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STROMA AND VESSEL CHARACTERISTICS IN CANCER; IMPACT ON PROGNOSIS AND RESPONSE TO TREATMENT

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STROMA AND VESSEL CHARACTERISTICS IN CANCER; IMPACT ON PROGNOSIS AND RESPONSE TO TREATMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my parents

Somewhere, something incredible is waiting to be known

Carl Sagan

ABSTRACT

A series of pre-clinical and clinical studies imply vessel and pericyte status as determinants of tumor growth, metastasis and response to treatment. These studies thus imply biomarker-potential of these features. Earlier studies of vessels and pericytes have largely applied semi-quantitative approaches. In the studies of this thesis, novel tools for quantification of vessel- and tumor stroma-related features were developed and applied to different sets of clinically well-annotated tumor collections.

Analyses of perivascular status in ovarian cancer and colorectal cancer identified independently expressed marker-defined subsets of perivascular cells with differential associations with survival. These studies also identified novel significant associations between specific oncogenic mutations and vascular phenotypes.

Studies analyzing stage II/III colon cancer samples, derived from a randomized adjuvant study, identified two novel stroma-related "metrics" that acted as markers for benefit of adjuvant chemotherapy. Firstly, high vessel density in the invasive region, but not tumor center, was identified as a marker that characterized patients benefiting from adjuvant treatment. Secondly, a digital-image-analyses-derived "metric", related to high complexity of the tumor stroma interface, also defined a group showing benefit of adjuvant treatment. Notably, both novel markers showed statistically significant interactions with treatment supporting their relevance as response-predictive markers.

Furthermore, cell-type-specific analyses of claudin-2 expression in colorectal cancer indicated that CAF-expression of this marker was specifically associated with benefit of oxaliplatin-based treatment for metastatic colorectal cancer.

Taken together, our findings suggest continued exploration and validation of stroma-derived features, for development of clinically meaningful prognostic and response-predictive tissue-based biomarkers.

LIST OF SCIENTIFIC PAPERS

I. **Mezheyeuski A**., Nerovnya A., Bich T., Tur G., Ostman A. and Portyanko A. (2015). ***Inter- and intra-tumoral relationships between vasculature characteristics, glut1 and budding in colorectal carcinoma***. Histology and histopathology 30, 1203-1211. Epub 2015 Mar 26

II. **Mezheyeuski A**., Lindh M.B.*, Guren T.K.*, Dragomir A., Pfeiffer P., Kure E.H., Ikdahl T., Skovlund E., Corvigno S., Strell C., Pietras K., Ponten F., Mulder J., Qvortrup C., Portyanko A., Tveit K.M., Glimelius B.#, Sorbye H.# and Ostman A. (2016). ***Survival-associated heterogeneity of marker-defined perivascular cells in colorectal cancer***. Oncotarget. [Epub ahead of print].

III. Corvigno S., Wisman G.B., **Mezheyeuski A**., van der Zee A.G., Nijman H.W., Avall-Lundqvist E., Ostman A. and Dahlstrand H. (2016). ***Markers of fibroblast-rich tumor stroma and perivascular cells in serous ovarian cancer: Inter- and intra-patient heterogeneity and impact on survival***. Oncotarget 7, 18573-18584.

IV. **Mezheyeuski A**., Hrynchyk I., Karberg M., Portyanko A., Ragnhammar P., Edler D., Glimelius B., Östman A. ***Image-analyses-quantitated tumor morphological complexity predicts prognosis and response to treatment in stage II-III colon cancer.*** Submitted

V. **Mezheyeuski A**., Hrynchyk I., Portyanko A., Ragnhammar P., Edler D., Glimelius B., Östman A. ***Vessel density in colon cancer: a predictive marker of benefit from adjuvant fluorouracil-based chemotherapy.*** Manuscript

VI. **Mezheyeuski A**., Strell C.*, Hrynchyk I.*, Kyrre T.K., Dragomir A., Pfeiffer P., Kure E.H., Sorbye H., Portyanko A., Glimelius B., Östman A. ***Survival-associated heterogeneity of claudin-2 expression in colon cancer.*** Manuscript

* equal contribution
# equal contribution

CONTENTS

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ALK	Anaplastic lymphoma receptor tyrosine kinase
APC	Adenomatous polyposis coli
ASCO	American Society of Clinical Oncology
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CAF	Cancer-associated fibroblast
CEA	Carcinoembryogenic antigen
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subtyping
CRC	Colorectal cancer
CSS	Cancer specific survival
DNA	Deoxyribonucleic acid
DSS	Disease specific survival
EDG1	Endothelial differentiation sphingolipid G-protein–coupled receptor 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ESMO	European Society for Medical Oncology
FAP	fibroblast-activating protein
FGF	Fibroblast growth factor
FU	Fluorouracil
HGF	Hepatocyte growth factor
HNPCC	Hereditary nonpolyposis colorectal cancer
IBD	Inflammatory bowel disease
IFP	Interstitial hypertension
IGF-1	Insulin like growth factor 1
IL	Interleukin
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-activated protein kinases

mCRC	Metastatic colorectal cancer
MSC	Mesenchymal stem cell
MSI	Microsatellite instability
MSS	Microsatellite stable
MVD	Microvessel density
MYC	Avian myelocytomatosis viral oncogene homolog
NK	Natural killers
OS	Overall survival
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PFS	Progression-free survival
PGE	prostaglandin E
PI3K	Phosphoinositide 3-kinase
PVC	Perivascular cell
S1P1	Sphingosine-1-phosphate-1
SDF-1	Stromal cell-derived factor 1
TGF	Transforming growth factor
TKI	Tyrosine kinase inhibitor
TNM	Tumor node metastasis
UICC	Union Internationale Contre le Cancer
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WNT	Wingless and INT
α-SMA	Anti-alpha smooth muscle Actin

1 COLORECTAL CANCER

1.1 GENERAL INTRODUCTION

Colorectal cancer (CRC) is a malignant tumor starting in the large intestine and rectum. More than 90% of colorectal carcinomas are adenocarcinomas, which originate from the epithelial cells of the colorectal mucosa (Sobin, Gospodarowicz, Wittekind, & International Union against Cancer., 2010). The current review will focus on this entity.

CRC is one of the most common malignant diseases in developed countries. In Europe 446000 new cases of CRC are diagnosed and 214000 deaths were registered during 2013 (Altobelli, Lattanzi, Paduano, Varassi, & di Orio, 2014). The etiology of CRC includes both genetic and environmental factors. Around 20% of CRC are linked to heritable gene variations (Grivennikov, 2013). CRC is also related to inflammatory bowel diseases (Terzic, Grivennikov, Karin, & Karin, 2010).

Staging of CRC is traditionally anatomically based. The most common system in practical usage is the TNM system, suggested by the American Joint Committee on Cancer (AJCC), which characterizes depth of tumor growth into the intestinal wall and adjacent structures (T), regional lymph node status (N) and presence of distant metastases (M). During tissue dissection at least 12 lymph nodes should be examined for adequate pN-staging (Hari et al., 2013). The TMN data is then combined into an overall Union Internationale Contre le Cancer (UICC) stage definition (Sobin et al., 2010). In addition, parameters such as grading, lymphatic, venous or perineural invasion, lymphoid inflammatory response as well as an involvement of resection margins have an important prognostic significance. For the mCRC an expanded RAS analyses was recommended by ESMO for the patients considered for EGFR therapy. Similarly, assessment of tumor MSI status is recommended (Van Cutsem et al., 2016).

1.2 RISK FACTORS

Despite the fact that the cause of CRC often remains unknown, distinct factors may increase the risk of its development.

If a first-degree relative has been diagnosed with colon cancer or colorectal polyps before the age of 60 or if two or more first-degree relatives have been diagnosed with colon cancer or colon polyps at any age, then the person is assumed to have family history of colon cancer, and significantly increased risk to develop CRC (Fuchs et al., 1994).

Behavioral/Lifestyle factors linked to cancer risk include obesity, diabetes, high consumption of alcohol, red meat and long-term cigarette smoking (Jarvinen, Knekt, Hakulinen, Rissanen, & Heliovaara, 2001; Kampman et al., 1999; Nguyen, Bent, Chen, & Terdiman, 2009).

Other known adverse risk factors are inflammatory bowel disease, high age, oriental race and male gender (Bernstein, Blanchard, Kliewer, & Wajda, 2001; Hahn, 1990; Jess, Rungoe, & Peyrin-Biroulet, 2012; Kaminski et al., 2014).

3-5 % of CRC are associated with the known hereditary syndromes Lynch syndrome/hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) (Abdel-Rahman, Mecklin, & Peltomaki, 2006; Burton, Hovick, & Peterson, 2012). Some rare syndromes, like Turcot, Muir-Torre, Peutz-Jeghers and juvenile polyposis syndromes are also associated with increased risk of CRC.

1.3 HISTOPATHOLOGY OF COLORECTAL ADENOCARCINOMA

The standard colorectal cancer histopathology reports contain a list of microscopic core data items, which are:

a. Histological tumour type.
b. Histological differentiation.
c. Maximum extent of local invasion (pT stage) and maximum distance of extramural spread.
d. Grade of tumour regression following pre-operative (neoadjuvant) therapy.
e. Resection margins (longitudinal and circumferential margins).
f. Lymph node status (number present, number involved, highest lymph node status).
g. Venous invasion.
h. Histologically confirmed distant metastatic disease.
i. Separate abnormalities.

The vast majority of all colorectal cancers are adenocarcinomas (also referred as 'adenocarcinomas not otherwise specified'). Other forms are relatively rare: mucinous carcinoma, signet ring cell carcinoma, adenosquamous carcinoma, primary squamous carcinoma, goblet cell carcinoids and mixed adenocarcinoma-neuroendocrine carcinoma, medullary carcinoma, undifferentiated carcinoma (Sobin et al., 2010).

Adenocarcinomas are gland-forming lesions. The extent of glandular appearances is a basis for tumor morphological grading. Adenocarcinomas has traditionally been divided into well, moderately and poorly differentiated. An alternative two-tiered grading system has been introduced, and presently recommended, and is composed of low grade (50% gland formation) and high grade (<50% gland formation) tumors (Sobin et al., 2010).

Mucinous adenocarcinoma is a type of colorectal carcinoma which is defined by having 50% of the tumor mass composed of extracellular mucin. They constitute about 10-15% of colorectal adenocarcinoma, and are most common in the right colon (Hugen, Brown, Glynne-Jones, de Wilt, & Nagtegaal, 2016). This histological sub-type is common in patients with inflammatory bowel diseases (IBD), such as Crohn's disease or ulcerative colitis (Hugen, van Beek, de Wilt, & Nagtegaal, 2014). Mucinous adenocarcinomas are often observed in Lynch syndrome patients and are microsatellite instable (MSI) (Hugen et al., 2015). They have a lower tendency to recur after surgery and longer overall survival (OS) (Hogan et al., 2014). In contrast, microsatellite stable (MSS) mucinous carcinomas more often disseminate (Nitsche et al., 2013; Papadopoulos et al., 2004). According to the WHO criteria, mucinous adenocarcinomas are considered poorly differentiated.

Signet-ring cell adenocarcinoma (<1 % of adenocarcinomas) is a type of CRC defined by 50% of tumor cells showing signet ring cell feature, which is represented by a prominent intracytoplasmic mucin deposition (H. Kang, O'Connell, Maggard, Sack, & Ko, 2005). Signet-ring cell adenocarcinomas are by definition poorly-differentiated and have bad prognosis (H. Kang et al., 2005).

Serrated adenocarcinoma (10 % of adenocarcinomas) arises from serrated precursor lesions, and is characterized by serrated morphology, mucinous differentiation, association with BRAF mutations, eosinophilic cytoplasm, vesicular nuclei and absence of dirty necrosis (Makinen et al., 2001). The prognosis is dependent on the molecular profile of the tumor. BRAF mutation and MSS cancers as well as proximal location, high tumor budding, lymphatic and perineural invasion are linked to poor prognosis in serrated adenocarcinoma (Pai et al., 2012; Samowitz et

al., 2005). In contrast, serrated cancers with MSI-H phenotype have a favorable prognosis (Samowitz et al., 2001).

Adenosquamous carcinoma and primary squamous carcinoma are very unusual types of CRC. Squamous epithelium can be differentiated from the mucosa near the dentate line, but the pathogenesis of squamous cell carcinoma in colon remains unclear (Frizelle, Hobday, Batts, & Nelson, 2001).

Medullary carcinoma is a distinctive type of CRC, which is associated with MSI-H phenotype. Morphologically it is poorly differentiated large cell carcinoma, which is build by closely packed nests and characterized by prominent infiltration by lymphocytes and good circumscription (Jass, 2007; Sobin et al., 2010).

1.4 MOLECULAR CLASSIFICATION OF CRC

A series of different molecular classification schemes for CRC have been proposed during the past decade. Among the most established molecular subgroups are the chromosomal instable group (CIN), the microsatellite instable (MSI) group and the group with CpG island methylator phenotype (CIMP) (Jass, 2007; Kanthan, Senger, & Kanthan, 2012).

CIN is the most common subtype of genetic instability in CRC (around 84%). It includes alterations in chromosomal number, chromosome rearrangements as well as chromosomal sequence changes and segregation defects. Almost all such tumors have a mutation in the APC/beta- catenin pathway, and 70% of CIN classified CRC have loss of 18q and deletion of SMAD2, SMAD4, or DCC, and 50% have KRAS mutations (Kanthan et al., 2012; Pino & Chung, 2010).

Around 15% of sporadic CRCs and most of Lynch syndrome-associated HNPCC are characterized by MSI (Moreira et al., 2012). MSI tumors are characterized by genomic alterations that result in defects in DNA mismatch repair enzymes and thus more deletion/insertion errors occur during DNA replication. EGFR, BAX, TGFbetaRII and BRAF are frequently mutated in these tumors (French et al., 2008). MSI cancers are poorly differentiated, mucinous, with marked intra-tumoral lymphatic infiltration, Crohn's-like peritumoral inflammation and have a low tendency to metastasize (Samowitz et al., 2001). If metastatic, they often have an aggressive clinical course.

CpG island methylation in CRC results in transcriptional silencing of tumor suppressor genes, which encode proteins controlling apoptosis, DNA repair, and cell-cycle (most often p16, MGMT, and hMLH1) (A. Goel et al., 2007). CIMP tumors can either be MSI or MSS.

During the past 25 years efforts to characterize the molecular pathways dysregulated in CRC have been made. Several critical pathways have been identified: WNT, RAS−MAPK, PI3K, TGF-β, P53 and DNA mismatch-repair pathways. Based on molecular characterization, including gene expression analyses, three to six molecular subtypes of CRC have been described (Cancer Genome Atlas, 2012; Jass, 2007; G. H. Kang, 2011; Marisa et al., 2013; Shen et al., 2007) and the most recent consensus classification (Guinney et al, Nat Med 2015). .

Tumors have also broadly been grouped based on the number of non-silent somatic mutations. Hyper-mutated tumors are characterized by MSI and frequent BRAF mutation. Among the rest, common molecular alterations include KRAS, APC, TP53, SMAD4 and PIK3CA (Cancer Genome Atlas, 2012; Kaemmerer, Klaus, Jeon, & Gassler, 2013).

In 2015 the Colorectal Cancer Subtyping Consortium generated a consensus molecular subtyping model (CMS) (Guinney et al., 2015). Four distinct subsets were defined by the unsupervised clustering of gene expression profiles from 4151 samples. CMS1 was largely consistent with previously described MSI-phenotype and 14 % of the patients. CMS2 group (37%) was characterized by MSS status, CIN, strong activation of WNT/MYC pathway, TP53 mutation and overexpression of EGFR. CMS1 cluster (13%) demonstrated frequent KRAS and PIK3CA mutations, low CIN and moderate activation of WNT/MYC pathway. CMS4 cluster (13%) was heterogeneous for CIN/MSI, had activated mesenchymal/TGF-beta pathway and NOTCH3/VEGFR2 overexpression.

1.5 PROGNOSTIC FACTORS IN CRC

There is a list of well-established prognostic factors in CRC. TNM (tumor node metastasis) staging remains the gold standard in clinical practice. Venous and lymphatic invasion, tumor grade, tumor budding and tumor border configuration are also clinically implemented prognostic factors (Zlobec & Lugli, 2008).

Most recently, the expression of specific oncogenes or loss of expression of tumor suppressor genes, such as KRAS, p53, and general genomic features such as CIN, MSI, CIMP have

invariably been linked to prognosis. Among these, only MSI has reproducibly been shown to act as an independent good prognostic factor for CRC patients (Hutchins et al., 2011; Popat, Hubner, & Houlston, 2005).

Marked T-lymphocyte infiltration, which is part of a tumour-specific host response, is believed to contribute to immune surveillance. Recent studies have shown associations between T-cell infiltration and good prognosis (Pages et al., 2005). Moreover, the prognostic significance of immune cell density and location may be even higher than classical TNM staging system (Galon et al., 2014; Mlecnik et al., 2016; Mlecnik, Bindea, Pages, & Galon, 2011). Clustering of early stage patients with regard to CD8+ and CD45RO+ cells presence in specific tumor regions revealed dramatic difference between groups in disease-free survival (DFS), disease specific survival (DSS) and OS, such that best survival rates was observed in the group of patients with high density of CD8+ and CD45RO+ cells (Pages et al., 2009).

1.6 TREATMENT AND PREDICTIVE FACTORS

Treatment for stage I-III colon cancer includes surgery with complete removal of the primary tumor and the regional lymph nodes. The 5-year survival for early-stage colon cancer in lymph node-negative cases (stage I-II) is as high as 80-90%. It is reduced however to 50-65% if lymph node metastases are present (stage III) (Bockelman, Engelmann, Kaprio, Hansen, & Glimelius, 2015; Zlobec & Lugli, 2008).

Stage I cases do not need adjuvant chemotherapy (Labianca et al., 2013).

It remains a subject of discussion if patients with lymph-node-negative disease could benefit from adjuvant chemotherapy (Bockelman et al., 2015; Dotan & Cohen, 2011; Fang, Efron, Berho, & Wexner, 2014). Adjuvant chemotherapy is normally not recommended for stage II patients (Labianca et al., 2013). It is however assumed that there are a number of high-risk patients in stage II group that could benefit from adjuvant treatment. Such high-risk criteria include number of sampled lymph nodes, low tumor differentiation and perforation of T4 stage (ASCO recommendations (Benson et al., 2004)). According to ESMO recommendations, high-risk criteria include also vascular invasion, lymphatic or perineural invasion, obstruction and high levels of carcinoembryonic antigen (Schmoll et al., 2012).

Adjuvant chemotherapeutic regimens for CRC are based on 5-fluorouracil (FU) therapy, which acts through a metabolite-mediated inhibition of thymidylate synthase. Fluorouracil is presently used together with leucovorin (LV), which increases remission rates and improves survival (Arkenau et al., 2003; Loffler et al., 1992). The MOSAIC study demonstrated the improvement in OS by the addition of oxaliplatin to 5-FU/LV (FOLFOX schema) (Andre et al., 2015). Another regimen, recommended by ESMO is XELOX (adjuvant capecitabine plus oxaliplatin) which has been shown to improve OS in stage III CRC (Schmoll et al., 2015).

Stage III patients generally receive adjuvant chemotherapy. The standard of the care of such patients after the surgical removal of the tumor include combination therapy with fluoropyrimidine and oxaliplatin (Andre et al., 2015). It is recognized, that a group of stage III patients with low-risk disease may not need addition of oxaliplatin to FU-based treatment. The criteria for the selection of such low-risk stage III group are still to be defined (Yamanaka et al., 2016).

Up to 25% of patients with CRC have synchronous distant metastases already at the time of diagnosis. Further, another 20-25% of the patients develop distance metastases at some point after (Andres et al., 2012; Cardona et al., 2013; Leporrier et al., 2006; Mayo et al., 2011). The only curative treatment for such patients includes surgical resection of the distant metastases which can lead to 5-year survival varying from 25 to 74% (House et al., 2010; Kanas et al., 2012). Surgical metastasectomy is not always possible, although practiced more and more (Adam et al., 2012).

5-FU and capecitabine are the main agents used in patients with metastatic CRC (mCRC) in combination with oxaliplatin (DNA replication and mRNA synthesis inhibitor) or irinotecan (DNA replication and transcription inhibitor) in regimens designated FOLFOX/CAPOX or FOLFIRI/CAPIRI, respectively (Cartwright, 2012; Jensen et al., 2012). Therapeutical treatment of mCRC may include targeted agents to block EGFR-receptor signaling or angiogenesis. cetuximab and panitumumab are EGFR inhibitors, which are approved in monotherapy or together with chemotherapy. Treatment is restricted to RAS wild-type tumors since KRAS mutated phenotype makes tumors resistant to anti-EGFR treatment (Amado et al., 2008; Douillard et al., 2013; Karapetis et al., 2008; Peeters et al., 2013). Another targeted therapy agent, bevacizumab targeting VEGF, has been approved for the use as first- and second-line treatment for mCRC in combination with chemotherapy (See part IV for more details). More recently, also other agents targeting angiogenesis have been approved (aflibercept and

ramucirumab) (See part IV). Regorafenib, a small molecule inhibiting angiogenesis, is used in patients with previously treated mCRC (See part IV).

2 FIBROBLASTS AND VASCULATURE IN CANCER DEVELOPMENT

Tumor behavior is governed not only by the genetic status of the malignant cells bit also by interactions between the malignant cells and cells of the tumor microenvironment. This chapter discusses the roles of fibroblasts and tumor vasculature in tumor progression as inferred from studies in different cancer models.

2.1 CANCER-ASSOCIATED FIBROBLASTS

Fibroblasts are the most abundant cells in the tumor stroma, and have been named carcinoma-associated fibroblasts or cancer-associated fibroblasts (CAFs). CAFs have phenotypic characteristics, which are different from normal fibroblasts. Recent studies have demonstrated functionally important roles of CAFs in many aspects of tumor biology.

2.1.1 Origin of CAFs and their characteristics

The origin of CAFs is unclear. The main suggested source of CAFs is tissue residing fibroblasts, whose transition is induced by TGF-β (C. R. Zhu, Guan, & Wu, 2000). Certain amount of the TGF-β is secreted by cancer cells, however, CAFs are known to be capable to secrete TGF-β themselves, creating a positive feedback loop (Kojima et al., 2010). Alternatively CAFs may develop from pericytes (Tubbs, Mortazavi, Shoja, Loukas, & Cohen-Gadol, 2012), hematopoietic stem cells (Speerschneider & Thomsen, 2013), adipose-derived stem cells (de Jongh, Bosma, Leenen, & Verhofstad, 2011), endothelial cells (S. Zhu, Bjorge, & Fujita, 2007) or bone marrow -derived mesenchymal stem cells (Petru, Jac, Sindelkova, & Polasek, 2011). Some studies have also implied cancer cells undergoing EMT as sources of CAFs. However, this notion has not been strongly supported by analyses of clinical samples

(Hsia & Marchlinski, 2002; Iwano et al., 2002; Kim et al., 2006; Radisky, Kenny, & Bissell, 2007; Zeisberg et al., 2003; Zeisberg & Kalluri, 2004).

In line with the concept of the tumor as "a wound that never heals" (Dvorak, 1986), CAFs share many features of activated fibroblasts in a healing wound including expression of marker proteins such as α-SMA and an increased production of extracellular matrix proteins (reviewed in (Kalluri, 2016)).

CAFs are heterogeneous and may express markers such as α-SMA, vimentin, endosialin, podoplanin, FSP-1, fibroblast-activating protein (FAP), platelet-derived growth factor receptors alpha and beta (PDGFR-α and PDGFR-β) (Anderberg et al., 2009; De Wever, Demetter, Mareel, & Bracke, 2008; Feig et al., 2013; Roberts et al., 2013; Sharon, Alon, Glanz, Servais, & Erez, 2013; Spaeth et al., 2009; Sugimoto, Mundel, Kieran, & Kalluri, 2006; Trimboli et al., 2009).

2.1.2 Roles of CAFs in tumor initiation and growth

CAFs are capable to stimulate tumor cell proliferation through secretion of various growth factors, hormones and cytokines (Ostman & Augsten, 2009). Among these are fibroblast growth factor (FGF), insulin like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and TGF-β (Cirri & Chiarugi, 2011; Kalluri & Zeisberg, 2006). Altered TGF-β-signaling in fibroblasts has been demonstrated to induce oncogenic transformation of adjacent epithelial cells and resulted in developing intra-epithelial neoplasia in the prostate and invasive squamous-cell carcinoma in the forestomach (Bhowmick et al., 2004). Not only tumor initiation but also tumor growth is supported by CAFs. CAFs, but not their normal counterparts, induced increased growth and morphological changes in immortalized prostatic epithelial cells (Olumi et al., 1999). Similarly, human CAFs promoted the growth of breast carcinoma cells in a xenograft tumor model, while this effect was not observed with normal mammary fibroblasts. The tumor growth was stimulated by paracrine effect of stromal cell-derived factor 1 (SDF-1), secreted by CAFs. Interestingly, SDF-1 was also involved in promoting tumor angiogenesis (Orimo et al., 2005).

2.1.3 CAFs and immune cell interactions

CAFs are important regulators of the tumor-initiated immune response of the host tissue. Findings supporting this concept were obtained in a study by Kraman et al using a syngeneic cancer model, allowing depletion of FAP-positive fibroblasts. Interestingly, vaccine-induced immune response and subsequent hypoxic necrosis of the tumor were observed exclusively in FAP-depleted animals. This process was dependent on interferon-γ and tumor necrosis factor-α. The study suggests a suppressive effect of the FAP-positive fibroblasts on the function of the adaptive immunity (Kraman et al., 2010). Another approach to target FAP was implemented by Wen and colleagues who applied a DNA vaccine directed against FAP. Vaccine treatment suppressed the growth of the murine model of colon cancer through a CD8(+) T-cell-dependent mechanism (Wen et al., 2010). Taken together these results imply immune-suppressive and tumor-supporting role of FAP-positive CAFs.

Another immune-modulatory mechanism involves the secretion of the TGF-β by CAFs. TGF-β secretion leads to immune suppression by activation of T-regulatory cells and inhibition of cytotoxic lymphocytes. TGF-β also regulates migration and activation of monocytes, macrophages, T-cells, NK cells and dendritic cells (Gruber, Marchese, & Kew, 1994; M. O. Li, Wan, Sanjabi, Robertson, & Flavell, 2006; Maghazachi & al-Aoukaty, 1993; Wrzesinski, Wan, & Flavell, 2007). TGF-β blockade in animal models increases efficacy of the T-cell receptor gene therapy (Bendle, Linnemann, Bies, Song, & Schumacher, 2013).

2.1.4 Roles of CAFs in metastasis

Metastasis formation is a multi-step process, which still remains poorly understood. CAFs have been shown to be involved in the metastatic process both through effects in the primary tumor and at the metastatic site.

During the first steps of the metastatic process cancer cells need to invade into peritumoral tissue and through the vessel wall (intravasation). Mesenchymal stem cells, used as CAF models, could promote metastasis in a co-injection breast cancer model by secretion of CCL5 (Karnoub et al., 2007). It has also been shown that fibroblasts can release exosomes, which mobilize pro-metastatic autocrine Wnt-PCP (WNT/planar cell polarity) signaling in tumor cells (Luga et al., 2012). Pro-migratory and invasive effects have also been demonstrated by PDGF-stimulated fibroblasts in studies combining tissue cultures with animal experiments (Pena et al.,

2013). CAFs may facilitate the invasiveness on the cancer cells not only by paracrine interaction but also by establishing tracks in the extracellular matrix and acting as leading cells in a process called collective migration (Gaggioli et al., 2007).

Fibroblasts are involved also in the formation of the metastatic niche. Breast cancer cells need periostin to be present in lung stroma in order to metastasize there (Malanchi et al., 2012). Periostin is found also in primary tumor stroma produced by CAFs and its expression in lung stroma can be induced by infiltrating breast cancer cells (Malanchi et al., 2012). S100A4(+) fibroblasts have been shown to needed for the production of the vascular endothelial growth factor (VEGF)-A and tenascin in order to establish an angiogenic and anti-apoptotic microenvironment at the metastatic site (O'Connell et al., 2011). Recently, it was reported that in a metastatic lung cancer model, tumor cells bring their primary stromal components to the metastatic site, which increase early metastatic growth (Duda et al., 2010). Similar findings have been made in a study where pancreatic stellate cells and pancreatic carcinoma cells were co-injected orthotopically in mice and the pancreatic stellate cell were found within the formed metastatic lesions (Xu et al., 2010).

2.2 CANCER-ASSOCIATED FIBROBLASTS IN CRC

The fibroblasts in the lamina propria of the normal colon are α-SMA negative. In the narrow pericriptal area however different types of cells are observed. They express α-SMA, smooth muscle myosin and vimentin but not desmin, which defines them as myofibroblasts (Adegboyega, Mifflin, DiMari, Saada, & Powell, 2002; Martin, Pujuguet, & Martin, 1996).

When the hyperplastic or adenomatous polyps develop the stromal fibroblasts of lamina propria also became α-SMA-positive (Adegboyega et al., 2002). Finally, in the stroma of the primary and metastatic CRC the myofibroblast-like cells build the major fraction of the stromal compartment (Herrera et al., 2013; Mueller et al., 2007).

The origin of the CAFs in CRC is similar to that of other solid cancers. The main source is probably residing fibroblasts. They get differentiated into CAFs upon stimulation with TGF-β, platelet-derived growth factor (PDGF), IL-4, IL-6, IGF-II, and prostaglandin E (PGE) (Conti & Thomas, 2011; Hawinkels et al., 2014; Kaler, Owusu, Augenlicht, & Klampfer, 2014; Peddareddigari, Wang, & Dubois, 2010). Mesenchymal stem cells have also been implied as a source of CRC CAFs (De Boeck et al., 2013; Peddareddigari et al., 2010). Other cell types

could also act as the source of CAFs in CRC, including adipocytes, hematopoietic stem cells and pericytes (Conti & Thomas, 2011; Kaler et al., 2014).

Colon CAFs play a major role in the development of the tumor desmoplasia (prominent fibrosis) and secrete factors such as HGF, EGF, IGF1/2, PDGF, PGE-2, FGF-1, and VEGF (De Boeck et al., 2013; Nakagawa et al., 2004; Peddareddigari et al., 2010). These factors mediate cancer cell proliferation, survival and invasion. Proteomic analysis has identified CAFs-associated pro-inflammatory and desmoplastic signatures in CRC (Torres et al., 2013).

Some experimental studies have demonstrated pro-metastatic effects of CAFs in CRC models. TGF-β production is frequently unregulated in advanced CRC, which in turn activate CAF autocrine regulatory loop and initiate the secretion of TGF-β1 by CAFs (Hawinkels et al., 2014). The TGF-β signaling by stromal sells increases the efficacy of metastatic formation (Calon et al., 2012). Activation of PDGF-β receptors in CAFs promotes migration and invasion of co-cultured CRC cells through PDGF-induced production and secretion of glycoprotein stanniocalcin-1. This pathway activation has been associated with metastases and progression in a CRC xenograft model (Pena et al., 2013).

It has also been shown that bone marrow-derived mesenchymal stem cells (MSCs) are capable to migrate towards the tumor stroma and to differentiate into CAFs (Shinagawa et al., 2010). Co-injection of MSCs in mice together with CRC cells resulted in increased tumor growth and metastasis formation, which could be prevented through PDGF-β receptor blockade by imatinib (Shinagawa et al., 2013).

2.3 CANCER-ASSOCIATED FIBROBLASTS AND RESPONSE TO TREATMENT

2.3.1 Drug uptake

Efficacy of systemic chemotherapy requires that the active substance is delivered to the cancer cells. One of the barriers for tumor drug delivery is the tumor interstitial hypertension (IFP), which prevents transvascular transport (Curti, 1993; Heldin, Rubin, Pietras, & Ostman, 2004). Reduction of IFP by enzymatic ablation of fibroblast-derived hyaluronan re-expanded the vasculature and improved drug delivery (Provenzano et al., 2012). Similarly, usage of different PDGF-β receptor-antagonists, targeting CAFs, reduced IFP and improved transcapillary

transport and tumor uptake of cytotoxic drugs and radioimmunotherapeutic antibodies (Baranowska-Kortylewicz et al., 2005; Pietras et al., 2001). In similar animal experiments, inhibition of PDGF receptor signaling improved the therapeutic effects of paclitaxel and 5-fluorouracil (Pietras et al., 2002). The notion of fibroblast-targeting as a mean to improve tumor drug-uptake has also been supported by studies using hedgehog-inhibitors targeting the stroma in models of pancreatic cancer (Olive et al., 2009).

2.3.2 Drug sensitivity

In addition to modulation of drug uptake, CAFs can also negatively affect drug sensitivity by providing paracrine signals which reduce the cancer cells' drug sensitivity (Johansson et al., 2012; Ostman, 2012; Pontiggia et al., 2012; Straussman et al., 2012). Specific effects of CAFs on the chemotherapy sensitivity of cancer-initiating cells in CRC have also been demonstrated through mechanisms involving secretion of specific cytokines and chemokines, including interleukin-17A (Lotti et al., 2013). Furthermore, HGF secreted by CAFs protects cancer cells treated with cetuximab (Liska, Chen, Bachleitner-Hofmann, Christensen, & Weiser, 2011). In agreement with these model findings, serum levels of HGF has been suggested as an indicator of the sensitivity to the anti-EGFR treatment in KRAS wild-type mCRC (Takahashi et al., 2014). CXCL12 and CXCR4-positive cancer cells is thought to have a protective effect against cytotoxic drugs in prostate cancer and acute myeloid leukemia (Domanska et al., 2012; Ishikawa et al., 2007; Nervi et al., 2009).

Moreover, it has been shown that the application of BRAF, ALK or EGFR kinase inhibitors initiates a damage response and the production of a so-called therapy-induced secretome. This leads to the establishment of a tumor microenvironment, which facilitate the development and expansion of drug-resistant clones of cancer cells (Obenauf et al., 2015). Furthermore, CXCL12 and CXCR4-positive cancer cells have a protective effect against cytotoxic drugs in prostate cancer and acute myeloid leukemia (Domanska et al., 2012; Ishikawa et al., 2007; Nervi et al., 2009).

Concerning effects of immune-therapy, FAP-positive fibroblasts have been shown to mediate tumor resistance to anti-PD-L1 therapy in pancreatic cancer mice model. The immunosuppression driven by FAP-positive CAFs was CXCL12/CXCR4-dependent and could therefore be prevented by CXCR4 receptor inhibitor (Feig et al., 2013).

2.3.3 Radiotherapy

CAFs are also able to modulate the sensitivity to radiotherapy. In agreement with experimental findings, the expression of FAP-α and SDF-1 predicted tumor re-growth and recurrence in patients with rectal cancer after pre-operative chemo-radiation therapy (Saigusa et al., 2011). In another study the resistance to radiotherapy in rectal cancer was also predicted by a CAF signature (Isella et al., 2015).

2.4 VASCULATURE

2.4.1 Vessels

During embryogenesis vessel formation is executed by two distinct mechanisms: formation of new endothelial cells and their assembly into tubes in a process called vasculogenesis, and new vessel sprouting from existing ones in a process called angiogenesis.

In adults, angiogenesis can be reactivated in settings such as wound healing or other pathological conditions. Such a neovascularization process is based on capillary sprouting, which involves integrated actions of three types of endothelial cell behavior: migration of active tip cells which direct the growth of the new sprout, proliferation of stalk cells and maturation of so-called phalanx cells which constitute the mature part of the vessel (Mazzone et al., 2009; Potente, Gerhardt, & Carmeliet, 2011).

The key regulator of this process is VEGF, which modulate the behavior of the tip cells (Phng & Gerhardt, 2009; Potente et al., 2011). Another key pathway for angiogenesis is Notch signaling (Phng & Gerhardt, 2009). Other angiogenesis regulators are angiopoietin 1 and 2 (Welti, Loges, Dimmeler, & Carmeliet, 2013). Normally the neovascularization process is tightly regulated and is turned off after playing its physiological role.

Tumors exploit physiological angiogenesis programs for induction of new vessel formation. The neovascularization is an essential stroma-related process for tumor progression which is considered to be a crucial step in cancerogenesis (Raica, Cimpean, & Ribatti, 2009). Cancer-associated vasculature is related not only to primary tumor growth, but also impacts on cancer cell intravasation and establishment of distant metastases (Bockhorn, Jain, & Munn, 2007).

Tumor vessels develop in response to angiogenic chemokines, produced by both stromal and cancer cells (Mosch, Reissenweber, Neuber, & Pietzsch, 2010). In cancer tissue VEGF-A secretion can be activated by e.g. altered oncogene signaling or hypoxia (Carmeliet, 2005; Kowanetz & Ferrara, 2006).

In the normal vasculature, distinct vessel types, organized in a hierarchical manner, can be easily distinguished: arteries, arterioles, capillaries, venules, and veins. Tumor-associated vasculature, in contrast, is composed of vessels, with an abnormal structure. Tumor vessels are larger than their normal counterparts, irregularly branched and leaky. Vessel leakiness leads to increased interstitial fluid pressure (IFP) (Stohrer, Boucher, Stangassinger, & Jain, 2000), associated with tumoral and peritumoral edema (Jain, Tong, & Munn, 2007). Because of the vessel abnormality and ineffective hierarchical organization of the vessel network, the nutrient and oxygen supply as well as cell waste removal is altered. Such disturbed perfusion has multiple adverse consequences. It results in alteration of tissue physiology including hypoxia and low pH within the tumor tissue (McDonald & Choyke, 2003; Stubbs, McSheehy, Griffiths, & Bashford, 2000). Hypoxia, in turn, promotes genetic instability, immunosuppression, a cancer stem-cell-like phenotype of tumor cells and epithelial-mesenchymal transition (Facciabene et al., 2011; Keith, Johnson, & Simon, 2012). Altered perfusion also reduces access of drugs in poorly perfused regions of the tumor.

2.4.2 Pericytes

According to Zimmermann, who introduced this term in 1923, “pericytes” are adventitial cells located within the basement membrane of capillaries and postcapillary venules (Zimmermann, 1923). Pericytes provide support to the endothelial cells by stabilizing the vessel wall and participate in the regulation of the blood flow. Precapillary arterioles are surrounded by smooth muscle cells, which are not imbedded in the basement membrane of the endothelial cells.

A number of markers have been used to identify pericytes. Morikawa et al showed in different mouse tumors models that tumor-associated pericytes express α-SMA and desmin, unlike normal pericytes, which express desmin but not α-SMA (Morikawa et al., 2002). Other markers include PDGFR-α, PDGFR-β, NG2, CD146 and RGS5 (Arnold et al., 2014; Cooke, LeBleu, Keskin, Khan, O'Conne, et al., 2012; Lindahl, Johansson, Leveen, & Betsholtz, 1997; Yao et al., 2007). However these markers are not exclusively expressed by pericytes (Diaz-Flores et

al., 2009; Schlingemann et al., 1991). Also different functional activities of the pericyte subpopulations with different marker expression profiles have been reported (Birbrair, Zhang, et al., 2013a, 2013b; Birbrair, Zhang, Wang, Messi, Mintz, et al., 2013).

Pericytes are important players in vascular development and functions include stabilization of the vessel wall and regulation of its permeability (Bergers & Song, 2005). They are involved in vessel maturation and function by reciprocal interaction with endothelial cells via grow factors and receptors like TGF-β, sphingosine-1-phosphate-1 (S1P1) and endothelial differentiation sphingolipid G-protein– coupled receptor 1 (EDG1), platelet-derived growth factor-β (PDGFβ/PDGFR-β), Ang1 and Tie2 (Armulik, Genove, & Betsholtz, 2011; Geevarghese & Herman, 2014; Jain, 2003). Interestingly, pericyte subsets have been recognized as cells-of-origin for fibroblast-like cells in scarring tissue, fibrosis and gliosis (Dulauroy, Di Carlo, Langa, Eberl, & Peduto, 2012; Goritz et al., 2011; Humphreys et al., 2010; LeBleu et al., 2013; Lin, Kisseleva, Brenner, & Duffield, 2008).

Tumor perivascular cells (PVCs), including both perivascular smooth muscle cells and pericytes, demonstrate abnormal structural characteristics; they are commonly found to be detached from endothelial cells, and are characterized by irregular shape and the presence of abnormal cytoplasmic processes (S. Goel et al., 2011; Morikawa et al., 2002).

A number of experimental studies have demonstrated that pericytes can regulate tumor growth, as first indicated in studies where PDGF receptor β, and its ligand PDGF-B, were manipulated (Abramsson, Lindblom, & Betsholtz, 2003). Subsequent studies suggest complex and tumor-type-specific effects of PDGF-dependent pericytes on tumor growth. In the B16 melanoma model, up-regulation of PDGF-production in cancer cells resulted in increased pericyte abundance and enhanced tumor growth, which occurred in the absence of changes in vessel density (Furuhashi et al., 2004). Also in mouse breast cancer model pericyte depletion was followed by inhibition of the primary tumor growth (Cooke, LeBleu, Keskin, Khan, O'Connell, et al., 2012). However, when overexpression of PDGF-B ligand was induced in colorectal and pancreatic cancer models marked tumor growth inhibition was noted, together with increased pericyte coverage. This study also noted enhanced tumor growth following treatment with a PDGFR-inhibitor, which occurred together with reduced PVC coverage rate (McCarty et al., 2007).

Both stimulatory and inhibitory effects of PDGF-dependent pericytes have been noted in animal models exploring links between pericytes and metastasis. Xian et al reported an

enhanced metastatic potential in tumors with altered interaction between pericytes and endothelial cells (Xian et al., 2006). Pericyte depletion suppressed tumor growth but at the same time enhanced hypoxia and metastasis in established tumors in genetic mouse breast cancer model (Cooke, LeBleu, Keskin, Khan, O'Connell, et al., 2012). Similar effects were noted in a pancreatic cancer xenograft (Welen, Jennbacken, Tesan, & Damber, 2009).

Pericyte coverage can also regulate immune cell trafficking (Proebstl et al., 2012). It was shown, that in mice, deficient for the pericyte-specific gene rgs5, developed tumors with increased infiltration of CD8+/CD4+ T-cells (Hamzah et al., 2008). In another pericyte-deficient mouse model an increased transmigration of Gr1+/CD11b+ cells was observed in induced tumors (Hong et al., 2015).

2.5 VASCULATURE AND RESPONSE TO TREATMENT

Chaotic tumor vasculature, subsequent alterations in blood flow, vessel leakiness and increased IFP negatively impact on drug delivery to the tumor site (Raut et al., 2012). The distance from vessels to cancer cells have been shown to affect the delivery of the anti-cancer drug to the cancer cells (Tredan, Galmarini, Patel, & Tannock, 2007). Vasculature-related hypoxia also provides resistance to different treatment strategies like radiation, chemo- or immunotherapy (Chan & Bristow, 2010; Facciabene et al., 2011; Wilson & Hay, 2011).

These findings have led to the development of the concept of therapeutic “vessel-normalization”. According to this concept anti-angiogenic therapy can normalize vessels and improve the delivery of conventional anti-cancer drugs.(Jain, 2001). This notion has been supported by a series of animal studies which have demonstrated that anti-VEGF-treatment leads to a change in tumor vasculature towards a more normal vessel-like phenotype with proper pericyte coverage. This normalization of the vessel phenotype made systemic chemotherapy much more effective (Tong et al., 2004; Winkler et al., 2004; Yuan et al., 1996). Similar findings for breast, ovarian cancer and melanoma models have been reported (Dings et al., 2007). Interesting, all the studies also reported increase of α-SMA-positive perivascular cell (PVC) vessel coverage after bevacizumab treatment.

In addition to “drug-delivery”-related effects, the vasculature has also been implied as a direct regulator of cancer cell drug sensitivity. Endothelial cells interacting with tumor cells can support their stem cell-like properties through paracrine signaling (Calabrese et al., 2007;

Krishnamurthy et al., 2010). A similar mechanism leads to increased chemo-resistance in CRC (Lu et al., 2013).

Vessel features have also been implied as determinants of sensitivity to anti-VEGF-agents in studies where perivascular status has been shown to affect sensitive to anti-VEGF treatment (Helfrich et al., 2010; Nisancioglu, Betsholtz, & Genove, 2010).

3 PROGNOSTIC RELEVANCE OF CANCER-ASSOCIATED FIBROBLASTS AND VASCULATURE

Studies from the last decade have identified clinically relevant variations in the composition and features of tumor stroma in clinical samples. The following paragraphs give some examples of studies where different methodological approaches have been used to demonstrate prognostic significance of CAFs and different vascular features.

3.1 CANCER-ASSOCIATED FIBROBLASTS AND PROGNOSIS

3.1.1 Stroma amount

The amount of CAF-dominated tumor stroma has been shown to be associated with prognosis. The stroma/tumor ratio has been shown to be associated with survival for many cancer types including breast cancer (de Kruijf et al., 2011; Moorman, Vink, Heijmans, van der Palen, & Kouwenhoven, 2012), hepatocellular carcinoma (Lv et al., 2015), ovarian carcinoma (Labiche et al., 2010), cervical cancer (Liu et al., 2014) and CRC (Huijbers et al., 2013; Park, Richards, McMillan, Horgan, & Roxburgh, 2014; West et al., 2010).

3.1.2 Marker-defined CAF subsets

IHC studies using different CAF markers have been used to identify activated fibroblasts in tumor stroma. High α-SMA and PDGFR-β expression in tumor stroma is associated with shorter survival in breast cancer (Paulsson et al., 2009; Surowiak et al., 2007) and prostate

cancer (Ayala et al., 2003; Hagglof et al., 2010). FAP and PDGDR-β has been shown to be associated with short survival in pancreatic cancer (Kawase et al., 2015; Yuzawa, Kano, Einama, & Nishihara, 2012). Stromal PDGDR-β is associated with short survival in ovarian cancer (Corvigno et al., 2016). There are conflicting results about the association of the expression of α-SMA in pancreatic cancer survival. In one study it was associated with shorter survival (Sinn et al., 2014). However this finding was not confirmed in other studies (Ozdemir et al., 2014; Yuzawa et al., 2012).

Notably, not all CAF markers are associated with worse prognosis. In some cases opposite results were reported. For example, FAP expression was associated with improved DFS and OS in breast cancer (Ariga, Sato, Ohuchi, Nagura, & Ohtani, 2001) . Similarly, FAP-1 associated with longer CSS in in squamous cell carcinoma of lung (Kilvaer et al., 2015). These findings are compatible with the overall concept of the existence of distinct marker-defined subsets of CAFs which are associated with different functions (Ostman, 2014).

3.1.3 Gene expression-based CAF subsets

The first study linking a stroma/fibroblast-related gene signature to prognosis was presented by Finak et al. This study reported a stroma-derived gene-expression signature, which was capable to identify breast cancer patients with poor outcome (Finak et al., 2008). Subsequent studies have identified additional prognostic CAF-related gene signatures. 11 genes were found to be differently expressed in normal fibroblasts and in CAFs from non-small cell lung cancer. These genes showed prognostic relevance when analyzed in multiple clinical datasets (Navab et al., 2011). A pro-inflammatory breast CAF gene signature has been shown to differ between molecular subsets of breast cancer (Erez, Truitt, Olson, Arron, & Hanahan, 2010). Furthermore, a signature related to PDGF-BB-activated fibroblasts showed independent prognostic capacity when analyzed in multiple breast cancer cohorts (Frings et al., 2013).

3.2 CANCER-ASSOCIATED FIBROBLASTS AND PROGNOSIS IN CRC

Similar to some other tumor types the stroma/tumor ratio has been reported to be prognostic in CRC (Huijbers et al., 2013; Park et al., 2014; West et al., 2010). Analyses of clinical samples

have also provided some support for relationship between marker-defined CAF subsets and prognosis and metastasis. Both PDGF beta and alpha receptors co-expression is linked to lymphatic dissemination (Wehler et al., 2008). Vimentin and α-SMA expression levels have also been found to be associated with worse prognosis (Ngan et al., 2007; Tsujino et al., 2007). High expression of FAP in the stroma of CRC is associated with more aggressive disease development and frequency of local recurrence or distant metastases (Henry et al., 2007). In another study FAP-α and SDF-1 expression was found to be associated with tumor re-growth and recurrence in rectal cancer after pre-operative chemo-radiation (Saigusa et al., 2011).

Gene expression in CAFs has also been analyzed in CRC. A 19-gene classifier have been defined which could accurately identify a group of low-risk stage II T4N0 patients low-risk patients (Berdiel-Acer, Cuadras, et al., 2014). In another study the same group reported a CAF-associated 5-gene classifier, which identified high-risk stage II/III CRC (Berdiel-Acer, Berenguer, et al., 2014).

Integrated analysis of gene expression profiles in CRC resulted in molecular classification of Consensus Molecular Subtype (Guinney et al., 2015) (also reviewed in part 1). The mesenchymal subtype (CMS4) has been reported to have the worst prognosis. However, several recent papers have suggested, that the unique CMS4 signature may be partly originating from the stromal elements, rather than from cancer cells (Calon et al., 2015; Isella et al., 2015). In a follow-up study Dunna et al emphasized a particular role of CAFs in the higher transcription levels of mesenchymal-associated genes (Dunne et al., 2016).

3.3 VASCULATURE AND PROGNOSIS

3.3.1 Micro-vessel density

An impressive amount of studies on the prognostic capacities of the micro-vessel density (MVD) have been performed during the past 15 years. Meta-analyses showed that high MVD is associated with poor survival in CRC (RFS and OS) (Des Guetz et al., 2006), ovarian cancer (PFS and OS) (He, Wang, & Zhao, 2015), hepatocellular carcinoma (RFS and OS) (Y. Li, Ma, Zhang, Liu, & Liu, 2014), bladder cancer (DFS, OS and CSS) (Huang et al., 2014), breast cancer (RFS and OS) (Uzzan, Nicolas, Cucherat, & Perret, 2004). Interestingly, similar meta-analytical approaches did not confirm the prognostic effect of MVD in renal cell carcinoma

(Cheng et al., 2014; Zhang, Ji, Yan, Liu, & Shi, 2014) and head and neck cancer (M. Yu et al., 2014).

3.3.2 Vascular proliferation

J Folkman suggested in 1971 that malignant tumors are dependent on the angiogenesis process to mountain sufficient blood supply (Folkman, 1971). In addition to MVD, the vessel proliferation index or the MVD restricted only to proliferation vessels may thus act as a better and/or independent prognostic factor. This notion has been explored in a series of studies which indeed has shown that vascular proliferation index is associated with survival in prostate, breast and endometrial cancer (Arnes et al., 2012; Gravdal, Halvorsen, Haukaas, & Akslen, 2009; Kruger et al., 2013; Stefansson, Salvesen, & Akslen, 2006).

3.3.3 VEGF

Similarly to high MVD, VEGF expression was linked to shorter survival in gastric cancer (VEGF-A and VEGF-D for OS but not VEGF-C) (Ji, Wang, Li, & Wang, 2014; Peng et al., 2012), hepatocellular carcinoma (OS) (Zhan, Qian, & Yu, 2013), prostate cancer (OS) (Wang, Peng, & Li, 2012), ovarian cancer (PFS and OS) (L. Yu, Deng, Li, Zhang, & Hu, 2013), head and neck cancer (PFS and OS) (Zang et al., 2013), lung cancer (Zhan et al., 2009) and CRC (PFS and OS) (He et al., 2015).

3.3.4 Pericytes

A number of clinical studies have investigated the impact of the perivascular cell status on prognosis. High pericyte coverage was associated with adverse prognosis in renal cell carcinoma, ovarian carcinoma and bladder cancer (Cao et al., 2013; Corvigno et al., 2016; O'Keeffe et al., 2008). In contrast, in CRC low pericyte coverage was linked to unfavorable outcome (Mezheyeuski et al., 2016; Yonenaga et al., 2005).

3.4 VASCULATURE AND PROGNOSIS IN CRC

3.4.1 MVD and VEGF

Micro-vessel density (MVD) has been extensively studied as prognostic factor in CRC. Increased MVD has been reported in CRC and adenomas compared to normal mucosa (Bossi et al., 1995; White et al., 2002). Also high expression of VEGF-C and VEGF-D has been observed in CRC more often than in adenomas or normal colon mucosa (Hu et al., 2007; White et al., 2002). Interestingly, no correlation was found between VEGF-D and MVD (White et al., 2002). VEGF-C expression is correlated with tumor clinical stage, lymph node metastases and depth of invasion (Hanrahan et al., 2003; Hu et al., 2007).

Des Guetz et al performed a meta-analysis and concluded that MVD is associated with poor RFS and OS in CRC. The same results were reported regarding the expression of VEGF (Des Guetz et al., 2006). It should be noted however, that despite such output of the meta-analysis, the individual studies were very heterogeneous with regard to both cohort selection and survival associations. Additionally, different markers were used to detect endothelial cells and different scoring methods were employed.

3.4.2 Pericytes

Pericyte coverage evaluated by scoring α-SMA-positive perivascular cells was associated with metastases and adverse prognosis in CRC (Yonenaga et al., 2005). These data are in line with experimental study of Mc Carty et al, who demonstrated the inhibition or stimulation of the growth of CRC in a mice model when PC coverage was increased or decreased by administration of PDGF-BB ligand or PDGF-inhibitor, respectively (McCarty et al., 2007).

4 CANCER-ASSOCIATED FIBROBLASTS AND VASCULATURE AS TARGETS FOR TREATMENT

4.1 TARGETING OF CANCER-ASSOCIATED FIBROBLASTS

There are no approved drugs that act exclusively by CAF-targeting. Notably, many of the multi-kinase inhibitor in the "vascular-targeting"-section below act as inhibitors of PDGF receptors which are well-documented regulators of CAFs. To what extent these activities contribute to the therapeutic efficacy presently remains unknown. Among other CAF-related targets, FAP and TGF-beta are subjected to clinical exploration.

FAP monoclonal antibodies have been used used for the treatment mCRC patients prior to surgery (Welt et al., 1994). This study was performed on 17 patients with the main aim to investigate toxicity, biodistribution and imaging capacities of the agent (iodin-131 labeling allowed usage of the planar and single-photon emission tomography scanner). Because of low toxicity and preferential uptake of the antibody by the tumor stroma, a phase I study used the humanized FAP monoclonal antibody sibrotuzumab was conducted (Scott et al., 2003). However, no objective tumor response was observed.

A phase II trial study in lung cancer with TGF-β2 vaccine (belagenpumatucel-L) demonstrated a dose-related survival difference in patients (Nemunaitis et al., 2006). Another trial using FANG vaccine (plasmid encoding granulocyte-macrophage colony-stimulating factor and bifunctional short hairpin RNAi targeting furin convertase, needed for TGFβ1 and TGFβ2 activation) reported increased immune response and improved disease control (Senzer et al., 2012). Human anti-TGFβ1/2/3 (fresolimumab) and anti-TGFβ1 (TβM1) were tested in phase I clinical trials in patients with advanced melanoma or renal carcinoma (Morris et al., 2014) and a mixed cohort of metastatic cancers (Cohn et al., 2014). Anyhow, both studies failed to demonstrate improvement in clinical outcome in treated patients, probably due to small sample size.

4.2 TARGETING OF TUMOR VASCULATURE

Following studies in tumor biology and experimental therapy studies in animal models, a number of anti-angiogenic drugs have been developed and shown efficacy in phase III studies. The majority of these drugs are targeting the VEGF system but, as outlined below, also the Angiopoeitin/Tie is tested as target. Based on their molecular mechanism of action the anti-angiogenic drugs are broadly divided into large molecules (antibodies and soluble receptors) and low-molecular weight kinase inhibitors. The following paragraphs discuss some of these drugs and their indications.

4.2.1 Anti-angiogenic antibodies, soluble receptors and peptibodies

Protein/petide-based drugs affecting angiogenesis may bind to either to angiogenic ligands or their receptor. Drugs in this category are bevacizumab, ramucirumab, aflibercept and trebananib.

Bevacizumab (Avastin) is anti-VEGF antibody, which is approved for a number of indications where it is used in combination therapy with chemotherapy or interferon-alpha. Positive phase III studies, in combination with chemotherapy, have been reported e.g. in colorectal, lung, ovarian and cervical cancer (Bennouna et al., 2013; Giantonio et al., 2007; Hurwitz et al., 2013; Penson et al., 2015; Poveda et al., 2015; Pujade-Lauraine et al., 2014; Sandler et al., 2006). Efficacy together with interferon-alpha has been shown in kidney cancer (Escudier, Cosaert, & Pisa, 2008).

Ramucirumab is a human monoclonal antibody against VEGFR2. The drug is approved for use in combination with FOLFIRI regimen for those mCRC patients who have disease progression after a first-line therapy with bevacizumab, oxaliplatin, and fluoropyrimidine. Ramucirumab treated group demonstrated improved OS in a phase III randomized trial (Tabernero et al., 2015). Ramucirumab is has been also shown to increase survival of patients with advanced or metastatic non-small cell lung cancer, advanced gastric or gastro-oesophageal junction cancer (Fuchs et al., 2014; Garon et al., 2012).

Another VEGF-targeting drug is **Aflibercept** (Zaltrap), which is a recombinant fusion protein composed of VEGF-binding portions from the extracellular domains of human VEGFR 1 and 2, fused to the Fc portion of human immunoglobulin IgG1. It blocks the activity the activity of

VEGF-A, VEGF-B, and PlGF (Lockhart et al., 2010). According to VELOUR trial aflibercept improved OS when it was given in combination with 5-fluorouracil, leucovorin and irinotecan for the treatment of patients with mCRC (Tabernero et al., 2014).

Trebananib peptibody is a first in the class agent which inhibits angiogenesis by blocking the interaction between angiopoietin-1/2 and Tie2. According to the TRINOVA-1 phase III study trebananib significantly prolonged PFS in ovarian cancer patients (Monk et al., 2016).

4.2.2 Small molecules

Small chemical compound-based agents are less specific then antibody-based drugs. Among the anti-angiogenic small-molecule drugs, all block VEGFR with additional activities against other angiogenesis-related tyrosine kinase inhibitors such as PDGFR.

Sunitinib, sorafenib and pazopanib are examples of small-molecule TKIs, targeting VEGFs and PDGF receptors (Abdollahi & Folkman, 2010) 24785224. Indications for these TKIs include metastatic renal cell carcinoma, gastrointestinal stromal tumors and hepatocellular cancer (Escudier et al., 2009; Goodman et al., 2007; Llovet et al., 2008; Motzer et al., 2009; Sternberg et al., 2010).

Regorafenib is another TKI blocking BRAF, VEGFR-1, -2, -3, KIT, TIE-2, PDGFR-β, FGFR-1, RET and RAF-1 (Wilhelm et al., 2011). A phase III study showed efficacy of regorafenib in chemo-refractory mCRC (Grothey et al., 2013; Majithia & Grothey, 2016). The drug has also showed efficacy in a phase III trial in patients with gastrointestinal stromal tumor, which were unsuccessfully treated with imatinib and sunitinib. Significantly prolonged PFS was observed in regorafenib group in comparison to placebo group (Schroeder, Li, Cranmer, Jones, & Pollack, 2016).

5 PRESENT INVESTIGATION

5.1 AIMS

The general aim of this thesis was to explore the possibility that a careful analyses and description of the tumor microenvironment can uncover novel features related to survival. Specific aims were:

- to investigate the prognostic and response-predictive potential of vascular and CAF features in ovarian cancer and different stages of colorectal cancer
- to explore expression pattern and potential clinical relevance of the tight junction protein claudin-2 in colorectal cancer
- to analyze the prognostic and response-predictive relevance of the tumor/stroma interface in colon cancer as determined by a semi-automated digital-image analyses approach

5.2 RESULTS

Article I

Inter- and intra-tumoral relationships between vasculature characteristics, GLUT1 and budding in colorectal carcinoma

Vascular characteristics, hypoxia and tumor budding are features that have been implied in the biology and prognosis of colorectal cancer. Internal relationships and the inter- and intra-tumoral variation of these tumor properties remain to be determined. In this study we characterized blood vessel status in different areas of CRC and in the peritumoral fibroblastic stroma. Analyses of these characteristics were supplemented by characterization of budding and hypoxia.

Analyses revealed significantly lower values of vessel perimeter (VP) and vessel lumen area (VL) at the invasive front and surrounding stroma as compared to the tumor center. Also, the number of vessels (VN) in the peritumoral stroma was higher than in the center. Thus, tumor center displays larger and fewer vessels as compared to the tumor periphery.

GLUT1 expression was correlated directly with VN ($r=0.351$, $p=0.028$) and inversely with VL and VP ($r=-0.432$, $p=0.006$ and $r=-0.484$, $p=0.002$) at the invasive front. Moreover, GLUT1 expression, VP at the invasive front, and VN in the surrounding peritumoral stroma, were

associated with budding score (r=0.574, p<0.000, r=-0.340, p=0,034 and r=-389, p=0.025 respectively). Furthermore, GLUT1, budding score, vessel number in peritumoral stroma, and vessel size in the invasive front, were significantly different in tumors with or without lymph node metastasis.

This study thereby identified previously unrecognized relationships between localization-specific vascular characteristics, hypoxia and tumor budding. The findings suggest potential functional relationships, which should be further explored, and also highlight the inter-tumoral variations in vasculature, which is highly relevant for ongoing efforts to identify vessel-based biomarkers.

Article II

Survival-associated heterogeneity of marker-defined perivascular cells in colorectal cancer

Based on cancer model studies perivascular cells (PC) have been implied as regulators of metastasis and immune cell activity. Perivascular heterogeneity in clinical samples, and associations with other tumor features and outcome, remain largely unknown.

The study developed a novel method for digital quantitative analyses of vessel characteristics and PC, which was applied to two collections of human metastatic colorectal cancer (mCRC).

Initial analyses identified marker-defined subsets of PC, including cells expressing PDGFR-β or α-SMA or both markers. PC subsets were largely independently expressed in a manner unrelated to vessel density and size. Association studies implied specific oncogenic mutations in malignant cells as determinants of PC status. Semi-quantitative and digital-image-analyses-based scoring of the NORDIC-VII cohort identified significant associations between low expression of perivascular PDGFR-α and -β and shorter overall survival. Analyses of the SPCRC cohort confirmed these findings. Perivascular PDGFR-α and -β remained independent factors for survival in multivariate analyses.

Overall, the study identified host vasculature and oncogenic status as determinants of tumor perivascular features. Perivascular PDGFR-α and -β were identified as novel independent markers predicting survival in mCRC.

Article III

Markers of fibroblast-rich tumor stroma and perivascular cells in serous ovarian cancer: inter- and intra-patient heterogeneity and impact on survival

Inter- and intra-patient variations in tumor microenvironment of serous ovarian cancer are largely unexplored. This study explored potential co-regulation of tumor stroma characteristics, analyzed their concordance in primary and metastatic lesions, and studied their impact on survival.

A tissue microarray (TMA) with 186 tumors and 91 matched metastases was subjected to immunohistochemistry double staining with endothelial cell marker CD34 and the fibroblast and pericyte markers α-SMA, PDGFβR and desmin. Images were digitally analyzed to yield "metrics" related to vasculature and stroma features.

Intra-case analyses showed that PDGFβR in perivascular cells and fibroblasts were strongly correlated. Similar findings were observed concerning α-SMA. Most stroma characteristics showed large variations in intra-case comparisons of primary tumors and metastasis. Large PDGFβR-positive stroma fraction and high PDGFβFR positive perivascular intensity were both significantly associated with shorter survival in uni- and multi-variate analyses (HR 1.7, 95% CI 1.1-2.5; HR 1.7, 95% CI 1.1-2.8).

In conclusion, we found PDGFβR- and α-SMA-expression to be largely independent of each other but concordantly activated in perivascular cells and in fibroblasts within the primary tumor. Stromal characteristics differed between primary tumors and metastases. Survival association studies suggest that PDGFβR in perivascular cells and in fibroblasts may be novel prognostic markers in serous ovarian cancer

Article IV

Image-analyses-quantitated tumor morphological complexity predicts prognosis and response to treatment in stage II-III colon cancer

The optimal selection of patients for adjuvant chemotherapy in stage II-III colon cancer remains challenging and needs to be improved. The complexity of the histo-morphology reflects underlying tumor biology. Automated image analyses, using fractal geometry-derived algorithms, can quantitate different aspects of histo-morphological metrics. This study investigated the relationship between "multifractal (MF) metrics", related to histo-morphological complexity, and the natural course of the disease as well as the response to adjuvant chemotherapy.

Multifractal analysis was applied to images of pan-cytokeratin-stained tissue sections from 291 stage II/III colon cancer tumors derived from a randomized study investigating efficacy of surgery versus surgery plus adjuvant treatment. Two distinct characteristics, local irregularity (αmax) and global irregularity (f(α)max), related to the complexity of the tumor histo-morphology were quantitated in each tumor. Status of these two MF metrics was then correlated with patient characteristics and survival.

In the surgery-alone group, related to the post-surgery natural course of the disease, both MF metrics were significantly associated with disease-free survival (DFS). The MF metrics were also associated with DFS in a multivariate analysis that included standard clinical characteristics. Analyses of the chemotherapy-benefit-predictive capacity of the MF metrics identified significant benefit of treatment in the "αmax -high" sub-group (p=0.019), not seen in the "αmax -low" sub-group ". Interactions between treatment and the αmax -metric was demonstrated by formal interaction test (p=0.023).

From these findings it is concluded that MF metrics might be independent markers for poor prognosis in the natural course of stage II-III colon cancer. The αmax -metric appear as a strong candidate for further evaluation as marker for benefit of treatment. In general terms the study suggests that MF metrics should be further explored as easy-to-implement markers guiding decisions regarding the use of adjuvant chemotherapy in colon cancer.

Article V

Vessel density in colon cancer: a predictive marker of benefit from adjuvant fluorouracil-based chemotherapy

Vessel characteristics have previously been analyzed with regard to potential prognostic and predictive capacity in stage II-III colon cancer with inconclusive results. This study extends previous efforts by reporting results from an analysis where vessel density (VD) was digitally quantitated separately in the tumor center and in the invasive margin in a randomized study-derived stage II-III colon cancer collection, allowing stringent separation of prognostic and response-predictive associations.

Tumor sections from 285 stage II-III colon cancer patients from a randomized trial evaluating fluorouracil (5-FU)-based adjuvant chemotherapy were used. The sections were stained with PDGFR-β and CD34 and subjected to digital image analyses for VD quantification performed separately in tumor center and invasive margin.

VD was not correlated with survival in the subgroup treated with surgery alone. In the subgroup of adjuvant-treated patients high VD in invasive margin, but not in tumor center, was associated with longer time-to-recurrence (TTR) ($p=0.001$). The association between high VD and improved outcome after adjuvant therapy was confirmed by multivariate analysis including other markers which have been associated with chemotherapy-sensitivity such as thymidylate synthase (TS) and MRR status ($P =0.007$). Interactions between treatment and VD were also demonstrated by formal interaction test ($p=0.011$).

The study, based on analyses of a well-annotated phase III-study-derived tumor collection, indicates that high VD in invasive margin acts as a response-predictive factor for adjuvant 5-FU-based chemotherapy in stage II-III colon cancer.

Article VI

Survival-associated heterogeneity of claudin-2 expression in colorectal cancer

Claudin-2 is a member of the claudin super-family trans-membrane proteins which are important components of tight junctions in epithelial calls. Elevated claudin-2 expression has been reported in colorectal cancer (CRC). Claudin-2 is also expressed in other cell types than epithelial cells. The aim of this study was to analyze the expression patterns of claudin-2 in human CRC and analyze its association with clinical characteristics including survival.

TMAs of primary tumors from two collections of human metastatic CRC (mCRC) cohorts were used. Claudin-2 IHC staining was evaluated in a semi-quantitative manner in different regions and cell types. Claudin-2 expression was also analyzed by immuno-fluorescence in primary cultures of human CRC cancer-associated fibroblasts (CAFs).

Initial analyses identified previously unrecognized expression of claudin-2 in CAFs of human CRC. Immunofluorescence analyses of primary cell cultures of CAFs confirmed claudin-2 expression in a fraction of fibroblasts from CRC tissue. Analyses of the population-based SPCRC mCRC cohort revealed that expression of the claudin-2 in CAFs of the invasive margin was associated with KRAS mutations, but not BRAF mutations. Claudin-2 expression in CAFs of the invasive margin, but not in the tumor center, was significantly associated with shorter progression-free survival (PFS). Subsequent subgroup analyses demonstrated that the survival associations occurred among cases that received 5-FU-oxaliplatin combination treatment (12.8 mo PFS for claudin-2-low vs 7.9 mo for claudin-2 high, log-rank p=0.001), but not in patients receiving 5-FU alone. Independent support indicating a relationship between CAF claudin-2 expression and sensitivity to 5-FU-oxaliplatin treatment was obtained by analyses of the NORDIC-VII cohort. Also in this cohort, where all cases received the 5-FU/oxaliplatin, significant associations were detected between high CAF claudin-2 expression and shorter survival.

The study thereby identified novel stromal expression of claudin-2 in CAFs of human CRC. Significant association between high claudin-2 expression in CAFs and shorter survival in FU/oxaliplatin-treated mCRC suggest CAF claudin-2 as a novel biomarker for benefit of FU/oxaliplatin.

5.3 CONCLUSIONS AND FUTURE PERSPECTIVES

The three first studies (I-III) and the study V were aimed to investigate a potential clinical relevance of tumor stroma and vasculature properties.

Our early investigations (study I) indicated an importance of the spatial localization of the tumor area, which undergoes examination. Study V confirmed this finding, by defining the vessel characteristics of the tumor invasive margin as being associated with clinical

characteristics and patient survival in contrast to those collected from tumor center. This information could be of high importance for the future studies, and could impact on the decisions of the design of tissue collection/usage in biomarker-based research.

Studies II and III were aimed to evaluate prognostic importance of such vessel characteristics, like vessel size and density, as well as of the marker-defined subsets of CAFs and perivascular cells. The approach identified PDGFR-β-positive pericytes (mCRC and ovarian cancer) and PDGFR-β-positive CAFs (ovarian cancer) as strong independent prognostic factors. The findings were validated for colorectal cancer on an independent cohort (study II). The ovarian cancer findings remain to be validated in independent cohorts. These two studies also reported association between mutational status of the tumor and its vessel density and perivascular coverage. Interestingly, it has been reported earlier that BRAF-mutations can be associated with increased VEGF-A production (Bottos et al., 2012). These findings suggest that there are specific angiogenic programs associated with different oncogenic mutations. This notion should be possible to explore in animal models where vessel features are characterized in tumor models with well-characterized status of oncogenic drivers. .

It should be noted, that the nature of the patient cohorts, used in studies II and III, prevents a clear distinction prognostic and treatment-related associations of the markers. Future studies should address this by separate analyses of cohorts where prognostic and response-predictive associations can be analyzed separately.

Study V identified vessel density in the tumor tissue as a factor predictive for the efficacy of the fluorouracil-based therapy applied for stage II and III colon cancer patients in adjuvant settings. The study was performed on the material form the randomized phase III trial for the adjuvant treatment, which took place in 1991-1997. To strengthen the result, it should be validated on independent patient cohort which includes present state-of-the-art surgical techniques and treatment regimens. Animal models mimicking adjuvant treatment have been developed (Srivastava et al., 2014). Such models should be possible to use to continue further mechanistic studies aiming to better understand the intriguing associations between vessel density and benefit of treatment. In this context recently described tumor models allowing pericyte manipulations should also be useful (Hong et al., 2015).

Study IV re-evaluates the significance of the tumor morphology as a prognostic and predictive factor in stage II and III colon cancer. This study applied a computer-based image analysis

approach to quantify the complexity of the tumor architecture at the invasive margin. Findings suggest that a computer-based approach may utilize the information of the tumor morphology in a better way than visual evaluation by human eye. Such digital scores when applied to the tumor samples form the randomized phase III trial for the adjuvant treatment (see above) defined patient subgroups which show differential benefit from adjuvant chemotherapy.. The predictive value of the digital scores should be validated in an independent patient cohort with a modern adjuvant treatment regimen. Future studies should also specifically look at the relevance of this marker in stage II and stage III cases. Obviously, it will also be interesting to explore these new metrics in other tumor types.

Studies I, IV and V all noted that the local origin of marker signals impacted on their score and clinical relevance. This intra-tumoral spatial variation has been demonstrated also in in other studies using different markers including hypoxia markers and immune cell infiltrates (Pages et al., 2009; Rajaganeshan et al., 2009). This should be considered in upcoming biomarker studies.

The sixth study provides novel evidence linking CAF-derived claudin-2 to reduced benefit of oxaliplatin. These findings should be further developed in experimental models where the impact of claudin-2-high- or –low CAFs analyzed with regard to oxaliliplatin-sensitivity of co-cultured CRC cells. Furthermore, the indications of claudin-2 expression in subsets of macrophages can be expanded in studies where claudin-2 –positive macrophages are functionally profiled.

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