostatic adenocarcinoma. *J Clin Pathol* 59(3): 285-8

Clasper S, Royston D, Baban D, Cao Y, Ewers S, Butz S, Vestweber D, Jackson DG (2008) A novel gene expression profile in lymphatics associated with tumor growth and nodal metastasis. *Cancer Res* 68(18): 7293-303

Coghill AE, Newcomb PA, Campbell PT, Burnett-Hartman AN, Adams SV, Poole EM, Potter JD, Ulrich CM (2011) Prediagnostic non-steroidal anti-inflammatory drug use and survival after diagnosis of colorectal cancer. *Gut* 60(4): 491-8

Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams M (1990) The adenosine/neutrophil paradox resolved: human neutrophils possess both A1 and A2 receptors that promote chemotaxis and inhibit O2 generation, respectively. *J Clin Invest* 85(4): 1150-7

Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G (1992) Neutrophil adherence to endothelium is enhanced via adenosine A1 receptors and inhibited via adenosine A2 receptors. *J Immunol* 148(7): 2201-6

Csóka B, Selmeczy Z, Koscsó B, Németh ZH, Pacher P, Murray PJ, Kepka-Lenhart D, Morris SM, Gause WC, Leibovich SJ, Haskó G (2012) Adenosine promotes alternative macrophage activation via A2A and A2B receptors. *FASEB J* 26(1): 376-86

Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, Starling N (2010) Colorectal cancer. *Lancet* 375(9719): 1030-47

Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van Cutsem E (2004) Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 351(4): 337-45

Cuypers HT, Selten G, Quint W, Zijlstra M, Maandag ER, Boelens W, van Wezenbeek P, Melief C, Berns A (1984) Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell* 37(1): 141-50

Dadras SS, Paul T, Bertoncini J, Brown LF, Muzikansky A, Jackson DG, Ellwanger U, Garbe C, Mihm MC, Detmar M (2003) Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. *Am J Pathol* 162(6): 1951-60

De Roock W, De Vriendt V, Normanno N, Ciardiello F, Tejpar S (2011) KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. *Lancet Oncol* 12(6): 594-603

Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, Kuchroo VK, Strom TB, Robson SC (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204(6): 1257-65

Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, Butcher EC (2005) Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol* 6(9): 889-94

Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, Pienta KJ, Rubin MA, Chinnaiyan AM (2001) Delineation of prognostic biomarkers in prostate cancer. *Nature* 412(6849): 822-6

Dietel M, Ellis IO, Höfler H, Kreipe H, Moch H, Dankof A, Kölble K, Kristiansen G (2007) Comparison of automated silver enhanced in situ hybridisation (SISH) and fluorescence ISH (FISH) for the validation of HER2 gene status in breast carcinoma according to the guidelines of the American Society of Clinical Oncology and the College of American Pathologists. *Virchows Arch* 451(1): 19-25

Dimopoulos M, Spencer A, Attal M, Prince HM, Harousseau JL, Dmoszynska A, San Miguel J, Hellmann A, Facon T, Foà R, Corso A, Masliak Z, Olesnyckyj M, Yu Z, Patin J, Zeldis JB, Knight RD, Investigators MMS (2007) Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma. *N Engl J Med* 357(21): 2123-32

Domen J, van der Lugt NM, Acton D, Laird PW, Linders K, Berns A (1993a) Pim-1 levels determine the size of early B lymphoid compartments in bone marrow. *J Exp Med* 178(5): 1665-73

Domen J, van der Lugt NM, Laird PW, Saris CJ, Clarke AR, Hooper ML, Berns A (1993b) Impaired interleukin-3 response in Pim-1-deficient bone marrow-derived mast cells. *Blood* 82(5): 1445-52

Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocákova I, Ruff P, Błasińska-Morawiec M, Šmakal M, Canon JL, Rother M, Oliner KS, Wolf M, Gansert J (2010) Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 28(31): 4697-705

Du Y, Liu Y, Wang Y, He Y, Yang C, Gao F (2011) LYVE-1 enhances the adhesion of HS-578T cells to COS-7 cells via hyaluronan. *Clin Invest Med* 34(1): E45-54

Ducreux M, Malka D, Mendiboure J, Etienne PL, Texereau P, Auby D, Rougier P, Gasmi M, Castaing M, Abbas M, Michel P, Gargot D, Azzedine A, Lombard-Bohas C, Geoffroy P, Denis B, Pignon JP, Bedenne L, Bouché O, Group FFdCDFC (2011) Sequential versus combination chemotherapy for the treatment of advanced colorectal cancer (FFCD 2000-05): an open-label, randomised, phase 3 trial. *Lancet Oncol* 12(11): 1032-44

Dunn GP, Old LJ, Schreiber RD (2004) The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21(2): 137-48

Eckle T, Füllbier L, Wehrmann M, Khoury J, Mittelbronn M, Ibla J, Rosenberger P, Eltzschig HK (2007) Identification of ectonucleotidases CD39 and CD73 in innate protection during acute lung injury. *J Immunol* 178(12): 8127-37

Edwards NL, Gelfand EW, Burk L, Dosch HM, Fox IH (1979) Distribution of 5'-nucleotidase in human lymphoid tissues. *Proc Natl Acad Sci U S A* 76(7): 3474-6

Edwards NL, Magilavy DB, Cassidy JT, Fox IH (1978) Lymphocyte ecto-5'-nucleotidase deficiency in agammaglobulinemia. *Science* 201(4356): 628-30

Eichmann A, Yuan L, Breant C, Alitalo K, Koskinen PJ (2000) Developmental expression of pim kinases suggests functions also outside of the hematopoietic system. *Oncogene* 19(9): 1215-24

Eltzschig HK, Thompson LF, Karhausen J, Cotta RJ, Ibla JC, Robson SC, Colgan SP (2004) Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: coordination by extracellular nucleotide metabolism. *Blood* 104(13): 3986-92

Erdman SE, Sohn JJ, Rao VP, Nambiar PR, Ge Z, Fox JG, Schauer DB (2005) CD4+CD25+ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/+ mice. *Cancer Res* 65(10): 3998-4004

Erreni M, Mantovani A, Allavena P (2011) Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer. *Cancer Microenviron* 4(2): 141-54

Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* 61(5): 759-67

Ferlay J, Parkin DM, Steliarova-Foucher E (2010) Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer* 46(4): 765-81

Finn OJ (2008) Cancer immunology. *N Engl J Med* 358(25): 2704-15

Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1(1): 27-31

Forssell J, Oberg A, Henriksson ML, Stenling R, Jung A, Palmqvist R (2007) High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clin Cancer Res* 13(5): 1472-9

Fox CJ, Hammerman PS, Thompson CB (2005) The Pim kinases control rapamycin-resistant T cell survival and activation. *J Exp Med* 201(2): 259-66

Fujiwara T, Fukushi J, Yamamoto S, Matsumoto Y, Setsu N, Oda Y, Yamada H, Okada S, Watari K, Ono M, Kuwano M, Kamura S, Iida K, Okada Y, Koga M, Iwamoto Y (2011) Macrophage infiltration predicts a poor prognosis for human ewing sarcoma. *Am J Pathol* 179(3): 1157-70

Funada Y, Noguchi T, Kikuchi R, Takeno S, Uchida Y, Gabbert HE (2003) Prognostic significance of CD8+ T cell and macrophage peritumoral infiltration in colorectal cancer. *Oncol Rep* 10(2): 309-13

Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoué F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pagès F (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313(5795): 1960-4

Gan HK, Seruga B, Knox JJ (2009) Sunitinib in solid tumors. *Expert Opin Investig Drugs* 18(6): 821-34

Gao F, Lu YM, Cao ML, Liu YW, He YQ, Wang Y (2006) Expression and quantification of LYVE-1 in human colorectal cancer. *Clin Exp Med* 6(2): 65-71

George B, Kopetz S (2011) Predictive and prognostic markers in colorectal cancer. *Curr Oncol Rep* 13(3): 206-15

George ML, Tutton MG, Janssen F, Arnaout A, Abulafi AM, Eccles SA, Swift RI (2001) VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. *Neoplasia* 3(5): 420-7

Giantonio BJ, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, Schwartz MA, Benson AB, E3200 ECOGS (2007) Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. *J Clin Oncol* 25(12): 1539-44

Glimelius B, Påhlman L, Cervantes A, Group EGW (2010) Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 21 Suppl 5: v82-6

Goerdt S, Bhardwaj R, Sorg C (1993) Inducible expression of MS-1 high-molecular-weight protein by endothelial cells of continuous origin and by dendritic cells/macrophages in vivo and in vitro. *Am J Pathol* 142(5): 1409-22

Goerdt S, Orfanos CE (1999) Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity* 10(2): 137-42

Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. *Cell* 140(6): 883-99

Grothey A, Sargent D, Goldberg RM, Schmoll HJ (2004) Survival of patients with advanced colorectal cancer improves with the availability of fluorouracil-leucovorin, irinotecan, and oxaliplatin in the course of treatment. *J Clin Oncol* 22(7): 1209-14

Guo S, Mao X, Chen J, Huang B, Jin C, Xu Z, Qiu S (2010) Overexpression of Pim-1 in bladder cancer. *J Exp Clin Cancer Res* 29: 161

Günther K, Leier J, Henning G, Dimmler A, Weissbach R, Hohenberger W, Förster R (2005) Prediction of lymph node metastasis in colorectal carcinoma by expressionof chemokine receptor CCR7. *Int J Cancer* 116(5): 726-33

Halama N, Michel S, Kloor M, Zoernig I, Benner A, Spille A, Pommerencke T, von Knebel DM, Folprecht G, Luber B, Feyen N, Martens UM, Beckhove P, Gnjatic S, Schirmacher P, Herpel E, Weitz J, Grabe N, Jaeger D (2011) Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer Res* 71(17): 5670-7

Hall FT, Freeman JL, Asa SL, Jackson DG, Beasley NJ (2003) Intratumoral lymphatics and lymph node metastases in papillary thyroid carcinoma. *Arch Otolaryngol Head Neck Surg* 129(7): 716-9

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100(1): 57-70

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5): 646-74

Hart ML, Grenz A, Gorzolla IC, Schittenhelm J, Dalton JH, Eltzschig HK (2011) Hypoxia-inducible factor-1α-dependent protection from intestinal ischemia/reperfusion injury involves ecto-5'-nucleotidase (CD73) and the A2B adenosine receptor. *J Immunol* 186(7): 4367-74

Hasita H, Komohara Y, Okabe H, Masuda T, Ohnishi K, Lei XF, Beppu T, Baba H, Takeya M Significance of alternatively activated macrophages in patients with intrahepatic cholangiocarcinoma. *Cancer Sci* 101(8): 1913-9

Hasita H, Komohara Y, Okabe H, Masuda T, Ohnishi K, Lei XF, Beppu T, Baba H, Takeya M (2010) Significance of alternatively activated macrophages in patients with intrahepatic cholangiocarcinoma. *Cancer Sci* 101(8): 1913-9

Hecht JR, Mitchell E, Chidiac T, Scroggin C, Hagenstad C, Spigel D, Marshall J, Cohn A, McCollum D, Stella P, Deeter R, Shahin S, Amado RG (2009) A randomized phase IIIB trial of chemotherapy, bevacizumab, and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer. *J Clin Oncol* 27(5): 672-80

Heim S, Mandahl N, Mitelman F (1988) Genetic convergence and divergence in tumor progression. *Cancer Res* 48(21): 5911-6

Henttinen T, Jalkanen S, Yegutkin GG (2003) Adherent leukocytes prevent adenosine formation and impair endothelial barrier function by Ecto-5'-nucleotidase/CD73-dependent mechanism. *J Biol Chem* 278(27): 24888-95

Hewish M, Lord CJ, Martin SA, Cunningham D, Ashworth A (2010) Mismatch repair deficient colorectal cancer in the era of personalized treatment. *Nat Rev Clin Oncol* 7(4): 197-208

Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M (2007) VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* 109(3): 1010-7

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363(8): 711-23

Holopainen T, Bry M, Alitalo K, Saaristo A (2011) Perspectives on lymphangiogenesis and angiogenesis in cancer. *J Surg Oncol* 103(6): 484-8

Horiguchi A, Ito K, Sumitomo M, Kimura F, Asano T, Hayakawa M (2008) Intratumoral lymphatics and lymphatic invasion are associated with tumor aggressiveness and poor prognosis in renal cell carcinoma. *Urology* 71(5): 928-32

Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldon W, Altekruse SF, Kosary CL, Ruhl J,Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK (2011) SEER Cancer Statistics Review, 1975-2008, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975-2008/, based on November 2010 SEER data submission, posted to the SEER website, 2011.

Hubner RA, Riley RD, Billingham LJ, Popat S (2011) Excision repair cross-complementation group 1 (ERCC1) status and lung cancer outcomes: a meta-analysis of published studies and recommendations. *PLoS One* 6(10): e25164

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 350(23): 2335-42

Hurwitz HI, Fehrenbacher L, Hainsworth JD, Heim W, Berlin J, Holmgren E, Hambleton J, Novotny WF, Kabbinavar F (2005) Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer. *J Clin Oncol* 23(15): 3502-8

Irjala H, Alanen K, Grenman R, Heikkila P, Joensuu H, Jalkanen S (2003a) Mannose receptor (MR) and common lymphatic endothelial and vascular endothelial receptor (CLEVER)-1 direct the binding of cancer cells to the lymph vessel endothelium. *Cancer Res* 63(15): 4671-6

Irjala H, Elima K, Johansson EL, Merinen M, Kontula K, Alanen K, Grenman R, Salmi M, Jalkanen S (2003b) The same endothelial receptor controls lymphocyte traffic both in vascular and lymphatic vessels. *Eur J Immunol* 33(3): 815-24

Irjala H, Johansson EL, Grenman R, Alanen K, Salmi M, Jalkanen S (2001) Mannose receptor is a novel ligand for L-selectin and mediates lymphocyte binding to lymphatic endothelium. *J Exp Med* 194(8): 1033-42

Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K, Vikkula M (2000) Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. *Am J Hum Genet* 67(2): 295-301

Ishikawa Y, Akishima-Fukasawa Y, Ito K, Akasaka Y, Yokoo T, Ishii T, Behavior TSGfCB (2008) Histopathologic determinants of regional lymph node metastasis in early colorectal cancer. *Cancer* 112(4): 924-33

Isola J, Tanner M, Forsyth A, Cooke TG, Watters AD, Bartlett JM (2004) Interlaboratory comparison of HER-2 oncogene amplification as detected by chromogenic and fluorescence in situ hybridization. *Clin Cancer Res* 10(14): 4793-8

Issa A, Le TX, Shoushtari AN, Shields JD, Swartz MA (2009) Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype. *Cancer Res* 69(1): 349-57

Jalkanen S, Salmi M (2008) VAP-1 and CD73, endothelial cell surface enzymes in leukocyte extravasation. *Arterioscler Thromb Vasc Biol* 28(1): 18-26

Ji RC (2006) Lymphatic endothelial cells, tumor lymphangiogenesis and metastasis: New insights into intratumoral and peritumoral lymphatics. *Cancer Metastasis Rev* 25(4): 677-94

Ji RC (2011) Macrophages are important mediators of either tumor- or inflammation-induced lymphangiogenesis. *Cell Mol Life Sci*

Jing H, Kase J, Dörr JR, Milanovic M, Lenze D, Grau M, Beuster G, Ji S, Reimann M, Lenz P, Hummel M, Dörken B, Lenz G, Scheidereit C, Schmitt CA, Lee S (2011) Opposing roles of NF-κB in anti-cancer treatment outcome unveiled by cross-species investigations. *Genes Dev* 25(20): 2137-46

Jones RG, Thompson CB (2009) Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev* 23(5): 537-48

Jonkers J, Korswagen HC, Acton D, Breuer M, Berns A (1997) Activation of a novel proto-oncogene, Frat1, contributes to progression of mouse T-cell lymphomas. *Embo J* 16(3): 441-50

Kang JC, Chen JS, Lee CH, Chang JJ, Shieh YS (2010) Intratumoral macrophage counts correlate with tumor progression in colorectal cancer. *J Surg Oncol* 102(3): 242-8

Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF, Investigators IS (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 363(5): 411-22

Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalcberg JR (2008) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 359(17): 1757-65

Karikoski M, Irjala H, Maksimow M, Miiluniemi M, Granfors K, Hernesniemi S, Elima K, Moldenhauer G, Schledzewski K, Kzhyshkowska J, Goerdt S, Salmi M, Jalkanen S (2009) Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. *Eur J Immunol* 39(12): 3477-87

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K (2004) Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 5(1): 74-80

Karpanen T, Wirzenius M, Mäkinen T, Veikkola T, Haisma HJ, Achen MG, Stacker SA, Pytowski B, Ylä-Herttuala S, Alitalo K (2006) Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. *Am J Pathol* 169(2): 708-18

Kerjaschki D, Huttary N, Raab I, Regele H, Bojarski-Nagy K, Bartel G, Kröber SM, Greinix H, Rosenmaier A, Karlhofer F, Wick N, Mazal PR (2006) Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. *Nat Med* 12(2): 230-4

Khorana AA, Ryan CK, Cox C, Eberly S, Sahasrabudhe DM (2003) Vascular endothelial growth factor, CD68, and epidermal growth factor receptor expression and survival in patients with Stage II and Stage III colon carcinoma: a role for the host response in prognosis. *Cancer* 97(4): 960-8

Kiss J, Yegutkin GG, Koskinen K, Savunen T, Jalkanen S, Salmi M (2007) IFN-beta protects from vascular leakage via up-regulation of CD73. *Eur J Immunol* 37(12): 3334-8

Klein U, Ghosh S (2011) The Two Faces of NF-κB Signaling in Cancer Development and Therapy. *Cancer Cell* 20(5): 556-8

Klintrup K, Mäkinen JM, Kauppila S, Väre PO, Melkko J, Tuominen H, Tuppurainen K, Mäkelä J, Karttunen TJ, Mäkinen MJ (2005) Inflammation and prognosis in colorectal cancer. *Eur J Cancer* 41(17): 2645-54

Kobie JJ, Shah PR, Yang L, Rebhahn JA, Fowell DJ, Mosmann TR (2006) T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J Immunol* 177(10): 6780-6

Kodumudi KN, Woan K, Gilvary DL, Sahakian E, Wei S, Djeu JY (2010) A novel chemoimmunomodulating property of docetaxel: suppression of myeloid-derived suppressor cells in tumor bearers. *Clin Cancer Res* 16(18): 4583-94

Komohara Y, Ohnishi K, Kuratsu J, Takeya M (2008) Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas. *J Pathol* 216(1): 15-24

Koopman M, Venderbosch S, Nagtegaal ID, van Krieken JH, Punt CJ (2009) A review on the use of molecular markers of cytotoxic therapy for colorectal cancer, what have we learned? *Eur J Cancer* 45(11): 1935-49

Koszalka P, Ozüyaman B, Huo Y, Zernecke A, Flögel U, Braun N, Buchheiser A, Decking UK, Smith ML, Sévigny J, Gear A, Weber AA, Molojavyi A, Ding Z, Weber C, Ley K, Zimmermann H, Gödecke A, Schrader J (2004) Targeted disruption of cd73/ecto-5'-nucleotidase alters thromboregulation and augments vascular inflammatory response. *Circ Res* 95(8): 814-21

Kubota Y, Takubo K, Shimizu T, Ohno H, Kishi K, Shibuya M, Saya H, Suda T (2009) M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis. *J Exp Med* 206(5): 1089-102

Kurahara H, Shinchi H, Mataki Y, Maemura K, Noma H, Kubo F, Sakoda M, Ueno S, Natsugoe S, Takao S (2011) Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. *J Surg Res* 167(2): e211-9

Kzhyshkowska J (2010) Multifunctional receptor stabilin-1 in homeostasis and disease. *ScientificWorldJournal* 10: 2039-53

Kzhyshkowska J, Gratchev A, Goerdt S (2006) Stabilin-1, a homeostatic scavenger receptor with multiple functions. *J Cell Mol Med* 10(3): 635-49

Labianca R, Nordlinger B, Beretta GD, Brouquet A, Cervantes A, Group EGW (2010) Primary colon cancer: ESMO Clinical Practice Guidelines for diagnosis, adjuvant treatment and follow-up. *Ann Oncol* 21 Suppl 5: v70-7

Lackner C, Jukic Z, Tsybrovskyy O, Jatzko G, Wette V, Hoefler G, Klimpfinger M, Denk H, Zatloukal K (2004) Prognostic relevance of tumour-associated macrophages and von Willebrand factor-positive microvessels in colorectal cancer. *Virchows Arch* 445(2): 160-7

Laghi L, Bianchi P, Miranda E, Balladore E, Pacetti V, Grizzi F, Allavena P, Torri V, Repici A, Santoro A, Mantovani A, Roncalli M, Malesci A (2009) CD3+ cells at the invasive margin of deeply invading (pT3-T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. *Lancet Oncol* 10(9): 877-84

Laird PW, van der Lugt NM, Clarke A, Domen J, Linders K, McWhir J, Berns A, Hooper M (1993) In vivo analysis of Pim-1 deficiency. *Nucleic Acids Res* 21(20): 4750-5

Laurent-Puig P, Cayre A, Manceau G, Buc E, Bachet JB, Lecomte T, Rougier P, Lievre A, Landi B, Boige V, Ducreux M, Ychou M, Bibeau F, Bouche O, Reid J, Stone S, Penault-Llorca F (2009) Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. *J Clin Oncol* 27(35): 5924-30

Lemmon MA, Schlessinger J (2010) Cell signaling by receptor tyrosine kinases. *Cell* 141(7): 1117-34

Leverson JD, Koskinen PJ, Orrico FC, Rainio EM, Jalkanen KJ, Dash AB, Eisenman RN, Ness SA (1998) Pim-1 kinase and p100 cooperate to enhance c-Myb activity. *Mol Cell* 2(4): 417-25

Ley K, Laudanna C, Cybulsky MI, Nourshargh S (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 7(9): 678-89

Li J, Loveland BE, Xing PX (2011a) Anti-Pim-1 mAb inhibits activation and proliferation of T lymphocytes and prolongs mouse skin allograft survival. *Cell Immunol* 272(1): 87-93

Li X, Liu B, Xiao J, Yuan Y, Ma J, Zhang Y (2011b) Roles of VEGF-C and Smad4 in the Lymphangiogenesis, Lymphatic Metastasis, and Prognosis in Colon Cancer. *J Gastrointest Surg* 15(11): 2001-10

Liang H, Hittelman W, Nagarajan L (1996) Ubiquitous expression and cell cycle regulation of the protein kinase PIM-1. *Arch Biochem Biophys* 330(2): 259-65

Liang P, Hong JW, Ubukata H, Liu HR, Watanabe Y, Katano M, Motohashi G, Kasuga T, Nakada I, Tabuchi T (2006) Increased density and diameter of lymphatic microvessels correlate with lymph node metastasis in early stage invasive colorectal carcinoma. *Virchows Arch* 448(5): 570-5

Liang P, Nakada I, Hong JW, Tabuchi T, Motohashi G, Takemura A, Nakachi T, Kasuga T (2007) Prognostic significance of immunohistochemically detected blood and lymphatic vessel invasion in colorectal carcinoma: its impact on prognosis. *Ann Surg Oncol* 14(2): 470-7

Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 66(8): 3992-5

Lilly M, Sandholm J, Cooper JJ, Koskinen PJ, Kraft A (1999) The PIM-1 serine kinase prolongs survival and inhibits apoptosis-related mitochondrial dysfunction in part through a bcl-2-dependent pathway. *Oncogene* 18(27): 4022-31

Lin M, Lin HZ, Ma SP, Ji P, Xie D, Yu JX (2011) Vascular endothelial growth factor-A and -C: expression and correlations with lymphatic metastasis and prognosis in colorectal cancer. *Med Oncol* 28(1): 151-8

Lin WW, Karin M (2007) A cytokine-mediated link between innate immunity, inflammation, and cancer. *J Clin Invest* 117(5): 1175-83

Lombardi L, Morelli F, Cinieri S, Santini D, Silvestris N, Fazio N, Orlando L, Tonini G, Colucci G, Maiello E (2010) Adjuvant colon cancer chemotherapy: where we are and where we'll go. *Cancer Treat Rev* 36 Suppl 3: S34-41

Longatto-Filho A, Pinheiro C, Ferreira L, Scapulatempo C, Alves VA, Baltazar F, Schmitt F (2008) Peritumoural, but not intratumoural, lymphatic vessel density and invasion correlate with colorectal carcinoma poor-outcome markers. *Virchows Arch* 452(2): 133-8

Loupakis F, Cremolini C, Salvatore L, Schirripa M, Lonardi S, Vaccaro V, Cuppone F, Giannarelli D, Zagonel V, Cognetti F, Tortora G, Falcone A, Bria E (2011) Clinical impact of anti-epidermal growth factor receptor monoclonal antibodies in first-line treatment of metastatic colorectal cancer: Meta-analytical estimation and implications for therapeutic strategies. *Cancer* Epub Aug 25

Lu Y, Yang Q, Du Y, Feng G, Yang C (2007a) Expression analysis of lymphangiogenic factors in human colorectal cancer with quantitative RT-PCR. *Cancer Invest* 25(6): 393-6

Lu Y, Yang Q, Du Y, Feng G, Yang C (2007b) Expression analysis of lymphangiogenic factors in human colorectal cancer with quantitative RT-PCR. *Cancer Invest* 25(6): 393-6

Mahalingam D, Espitia CM, Medina EC, Esquivel JA, Kelly KR, Bearss D, Choy G, Taverna P, Carew JS, Giles FJ, Nawrocki ST (2011) Targeting PIM kinase enhances the activity of sunitinib in renal cell carcinoma. *Br J Cancer* 105(10): 1563-73

Mahmoud SM, Lee AH, Paish EC, Macmillan RD, Ellis IO, Green AR (2011) Tumour-infiltrating macrophages and clinical outcome in breast cancer. *J Clin Pathol*

Maita H, Harada Y, Nagakubo D, Kitaura H, Ikeda M, Tamai K, Takahashi K, Ariga H, Iguchi-Ariga SM (2000) PAP-1, a novel target protein of phosphorylation by pim-1 kinase. *Eur J Biochem* 267(16): 5168-78

Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, Thornton R, Shei GJ, Card D, Keohane C, Rosenbach M, Hale J, Lynch CL, Rupprecht K, Parsons W, Rosen H (2002) Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* 296(5566): 346-9

Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. *Nature* 454(7203): 436-44

Mantovani A, Sica A (2010) Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol* 22(2): 231-7

Marttila-Ichihara F, Turja R, Miiluniemi M, Karikoski M, Maksimow M, Niemelä J, Martinez-Pomares L, Salmi M, Jalkanen S (2008) Macrophage mannose receptor on lymphatics controls cell trafficking. *Blood* 112(1): 64-72

Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, Van Rooijen N, Takenaka H, D'Amore PA, Stein-Streilein J, Losordo DW, Streilein JW (2005) Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest* 115(9): 2363-72

Matsui K, Breitender-Geleff S, Soleiman A, Kowalski H, Kerjaschki D (1999) Podoplanin, a novel 43-kDa membrane protein, controls the shape of podocytes. *Nephrol Dial Transplant* 14 Suppl 1: 9-11

Matsumoto K, Nakayama Y, Inoue Y, Minagawa N, Katsuki T, Shibao K, Tsurudome Y, Hirata K, Nagata N, Itoh H (2007) Lymphatic microvessel density is an independent prognostic factor in colorectal cancer. *Dis Colon Rectum* 50(3): 308-14

Maughan TS, Adams RA, Smith CG, Meade AM, Seymour MT, Wilson RH, Idziaszczyk S, Harris R, Fisher D, Kenny SL, Kay E, Mitchell JK, Madi A, Jasani B, James MD, Bridgewater J, Kennedy MJ, Claes B, Lambrechts D, Kaplan R, Cheadle JP, Investigators MCT (2011) Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet* 377(9783): 2103-14

Maula SM, Luukkaa M, Grenman R, Jackson D, Jalkanen S, Ristamaki R (2003) Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res* 63(8): 1920-6

McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, Diagnostics SSotN-EWGoC (2005) REporting recommendations for tumor MARKer prognostic studies (REMARK). *Nat Clin Pract Oncol* 2(8): 416-22

Mellor RH, Brice G, Stanton AW, French J, Smith A, Jeffery S, Levick JR, Burnand KG, Mortimer PS, Consortium LR (2007) Mutations in FOXC2 are strongly associated with primary valve failure in veins of the lower limb. *Circulation* 115(14): 1912-20

Michaloglou C, Vredeveld LC, Mooi WJ, Peeper DS (2008) BRAF(E600) in benign and malignant human tumours. *Oncogene* 27(7): 877-95

Mikkers H, Nawijn M, Allen J, Brouwers C, Verhoeven E, Jonkers J, Berns A (2004) Mice deficient for all PIM kinases display reduced body size and impaired responses to hematopoietic growth factors. *Mol Cell Biol* 24(13): 6104-15

Mochizuki T, Kitanaka C, Noguchi K, Muramatsu T, Asai A, Kuchino Y (1999) Physical and functional interactions between Pim-1 kinase and Cdc25A phosphatase. Implications for the Pim-1-mediated activation of the c-Myc signaling pathway. *J Biol Chem* 274(26): 18659-66

Mochizuki T, Kitanaka C, Noguchi K, Sugiyama A, Kagaya S, Chi S, Asai A, Kuchino Y (1997) Pim-1 kinase stimulates c-Myc-mediated death signaling upstream of caspase-3 (CPP32)-like protease activation. *Oncogene* 15(12): 1471-80

Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, Gambacorta M, Siena S, Bardelli A (2005) Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 6(5): 279-86

Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8(12): 958-69

Mouta Carreira C, Nasser SM, di Tomaso E, Padera TP, Boucher Y, Tomarev SI, Jain RK (2001) LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. *Cancer Res* 61(22): 8079-84

Movahedi K, Laoui D, Gysemans C, Baeten M, Stangé G, Van den Bossche J, Mack M, Pipeleers D, In't Veld P, De Baetselier P, Van Ginderachter JA (2010) Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res* 70(14): 5728-39

Mukaida N, Baba T (2012) Chemokines in tumor development and progression. *Exp Cell Res* 318(2): 95-102

Mulder K, Koski S, Scarfe A, Chu Q, King K, Spratlin J (2010) Antiangiogenic agents in advanced gastrointestinal malignancies: past, present and a novel future. *Oncotarget* 1(7): 515-29

Mumenthaler SM, Ng PY, Hodge A, Bearss D, Berk G, Kanekal S, Redkar S, Taverna P, Agus DB, Jain A (2009) Pharmacologic inhibition of Pim kinases alters prostate cancer cell growth and resensitizes chemoresistant cells to taxanes. *Mol Cancer Ther* 8(10): 2882-93

Murri AM, Hilmy M, Bell J, Wilson C, McNicol AM, Lannigan A, Doughty JC, McMillan DC (2008) The relationship between the systemic inflammatory response, tumour proliferative activity, T-lymphocytic and macrophage infiltration, microvessel density and survival in patients with primary operable breast cancer. *Br J Cancer* 99(7): 1013-9

Murugaiyan G, Saha B (2009) Protumor vs antitumor functions of IL-17. *J Immunol* 183(7): 4169-75

Nagahashi M, Ramachandran S, Rashid OM, Takabe K (2010) Lymphangiogenesis: a new player in cancer progression. *World J Gastroenterol* 16(32): 4003-12

Nagarajan L, Louie E, Tsujimoto Y, ar-Rushdi A, Huebner K, Croce CM (1986) Localization of the human pim oncogene (PIM) to a region of chromosome 6 involved in translocations in acute leukemias. *Proc Natl Acad Sci U S A* 83(8): 2556-60

Nagorsen D, Voigt S, Berg E, Stein H, Thiel E, Loddenkemper C (2007) Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. *J Transl Med* 5: 62

Nagtegaal ID, Marijnen CA, Kranenbarg EK, Mulder-Stapel A, Hermans J, van de Velde CJ, van Krieken JH (2001) Local and distant recurrences in rectal cancer patients are predicted by the nonspecific immune response; specific immune response has only a systemic effect--a histopathological and immunohistochemical study. *BMC Cancer* 1: 7

Nakamura Y, Yasuoka H, Tsujimoto M, Imabun S, Nakahara M, Nakao K, Nakamura M, Mori I, Kakudo K (2005) Lymph vessel density correlates with nodal status, VEGF-C expression, and prognosis in breast cancer. *Breast Cancer Res Treat* 91(2): 125-32

Nakayama Y, Nagashima N, Minagawa N, Inoue Y, Katsuki T, Onitsuka K, Sako T, Hirata K, Nagata N, Itoh H (2002) Relationships between tumor-associated macrophages and clinicopathological factors in patients with colorectal cancer. *Anticancer Res* 22(6C): 4291-6

Negrini S, Gorgoulis VG, Halazonetis TD (2010) Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* 11(3): 220-8

Nga ME, Swe NN, Chen KT, Shen L, Lilly MB, Chan SP, Salto-Tellez M, Das K (2010) PIM-1 kinase expression in adipocytic neoplasms: diagnostic and biological implications. *Int J Exp Pathol* 91(1): 34-43

Nguyen DX, Bos PD, Massagué J (2009) Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 9(4): 274-84

Niemelä J, Henttinen T, Yegutkin GG, Airas L, Kujari AM, Rajala P, Jalkanen S (2004) IFN-alpha induced adenosine production on the endothelium: a mechanism mediated by CD73 (ecto-5'-nucleotidase) up-regulation. *J Immunol* 172(3): 1646-53

Niemelä J, Ifergan I, Yegutkin GG, Jalkanen S, Prat A, Airas L (2008) IFN-beta regulates CD73 and adenosine expression at the blood-brain barrier. *Eur J Immunol* 38(10): 2718-26

Noffsinger AE (2009) Serrated polyps and colorectal cancer: new pathway to malignancy. *Annu Rev Pathol* 4: 343-64

Nordlinger B, Sorbye H, Glimelius B, Poston GJ, Schlag PM, Rougier P, Bechstein WO, Primrose JN, Walpole ET, Finch-Jones M, Jaeck D, Mirza D, Parks RW, Collette L, Praet M, Bethe U, Van Cutsem E, Scheithauer W, Gruenberger T, Group EG-ITC, UK CR, (ALM-CAO) ALu-tidCAO, (AGITG) AG-ITG, (FFCD) FFdCD (2008) Perioperative chemotherapy with FOLFOX4 and surgery versus surgery alone for resectable liver metastases from colorectal cancer (EORTC Intergroup trial 40983): a randomised controlled trial. *Lancet* 371(9617): 1007-16

Norrmén C, Tammela T, Petrova TV, Alitalo K (2011) Biological basis of therapeutic lymphangiogenesis. *Circulation* 123(12): 1335-51

Nosho K, Baba Y, Tanaka N, Shima K, Hayashi M, Meyerhardt JA, Giovannucci E, Dranoff G, Fuchs CS, Ogino S (2010) Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 222(4): 350-66

Oberg A, Samii S, Stenling R, Lindmark G (2002) Different occurrence of CD8+, CD45R0+, and CD68+ immune cells in regional lymph node metastases from colorectal cancer as potential prognostic predictors. *Int J Colorectal Dis* 17(1): 25-9

Ogino S, Galon J, Fuchs CS, Dranoff G (2011) Cancer immunology--analysis of host and tumor factors for personalized medicine. *Nat Rev Clin Oncol* 8(12): 711-9

Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MK, Huang X, Caldwell S, Liu K, Smith P, Chen JF, Jackson EK, Apasov S, Abrams S, Sitkovsky M (2006) A2A adenosine receptor protects tumors from antitumor T cells. *Proc Natl Acad Sci U S A* 103(35): 13132-7

Ohta A, Sitkovsky M (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414(6866): 916-20

Omachi T, Kawai Y, Mizuno R, Nomiyama T, Miyagawa S, Ohhashi T, Nakayama J (2007) Immunohistochemical demonstration of proliferating lymphatic vessels in colorectal carcinoma and its clinicopathological significance. *Cancer Lett* 246(1-2): 167-72

Ong SM, Tan YC, Beretta O, Jiang D, Yeap WH, Tai JJ, Wong WC, Yang H, Schwarz H, Lim KH, Koh PK, Ling KL, Wong SC (2012) Macrophages in human colorectal cancer are pro-inflammatory and prime T cells towards an anti-tumour type-1 inflammatory response. *Eur J Immunol* 42(1): 89-100

Paavonen K, Puolakkainen P, Jussila L, Jahkola T, Alitalo K (2000) Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathol* 156(5): 1499-504

Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, Munn LL, Jain RK (2002) Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 296(5574): 1883-6

Palani S, Maksimow M, Miiluniemi M, Auvinen K, Jalkanen S, Salmi M (2011) Stabilin-1/CLEVER-1, a type 2 macrophage marker, is an adhesion and scavenging molecule on human placental macrophages. *Eur J Immunol* 41(7): 2052-63

Parr C, Jiang WG (2003) Quantitative analysis of lymphangiogenic markers in human colorectal cancer. *Int J Oncol* 23(2): 533-9

Partanen TA, Alitalo K, Miettinen M (1999) Lack of lymphatic vascular specificity of vascular endothelial growth factor receptor 3 in 185 vascular tumors. *Cancer* 86(11): 2406-12

Paschos KA, Canovas D, Bird NC (2009) The role of cell adhesion molecules in the progression of colorectal cancer and the development of liver metastasis. *Cell Signal* 21(5): 665-74

Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, André T, Chan E, Lordick F, Punt CJ, Strickland AH, Wilson G, Ciuleanu TE, Roman L, Van Cutsem E, Tzekova V, Collins S, Oliner KS, Rong A, Gansert J (2010) Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. *J Clin Oncol* 28(31): 4706-13

Peltola K, Hollmen M, Maula SM, Rainio E, Ristamaki R, Luukkaa M, Sandholm J, Sundvall M, Elenius K, Koskinen PJ, Grenman R, Jalkanen S (2009) Pim-1 kinase expression predicts radiation response in squamocellular carcinoma of head and neck and is under the control of epidermal growth factor receptor. *Neoplasia* 11(7): 629-36

Peltola KJ, Paukku K, Aho TL, Ruuska M, Silvennoinen O, Koskinen PJ (2004) Pim-1 kinase inhibits STAT5-dependent transcription via its interactions with SOCS1 and SOCS3. *Blood* 103(10): 3744-50

Perez EA, Reinholz MM, Hillman DW, Tenner KS, Schroeder MJ, Davidson NE, Martino S, Sledge GW, Harris LN, Gralow JR, Dueck AC, Ketterling RP, Ingle JN, Lingle WL, Kaufman PA, Visscher DW, Jenkins RB (2010) HER2 and chromosome 17 effect on patient outcome in the N9831 adjuvant trastuzumab trial. *J Clin Oncol* 28(28): 4307-15

Personeni N, Fieuws S, Piessevaux H, De Hertogh G, De Schutter J, Biesmans B, De Roock W, Capoen A, Debiec-Rychter M, Van Laethem JL, Peeters M, Humblet Y, Van Cutsem E, Tejpar S (2008) Clinical usefulness of EGFR gene copy number as a predictive marker in colorectal cancer patients treated with cetuximab: a fluorescent in situ hybridization study. *Clin Cancer Res* 14(18): 5869-76

Petrova TV, Mäkinen T, Mäkelä TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Ylä-Herttuala S, Alitalo K (2002) Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J* 21(17): 4593-9

Politz O, Gratchev A, McCourt PA, Schledzewski K, Guillot P, Johansson S, Svineng G, Franke P, Kannicht C, Kzhyshkowska J, Longati P, Velten FW, Goerdt S (2002) Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues. *Biochem J* 362(Pt 1): 155-64

Popivanova BK, Kostadinova FI, Furuichi K, Shamekh MM, Kondo T, Wada T, Egashira K, Mukaida N (2009) Blockade of a chemokine, CCL2, reduces chronic colitis-associated carcinogenesis in mice. *Cancer Res* 69(19): 7884-92

Potente M, Gerhardt H, Carmeliet P (2011) Basic and therapeutic aspects of angiogenesis. *Cell* 146(6): 873-87

Price TJ, Hardingham JE, Lee CK, Weickhardt A, Townsend AR, Wrin JW, Chua A, Shivasami A, Cummins MM, Murone C, Tebbutt NC (2011) Impact of KRAS and BRAF Gene Mutation Status on Outcomes From the Phase III AGITG MAX Trial of Capecitabine Alone or in Combination With Bevacizumab and Mitomycin in Advanced Colorectal Cancer. *J Clin Oncol* 29(19): 2675-82

Pritchard CC, Grady WM (2011) Colorectal cancer molecular biology moves into clinical practice. *Gut* 60(1): 116-29

Qiu LX, Mao C, Zhang J, Zhu XD, Liao RY, Xue K, Li J, Chen Q (2010) Predictive and prognostic value of KRAS mutations in metastatic colorectal cancer patients treated with cetuximab: a meta-analysis of 22 studies. *Eur J Cancer* 46(15): 2781-7

Quasar Collaborative G, Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ (2007) Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. *Lancet* 370(9604): 2020-9

Rainio EM, Sandholm J, Koskinen PJ (2002) Cutting edge: Transcriptional activity of NFATc1 is enhanced by the Pim-1 kinase. *J Immunol* 168(4): 1524-7

Raynaud CM, Hernandez J, Llorca FP, Nuciforo P, Mathieu MC, Commo F, Delaloge S, Sabatier L, André F, Soria JC (2010) DNA damage repair and telomere length in normal breast, preneoplastic lesions, and invasive cancer. *Am J Clin Oncol* 33(4): 341-5

Reiser-Erkan C, Erkan M, Pan Z, Bekasi S, Giese NA, Streit S, Michalski CW, Friess H, Kleeff J (2008) Hypoxia-inducible proto-oncogene Pim-1 is a prognostic marker in pancreatic ductal adenocarcinoma. *Cancer Biol Ther* 7(9): 1352-9

Reutershan J, Vollmer I, Stark S, Wagner R, Ngamsri KC, Eltzschig HK (2009) Adenosine and inflammation: CD39 and CD73 are critical mediators in LPS-induced PMN trafficking into the lungs. *FASEB J* 23(2): 473-82

Rhodes DR, Sanda MG, Otte AP, Chinnaiyan AM, Rubin MA (2003) Multiplex biomarker approach for determining risk of prostate-specific antigen-defined recurrence of prostate cancer. *J Natl Cancer Inst* 95(9): 661-8

Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, Hamilton SR, Laurent-Puig P, Gryfe R, Shepherd LE, Tu D, Redston M, Gallinger S (2003) Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med* 349(3): 247-57

Robert C, Thomas L, Bondarenko I, O'Day S, M D JW, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ, Davidson N, Richards J, Maio M, Hauschild A, Miller WH, Gascon P, Lotem M, Harmankaya K, Ibrahim R, Francis S, Chen TT, Humphrey R, Hoos A, Wolchok JD (2011) Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 364(26): 2517-26

Roh M, Gary B, Song C, Said-Al-Naief N, Tousson A, Kraft A, Eltoum IE, Abdulkadir SA (2003) Overexpression of the oncogenic kinase Pim-1 leads to genomic instability. *Cancer Res* 63(23): 8079-84

Roithmaier S, Haydon AM, Loi S, Esmore D, Griffiths A, Bergin P, Williams TJ, Schwarz MA (2007) Incidence of malignancies in heart and/or lung transplant recipients: a single-institution experience. *J Heart Lung Transplant* 26(8): 845-9

Rosen H, Sanna G, Alfonso C (2003) Egress: a receptor-regulated step in lymphocyte trafficking. *Immunol Rev* 195: 160-77

Ross JS, Torres-Mora J, Wagle N, Jennings TA, Jones DM (2010) Biomarker-based prediction of response to therapy for colorectal cancer: current perspective. *Am J Clin Pathol* 134(3): 478-90

Roxburgh CS, McMillan DC (2011) The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer. *Cancer Treat Rev* Epub Sep 24

Royston D, Jackson DG (2009) Mechanisms of lymphatic metastasis in human colorectal adenocarcinoma. *J Pathol* 217(5): 608-19

Saad RS, Kordunsky L, Liu YL, Denning KL, Kandil HA, Silverman JF (2006) Lymphatic microvessel density as prognostic marker in colorectal cancer. *Mod Pathol* 19(10): 1317-23

Saif MW (2010) Colorectal cancer in review: the role of the EGFR pathway. *Expert Opin Investig Drugs* 19(3): 357-69

Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B (2009) Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 27(2): 186-92

Salmi M, Jalkanen S (2005) Cell-surface enzymes in control of leukocyte trafficking. *Nat Rev Immunol* 5(10): 760-71

Salmi M, Jalkanen S (2011) Homing-associated molecules CD73 and VAP-1 as targets to prevent harmful inflammations and cancer spread. *FEBS Lett* 585(11): 1543-50

Salmi M, Koskinen K, Henttinen T, Elima K, Jalkanen S (2004) CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. *Blood* 104(13): 3849-57

Saltz LB, Clarke S, Díaz-Rubio E, Scheithauer W, Figer A, Wong R, Koski S, Lichinitser M, Yang TS, Rivera F, Couture F, Sirzén F, Cassidy J (2008) Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. *J Clin Oncol* 26(12): 2013-9

Saltz LB, Meropol NJ, Loehrer PJ, Sr., Needle MN, Kopit J, Mayer RJ (2004) Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 22(7): 1201-8

Sargent D, Grothey A (2010) Adjuvant therapy for colon cancer: learning from the past to inform the future. *Ann Surg Oncol* 17(4): 947-9

Sargent DJ, Marsoni S, Monges G, Thibodeau SN, Labianca R, Hamilton SR, French AJ, Kabat B, Foster NR, Torri V, Ribic C, Grothey A, Moore M, Zaniboni A, Seitz JF, Sinicrope F, Gallinger S (2010) Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 28(20): 3219-26

Saris CJ, Domen J, Berns A (1991) The pim-1 oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. *Embo J* 10(3): 655-64

Sartore-Bianchi A, Fieuws S, Veronese S, Moroni M, Personeni N, Frattini M, Torri V, Cappuzzo F, Vander Borght S, Martin V, Skokan M, Santoro A, Gambacorta M, Tejpar S, Varella-Garcia M, Siena S (2011) Standardisation of EGFR FISH in colorectal cancer: results of an international interlaboratory reproducibility ring study. *J Clin Pathol* Epub Nov 30

Sartore-Bianchi A, Moroni M, Veronese S, Carnaghi C, Bajetta E, Luppi G, Sobrero A, Barone C, Cascinu S, Colucci G, Cortesi E, Nichelatti M, Gambacorta M, Siena S (2007) Epidermal growth factor receptor gene copy number and clinical outcome of metastatic colorectal cancer treated with panitumumab. *J Clin Oncol* 25(22): 3238-45

Sauer R, Becker H, Hohenberger W, Rödel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H, Raab R, Group GRCS (2004) Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med* 351(17): 1731-40

Savage ND, de Boer T, Walburg KV, Joosten SA, van Meijgaarden K, Geluk A, Ottenhoff TH (2008) Human anti-inflammatory macrophages induce Foxp3+ GITR+ CD25+ regulatory T cells, which suppress via membrane-bound TGFbeta-1. *J Immunol* 181(3): 2220-6

Scartozzi M, Bearzi I, Mandolesi A, Pierantoni C, Loupakis F, Zaniboni A, Negri F, Quadri A, Zorzi F, Galizia E, Berardi R, Biscotti T, Labianca R, Masi G, Falcone A, Cascinu S (2009) Epidermal Growth Factor Receptor (EGFR) gene copy number (GCN) correlates with clinical activity of irinotecan-cetuximab in K-RAS wild-type colorectal cancer: a fluorescence in situ (FISH) and chromogenic in situ hybridization (CISH) analysis. *BMC Cancer* 9: 303

Scartozzi M, Giampieri R, Maccaroni E, Mandolesi A, Giustini L, Silva R, Zaniboni A, Biscotti T, Biagetti S, Galizia E, Loupakis F, Falcone A, Bearzi I, Cascinu S (2011) Analysis of HER-3, insulin growth factor-1, nuclear factor-kB and epidermal growth factor receptor gene copy number in the prediction of clinical outcome for K-RAS wild-type colorectal cancer patients receiving irinotecan-cetuximab. *Ann Oncol* Epub Nov 23

Schmidt T, Karsunky H, Gau E, Zevnik B, Elsasser HP, Moroy T (1998) Zinc finger protein GFI-1 has low oncogenic potential but cooperates strongly with pim and myc genes in T-cell lymphomagenesis. *Oncogene* 17(20): 2661-7

Schoppmann SF, Bayer G, Aumayr K, Taucher S, Geleff S, Rudas M, Kubista E, Hausmaninger H, Samonigg H, Gnant M, Jakesz R, Horvat R (2004) Prognostic value of lymphangiogenesis and lymphovascular invasion in invasive breast cancer. *Ann Surg* 240(2): 306-12

Schroedl F, Brehmer A, Neuhuber WL, Kruse FE, May CA, Cursiefen C (2008) The normal human choroid is endowed with a significant number of lymphatic vessel endothelial hyaluronate receptor 1 (LYVE-1)-positive macrophages. *Invest Ophthalmol Vis Sci* 49(12): 5222-9

Schutz FA, Choueiri TK, Sternberg CN (2011) Pazopanib: Clinical development of a potent anti-angiogenic drug. *Crit Rev Oncol Hematol* 77(3): 163-71

Shah N, Pang B, Yeoh KG, Thorn S, Chen CS, Lilly MB, Salto-Tellez M (2008) Potential roles for the PIM1 kinase in human cancer - a molecular and therapeutic appraisal. *Eur J Cancer* 44(15): 2144-51

Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A (2008) Macrophage polarization in tumour progression. *Semin Cancer Biol* 18(5): 349-55

Siironen P, Ristimäki A, Narko K, Nordling S, Louhimo J, Andersson S, Haapiainen R, Haglund C (2006) VEGF-C and COX-2 expression in papillary thyroid cancer. *Endocr Relat Cancer* 13(2): 465-73

Sinicrope FA, Foster NR, Thibodeau SN, Marsoni S, Monges G, Labianca R, Kim GP, Yothers G, Allegra C, Moore MJ, Gallinger S, Sargent DJ (2011) DNA mismatch repair status and colon cancer recurrence and survival in clinical trials of 5-fluorouracil-based adjuvant therapy. *J Natl Cancer Inst* 103(11): 863-75

Sitkovsky MV, Ohta A (2005) The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? *Trends Immunol* 26(6): 299-304

Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, Detmar M (2001a) Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol* 159(3): 893-903

Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M (2001b) Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 7(2): 192-8

Sobrero AF, Maurel J, Fehrenbacher L, Scheithauer W, Abubakr YA, Lutz MP, Vega-Villegas ME, Eng C, Steinhauer EU, Prausova J, Lenz HJ, Borg C, Middleton G, Kroning H, Luppi G, Kisker O, Zubel A, Langer C, Kopit J, Burris HA, 3rd (2008) EPIC: phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer. *J Clin Oncol* 26(14): 2311-9

Sorrentino V, McKinney MD, Giorgi M, Geremia R, Fleissner E (1988) Expression of cellular protooncogenes in the mouse male germ line: a distinctive 2.4-kilobase pim-1 transcript is expressed in haploid postmeiotic cells. *Proc Natl Acad Sci U S A* 85(7): 2191-5

Soumaoro LT, Uetake H, Higuchi T, Takagi Y, Enomoto M, Sugihara K (2004) Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. *Clin Cancer Res* 10(24): 8465-71

Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG (2001) VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med* 7(2): 186-91

Stagg J, Divisekera U, Duret H, Sparwasser T, Teng MW, Darcy PK, Smyth MJ (2011) CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. *Cancer Res* 71(8): 2892-900

Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, Dwyer KM, Smyth MJ (2010) Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci U S A* 107(4): 1547-52

Stagg J, Smyth MJ (2010) Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene* 29(39): 5346-58

Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, Delaney A, Jones SJ, Iqbal J, Weisenburger DD, Bast MA, Rosenwald A, Muller-Hermelink HK, Rimsza LM, Campo E, Delabie J, Braziel RM, Cook JR, Tubbs RR, Jaffe ES, Lenz G, Connors JM, Staudt LM, Chan WC, Gascoyne RD (2010) Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *N Engl J Med* 362(10): 875-85

Stewart BE, Rice RH (1995) Differentiation-associated expression of the proto-oncogene pim-1 in cultured human keratinocytes. *J Invest Dermatol* 105(5): 699-703

Stintzing S, Fischer von Weikersthal L, Decker T, Vehling-Kaiser U, Jäger E, Heintges T, Stoll C, Giessen C, Modest DP, Neumann J, Jung A, Kirchner T, Scheithauer W, Heinemann V (2012) FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer-subgroup analysis of patients with KRAS: mutated tumours in the randomised German AIO study KRK-0306. *Ann Oncol* Epub Jan 4

Straume O, Jackson DG, Akslen LA (2003) Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. *Clin Cancer Res* 9(1): 250-6

Sundlisaeter E, Rosland GV, Hertel JK, Sakariassen PO, Almas B, Dicko A, Sondenaa K (2009) Increased lymphatic vascular density is seen before colorectal cancers reach stage II and growth factor FGF-2 is downregulated in tumor tissue compared with normal mucosa. *Apmis* 117(3): 212-21

Swann JB, Smyth MJ (2007) Immune surveillance of tumors. *J Clin Invest* 117(5): 1137-46

Takayama T, Miyanishi K, Hayashi T, Sato Y, Niitsu Y (2006) Colorectal cancer: genetics of development and metastasis. *J Gastroenterol* 41(3): 185-92

Takedachi M, Qu D, Ebisuno Y, Oohara H, Joachims ML, McGee ST, Maeda E, McEver RP, Tanaka T, Miyasaka M, Murakami S, Krahn T, Blackburn MR, Thompson LF (2008) CD73-generated adenosine restricts lymphocyte migration into draining lymph nodes. *J Immunol* 180(9): 6288-96

Tammela T, Alitalo K (2010) Lymphangiogenesis: Molecular mechanisms and future promise. *Cell* 140(4): 460-76

Tan SY, Fan Y, Luo HS, Shen ZX, Guo Y, Zhao LJ (2005) Prognostic significance of cell infiltrations of immunosurveillance in colorectal cancer. *World J Gastroenterol* 11(8): 1210-4

Tanner M, Gancberg D, Di Leo A, Larsimont D, Rouas G, Piccart MJ, Isola J (2000) Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. *Am J Pathol* 157(5): 1467-72

Taskinen M, Jantunen E, Kosma VM, Bono P, Karjalainen-Lindsberg ML, Leppä S (2010) Prognostic impact of CD31-positive microvessel density in follicular lymphoma patients treated with immunochemotherapy. *Eur J Cancer* 46(13): 2506-12

Terzić J, Grivennikov S, Karin E, Karin M (2010) Inflammation and colon cancer. *Gastroenterology* 138(6): 2101-2114.e5

Thiel A, Mrena J, Ristimäki A (2011) Cyclooxygenase-2 and gastric cancer. *Cancer Metastasis Rev* 30(3-4): 387-95

Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, Morote-Garcia JC, Colgan SP (2004) Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J Exp Med* 200(11): 1395-405

Thompson LF, O'Connor RD, Bastian JF (1984) Phenotype and function of engrafted maternal T cells in patients with severe combined immunodeficiency. *J Immunol* 133(5): 2513-7

Thompson LF, Ruedi JM, Glass A, Low MG, Lucas AH (1989) Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate. *J Immunol* 143(6): 1815-21

Thompson LF, Ruedi JM, O'Connor RD, Bastian JF (1986) Ecto-5'-nucleotidase expression during human B cell development. An explanation for the heterogeneity in B lymphocyte ecto-5'-nucleotidase activity in patients with hypogammaglobulinemia. *J Immunol* 137(8): 2496-500

Thomson LF, Ruedi JM, Glass A, Moldenhauer G, Moller P, Low MG, Klemens MR, Massaia M, Lucas AH (1990) Production and characterization of monoclonal antibodies to the glycosyl phosphatidylinositol-anchored lymphocyte differentiation antigen ecto-5'-nucleotidase (CD73). *Tissue Antigens* 35(1): 9-19

Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, Erdkamp FL, Vos AH, van Groeningen CJ, Sinnige HA, Richel DJ, Voest EE, Dijkstra JR, Vink-Borger ME, Antonini NF, Mol L, van Krieken JH, Dalesio O, Punt CJ (2009) Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. *N Engl J Med* 360(6): 563-72

Tournigand C, André T, Achille E, Lledo G, Flesh M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C, de Gramont A (2004) FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J Clin Oncol* 22(2): 229-37

Tournigand C, Cervantes A, Figer A, Lledo G, Flesch M, Buyse M, Mineur L, Carola E, Etienne PL, Rivera F, Chirivella I, Perez-Staub N, Louvet C, André T, Tabah-Fisch I, de Gramont A (2006) OPTIMOX1: a randomized study of FOLFOX4 or FOLFOX7 with oxaliplatin in a stop-and-Go fashion in advanced colorectal cancer--a GERCOR study. *J Clin Oncol* 24(3): 394-400

Tveit K, Guren T, Glimelius B, Pfeiffer P, Sorbye H, Pyrhonen S, Kure E, Ikdahl T, Skovlund E, Christoffersen T, Nordic Colorectal Cancer Biomodulation Group (2010) Randomized phase III study of 5-fluorouracil/folinate/oxaliplatin given continuosly or intermittently with or without cetuximab, as first-line treatment of metastatic colorectal cancer: the Nordic VII study (NCT00145314), by the nordic colorectal cancer biomodulation group. *35th ESMO congress, LBA20, Annals of oncol 2010 suppl 8, abstract.*

Vakkila J, Lotze MT (2004) Inflammation and necrosis promote tumour growth. *Nat Rev Immunol* 4(8): 641-8

Valdman A, Fang X, Pang ST, Ekman P, Egevad L (2004) Pim-1 expression in prostatic intraepithelial neoplasia and human prostate cancer. *Prostate* 60(4): 367-71

Valtola R, Salven P, Heikkilä P, Taipale J, Joensuu H, Rehn M, Pihlajaniemi T, Weich H, deWaal R, Alitalo K (1999) VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 154(5): 1381-90

Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pinter T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, Schlichting M, Nippgen J, Rougier P (2009a) Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 360(14): 1408-17

Van Cutsem E, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zubel A, Celik I, Rougier P, Ciardiello F (2011) Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 29(15): 2011-9

Van Cutsem E, Labianca R, Bodoky G, Barone C, Aranda E, Nordlinger B, Topham C, Tabernero J, André T, Sobrero AF, Mini E, Greil R, Di Costanzo F, Collette L, Cisar L, Zhang X, Khayat D, Bokemeyer C, Roth AD, Cunningham D (2009b) Randomized phase III trial comparing biweekly infusional fluorouracil/leucovorin alone or with irinotecan in the adjuvant treatment of stage III colon cancer: PETACC-3. *J Clin Oncol* 27(19): 3117-25

Van Cutsem E, Nordlinger B, Cervantes A, Group EGW (2010) Advanced colorectal cancer: ESMO Clinical Practice Guidelines for treatment. *Ann Oncol* 21 Suppl 5: v93-7

Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, Canon JL, Van Laethem JL, Maurel J, Richardson G, Wolf M, Amado RG (2007) Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* 25(13): 1658-64

Van der Auwera I, Cao Y, Tille JC, Pepper MS, Jackson DG, Fox SB, Harris AL, Dirix LY, Vermeulen PB (2006) First international consensus on the methodology of lymphangiogenesis quantification in solid human tumours. *Br J Cancer* 95(12): 1611-25

van der Houven van Oordt CW, Schouten TG, van Krieken JH, van Dierendonck JH, van der Eb AJ, Breuer ML (1998) X-ray-induced lymphomagenesis in E mu-pim-1 transgenic mice: an investigation of the co-operating molecular events. *Carcinogenesis* 19(5): 847-53

van Gijn W, Marijnen CA, Nagtegaal ID, Kranenbarg EM, Putter H, Wiggers T, Rutten HJ, Påhlman L, Glimelius B, van de Velde CJ, Group DCC (2011) Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol* 12(6): 575-82

van Lohuizen M, Verbeek S, Krimpenfort P, Domen J, Saris C, Radaszkiewicz T, Berns A (1989) Predisposition to lymphomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. *Cell* 56(4): 673-82

Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324(5930): 1029-33

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med* 319(9): 525-32

Wang L, Zhou X, Zhou T, Ma D, Chen S, Zhi X, Yin L, Shao Z, Ou Z, Zhou P (2008) Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. *J Cancer Res Clin Oncol* 134(3): 365-72

Wang Z, Bhattacharya N, Weaver M, Petersen K, Meyer M, Gapter L, Magnuson NS (2001) Pim-1: a serine/threonine kinase with a role in cell survival, proliferation, differentiation and tumorigenesis. *J Vet Sci* 2(3): 167-79

Warnecke-Eberz U, Bollschweiler E, Drebber U, Pohl A, Baldus SE, Hoelscher AH, Metzger R (2008) Frequent down-regulation of pim-1 mRNA expression in non-small cell lung cancer is associated with lymph node metastases. *Oncol Rep* 20(3): 619-24

Webster AD, North M, Allsop J, Asherson GL, Watts RW (1978) Purine metabolism in lymphocytes from patients with primary hypogammaglobulinaemia. *Clin Exp Immunol* 31(3): 456-63

Welch S, Spithoff K, Rumble RB, Maroun J, Group GCDS (2010) Bevacizumab combined with chemotherapy for patients with advanced colorectal cancer: a systematic review. *Ann Oncol* 21(6): 1152-62

White E, DiPaola RS (2009) The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res* 15(17): 5308-16

White JD, Hewett PW, Kosuge D, McCulloch T, Enholm BC, Carmichael J, Murray JC (2002) Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. *Cancer Res* 62(6): 1669-75

Wicki A, Christofori G (2007) The potential role of podoplanin in tumour invasion. *Br J Cancer* 96(1): 1-5

Wingett D, Reeves R, Magnuson NS (1992) Characterization of the testes-specific pim-1 transcript in rat. *Nucleic Acids Res* 20(12): 3183-9

Xu D, Allsop SA, Witherspoon SM, Snider JL, Yeh JJ, Fiordalisi JJ, White CD, Williams D, Cox AD, Baines AT (2011) The oncogenic kinase Pim-1 is modulated by K-Ras signaling and mediates transformed growth and radioresistance in human pancreatic ductal adenocarcinoma cells. *Carcinogenesis* 32(4): 488-95

Xu Y, Zhang T, Tang H, Zhang S, Liu M, Ren D, Niu Y (2005) Overexpression of PIM-1 is a potential biomarker in prostate carcinoma. *J Surg Oncol* 92(4): 326-30

Yan G, Zhou XY, Cai SJ, Zhang GH, Peng JJ, Du X (2008) Lymphangiogenic and angiogenic microvessel density in human primary sporadic colorectal carcinoma. *World J Gastroenterol* 14(1): 101-7

Yasuoka H, Nakamura Y, Zuo H, Tang W, Takamura Y, Miyauchi A, Nakamura M, Mori I, Kakudo K (2005) VEGF-D expression and lymph vessels play an important role for lymph node metastasis in papillary thyroid carcinoma. *Mod Pathol* 18(8): 1127-33

Ychou M, Raoul JL, Douillard JY, Gourgou-Bourgade S, Bugat R, Mineur L, Viret F, Becouarn Y, Bouché O, Gamelin E, Ducreux M, Conroy T, Seitz JF, Bedenne L, Kramar A (2009) A phase III randomised trial of LV5FU2 + irinotecan versus LV5FU2 alone in adjuvant high-risk colon cancer (FNCLCC Accord02/FFCD9802). *Ann Oncol* 20(4): 674-80

Yegutkin GG (2008) Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta* 1783(5): 673-94

Yegutkin GG, Henttinen T, Samburski SS, Spychala J, Jalkanen S (2002) The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells. *Biochem J* 367(Pt 1): 121-8

Yegutkin GG, Marttila-Ichihara F, Karikoski M, Niemelä J, Laurila JP, Elima K, Jalkanen S, Salmi M (2011) Altered purinergic signaling in CD73-deficient mice inhibits tumor progression. *Eur J Immunol* 41(5): 1231-41

Yothers G, O'Connell MJ, Allegra CJ, Kuebler JP, Colangelo LH, Petrelli NJ, Wolmark N (2011) Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. *J Clin Oncol* 29(28): 3768-74

Zaki MA, Wada N, Ikeda J, Shibayama H, Hashimoto K, Yamagami T, Tatsumi Y, Tsukaguchi M, Take H, Tsudo M, Morii E, Aozasa K (2011) Prognostic implication of types of tumor-associated macrophages in Hodgkin lymphoma. *Virchows Arch* 459(4): 361-6

Zernecke A, Bidzhekov K, Ozüyaman B, Fraemohs L, Liehn EA, Lüscher-Firzlaff JM, Lüscher B, Schrader J, Weber C (2006) CD73/ecto-5'-nucleotidase protects against vascular inflammation and neointima formation. *Circulation* 113(17): 2120-7

Zhang BC, Gao J, Wang J, Rao ZG, Wang BC, Gao JF (2011) Tumor-associated macrophages infiltration is associated with peritumoral lymphangiogenesis and poor prognosis in lung adenocarcinoma. *Med Oncol* 28(4): 1447-52

Zhong D, Li Y, Peng Q, Zhou J, Zhou Q, Zhang R, Liang H (2009) Expression of Tiam1 and VEGF-C correlates with lymphangiogenesis in human colorectal carcinoma. *Cancer Biol Ther* 8(8): 689-95

Zhou Q, Peng RQ, Wu XJ, Xia Q, Hou JH, Ding Y, Zhou QM, Zhang X, Pang ZZ, Wan DS, Zeng YX, Zhang XS (2010) The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. *J Transl Med* 8: 13

Zhou X, Zhi X, Zhou P, Chen S, Zhao F, Shao Z, Ou Z, Yin L (2007) Effects of ecto-5'-nucleotidase on human breast cancer cell growth in vitro and in vivo. *Oncol Rep* 17(6): 1341-6

Zimmermann H (1992) 5'-Nucleotidase: molecular structure and functional aspects. *Biochem J* 285 (Pt 2): 345-65

Zippo A, De Robertis A, Bardelli M, Galvagni F, Oliviero S (2004) Identification of Flk-1 target genes in vasculogenesis: Pim-1 is required for endothelial and mural cell differentiation in vitro. *Blood* 103(12): 4536-44

Zlobec I, Minoo P, Terracciano L, Baker K, Lugli A (2011) Characterization of the immunological microenvironment of tumour buds and its impact on prognosis in mismatch repair-proficient and -deficient colorectal cancers. *Histopathology* 59(3): 482-95