From the Department of Oncology – Pathology
Karolinska Institutet, Stockholm, Sweden

TUMOR MICROENVIRONMENT DERIVED BIOMARKERS IN RENAL CELL CANCER

Magnus Frödin-Bolling

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Magnus Frödin-Bolling

Principal Supervisor:
Dr. Per Sandström
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor(s):
Professor Arne Östman
Karolinska Institutet
Department of Oncology-Pathology

Assoc. Prof. Anders Ullén
Karolinska Institutet
Department of Oncology – Pathology

Assoc. Prof. Thomas Hatschek
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Tim Eisen
University of Cambridge
Department of Oncology

Examination Board:
Assoc. Prof. Anna Dimberg
Uppsala University
Department of Immunology, Genetics and Pathology

Professor Gunilla Enblad
Uppsala University
Department of Immunology, Genetics and Pathology

Assoc. Prof. Christofer Juhlin
Karolinska Institutet
Department of Oncology-Pathology

Till mormor

“Burning the candle in both ends gives a bright light”

- *Christoffer Hitchens*

ABSTRACT

Renal cell carcinoma (RCC) is the 13th most common malignancy worldwide, and constitutes around 2% of all malignant tumors. The entity renal cell carcinoma comprises a heterogenous group of malignant tumors that originates from the epithelial cells in the renal proximal tubule. The most frequently occurring subtype is clear cell renal cell carcinoma which is characterized by a mutation in the von-Hippel-Lindau gene leading to accumulation of hypoxia inducible factor and subsequent upregulation of growth factors involved in angiogenesis. RCC is inherently resistant to conventional chemotherapy, and thus radical surgery before metastasis has occurred still is the best chance for permanent cure. However, in recent years, the introduction of various targeted therapies and immune-modulators have changed the picture, and there are now numerous options which increases the hope for patients with metastatic disease.

In this thesis, we investigated the tumor microenvironment to identify factors with impact on prognosis and response to anti-angiogenic therapy in patients with mRCC. We found that both high perivascular expression of PDGFR-β as well as high heterogeneity of perivascular PDGFR-β was significantly associated with shorter survival. In order to make an in-depth characterization of the tumor microenvironment, we compared vascular, perivascular and stromal features in renal, colorectal and ovarian cancer. This revealed significant differences regarding several metrics, but also similarities. We also studied the impact on tumor infiltrating B-lymphocytes in RCC and found that high infiltration conveyed a worse prognosis, counter to what is seen in many other tumor types, suggesting that high levels B-cells in RCC rather dampens the anti-tumor immune response than indicates an activated immune system. In the last paper, we investigated the role of intra-tumoral vessel size for response to anti-angiogenic treatment and found that tumors dominated by medium sized vessels was more sensitive to sunitinib.

In summary, our findings indicate that the tumor microenvironment influences prognosis as well as response to treatment in a context dependent manner, and that this prompts further investigation within this field.

LIST OF SCIENTIFIC PAPERS

I. **MAGNUS FRÖDIN**, Artur Mezheyeuski, Sara Corvigno, Ulrika Harmenberg, Per Sandström, Lars Egevad1, Martin Johansson, and Arne Östman (2016)
Perivascular PDGFR-β is an independent marker for prognosis in renal cell carcinoma
British Journal of Cancer, 2016, 116, 195-201

II. Corvigno, Sara ; **FRÖDIN, MAGNUS** ; Wisman, G Bea A ; Nijman, Hans W ; Van Der Zee, Ate Gj ; Jirström, Karin ; Nodin, Björn ; Hrynchyk, Ina ; Edler, David ; Ragnhammar, Peter ; Johansson, Martin ; Dahlstrand, Hanna ; Mezheyeuski, Artur ; Östman, Arne (2017)
Multi-parametric profiling of renal cell, colorectal, and ovarian cancer identifies tumour-type-specific stroma phenotypes and a novel vascular biomarker
Journal of Pathology: Clinical research, 2017, 3(3), 214-224

III. Elin Sjöberg, **MAGNUS FRÖDIN**, Artur Mezheyeusky, Martin Johansson, Ulrika Harmenberg, Lars Egevad, Per Sandström, Arne Östman (2017)
Identification of a CD20/ MS4A1-high minority-group of renal cell cancer associated with poor prognosis
Manuscript

IV. **MAGNUS FRÖDIN**, Artur Mezheyeuski, Katriina Peltola, Petri Bono, Ulrika Harmenberg, Martin Johansson, Lars Egevad, Arne Östman, Per Sandström (2017)
Vessel diameter predicts response to sunitinib in mRCC
Manuscript

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LIST OF ABBREVIATIONS

α-SMA	alpha- Smooth muscle actin
Ang	Angiopoeitin
ATP	Adenosine triphosphate
Akt	Serine/threonine specific protein kinase
Bcr	B-cell receptor
Breg	Regulatory B-cell
CAF	Cancer associated fibroblast
CD	Cluster of differentiation
CI	Confidence interval
CT	Computed tomography
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
ECM	Extracellular matrix
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
EMT	Epithelial to mesenchymal transition
FAP	Fibroblast activating protein
FGF	Fibroblast growth factor
FLCN	Folliculin
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HR	Hazard ratio
IFN	Interferon
IGF	Insulin growth factor
IL	Interleukin
IQR	Interquartile range
KRAS	Kirsten rat sarcoma viral oncogene homolog
MDSC	Myeloid derived suppressor cell
MEK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase

mRCC	Metastatic renal cell carcinoma
mTOR	Mammalian target of rapamycin
MVD	Micro-vessel density
OD	Optical density
OS	Overall survival
PD	Programmed cell death protein
PD-L	Programmed cell death protein-ligand
PDGF	Platelet derived growth factor
PDGFR-β	Platelet derived growth factor receptor β
PFS	Progression free-survival
PI3K	Phospahtidylinositol -3-kinase
PTEN	Phosphatase and tensin homolog
PVI	Perivascular intensity
RCC	Renal cell carcinoma
RECIST	Response evaluation criteria in solid tumors
TGF-β	Transforming growth factor β
TIL	Tumor infiltrating lymphocytes
Th	T-helper
TKI	Tyrosine kinase inhibitor
TMA	Tissue microarray
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor α
Treg	Regulatory T-cell
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wingless-related integration site

1 RENAL CELL CARCINOMA

1.1 General introduction

Renal cell carcinoma (RCC) is a heterogenous group of malignant tumors that originates from the renal proximal tubule and around 270000 new cases of RCC are diagnosed every year making it number 13 on the list of most common malignancies in the world [1]. Many patients are cured by surgery alone but 25-30 % of patients have metastasis on presentation, making it a challenge to health care systems worldwide [2]. In 2008, 116 000 deaths were attributed to this disease and mortality rates were highest in Australia, New Zealand and North America, while Europe together with Africa and Asia reported the lower mortality rates [3]. Incidence of RCC is twice as high in men than in women, for reasons not yet fully understood [3]. In Sweden, around 1000 new cases per year are diagnosed, which constitutes 2.3% of male cancers and 1.5% of female cancers (National Swedish Cancer Registry). Most cases are diagnosed in people above 60 years of age, ca 80 cases per year is found in people younger than 50 years. The percentage of incidentally detected kidney cancers has risen, due to the more widespread use of ultrasound, MRI and CT.

1.2 Risk factors

The most thoroughly studied lifestyle risk factor for RCC is cigarette smoking. A meta-analysis investigating five cohort studies and 19 case-control studies revealed that ever smoking increases the risk compared to never smoking but the association was not as strong as with cigarette smoking and lung cancer, even if a dose-response relation was found [4]. Increased body weight is also considered a risk factor for RCC, a notion supported by both case-control and cohort studies were a correlation between higher body mass index (BMI) and risk of developing RCC. For or every 5 kg/m2 increase in BMI there was an increased risk of 1.24 in men and 1.34 in women [5]. The stronger association with BMI and risk for RCC seen in women is not fully explained, and the mechanisms by which obesity increases the risk are not fully elucidated, but one possible contributing factor is increased levels of blood glucose and IGF-1 which is known to have impact on tumor growth [6].

1.3 Histopathology of RCC

1.3.1 Clear cell carcinoma

The most prevalent subtype of RCC is clear cell renal carcinoma (ccRCC) which accounts for about 75-85 % of all RCC cases and has its origin in the proximal tubule. Clear cell carcinoma is characterized by loss of the tumor suppressor von Hippel-Lindau gene [7]. The inactivation of VHL is possible by mutation, deletion or methylation. Mutations of the VHL gene found in sporadic RCC differ from those seen in RCC associated with inherited VHL disease. In sporadic RCC, 45% of the mutations are clustered in the second exon, although abnormalities are seen in all three exons. Large deletions are not observed, and 48% of the mutations are micro-deletions or insertions, resulting in frame shifts of the protein-coding sequence [8].

Under normal oxygen levels, the regulatory protein hypoxia inducible factor-α (HIF-α) is hydroxylated, which makes it bind to the VHL-protein. The VHL-protein is in turn a part of a ubiquitin-ligase complex (E3) that targets HIF for degradation [8]. In the case of inactivating VHL mutations, HIF is instead accumulating, leading to upregulation of VEGF, PDGF-beta and TGF-alpha [9]. This in turn leads to very high levels these growth factors which is the rationale for targeted therapy with TKIs targeting receptors of these growth factors.

1.3.2 Papillary carcinoma

Papillary RCCs constitutes ca 10 % of all RCCs and are suggested to be of renal proximal tubular origin [10]. Two subtypes are described, type-I and type-2. Type-1 papillary RCC is less frequent, associated with c-MET mutations and is seen in the hereditary papillary renal cancer syndrome and only occasionally in sporadic papillary RCC (with or without MET mutations) [11]. Key histological features include pale cytoplasm around the basement membrane of papillary cones. Psammoma bodies and foam cell-like macrophages is also commonly found [11]. Type-2 papillary RCC does not harbor c-MET mutations and is more commonly seen in sporadic cases [12]. Type-2 also in general have eosinophilic cytoplasm and nuclei described as “pseudostratified” [11].

1.3.3 Chromophobic carcinoma

Chromophobe RCC is less common than clear cell and papillary cancers, and is seen in about 5 % of all RCCs [13]. Chromophobe RCC arises in the intercalated cells of the

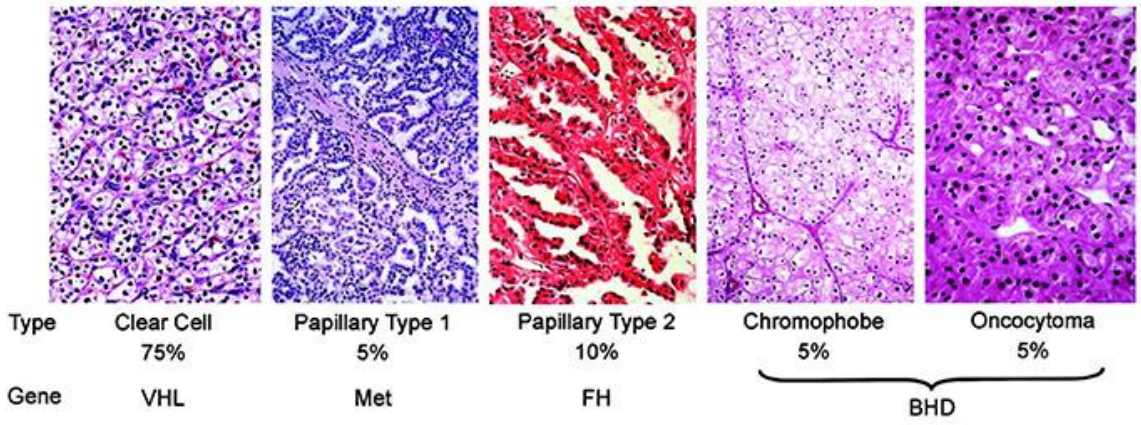
cortical collecting ducts [13]. On the cytogenetical level, chromophobe RCC shows widespread loss of heterozygosity of chromosomes 1, 2, 6, 10, 13, 17 and 21 and hypoploidy due to non-random multiple chromosome losses [12]. These multiple losses have made it difficult to define specific genes essential to the development of chromophobe RCC but mutations in the FLCN- gene is described [14]. This gene is mutated in the Birth-Hogg-Dubé hereditary syndrome. This is an autosomal dominant disease leading to spontaneous pneumothorax, pulmonary cysts, dermal fibrofolliculomas and renal carcinomas [15].

1.3.4 Oncocytoma

Oncocytomas are commonly considered as benign renal neoplasms, but cases of metastatic tumors have been described [16]. When metastasis has occurred, these tumors usually have been re-classified as chromophobe carcinomas. Similar to chromophobic carcinomas, oncocytomas are related to the hereditary Birth-Hogg-Dubé-syndrome and mutations in the FLCN-gene [14].

1.3.5. Collecting Duct Carcinoma

The collecting duct carcinoma is the rarest form (1-2% of all cases), and it is derived from the collecting duct epithelia [11]. Data regarding the genetic aberrations of collecting duct carcinoma are scarce.



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1.4 Molecular subsets of RCC

Since clear cell carcinoma is the most prevalent form of RCC, this chapter will focus on ccRCC. The most common genetic alteration in ccRCC is the inactivation of the VHL gene on chromosome p3. But also genes involved in maintenance of chromatin status (such as PBRM1) plays an important role. In a comprehensive report from Nature, 2013, 400 tumors were analysed using genetic platforms. In this analysis, they found 19 genes that showed significant grade of mutation. One of the most frequently mutated pathways was the PI3K/Akt-pathway, making inhibition of the several steps involved a potential therapeutic target. Further was DNA hypomethylation often seen, which was described as associated with mutations in H3K36 methyltransferase SETD2 [17]. Aggressive tumors showed signs of a metabolic shifting and downregulation of genes involved in the citric acid cycle. Other reported findings were decreased levels of the tumor suppressor protein PTEN, which negatively regulates the Akt-pathway and upregulation of genes involved in pentose and glutamine transport [17].

1.5 Prognostic factors

The most widely used prognostic factors in RCC are based on anatomical features like size of the primary tumor, lymphnode involvement and metastasis-status (TNM classification) in combination with histological characteristics (Fuhrman grade, subtype) and clinical status (symptoms and performance status). When used alone, none of these features have very high accuracy. Therefore, combined scoring models are used in the clinic. Two scoring models previously used are the Leibovic score [18] and the Memorial Sloane Kettering Cancer Center risk group score (MSKCC) [19]. Today, the most widely used score is the HENG-score, which take into consideration time from diagnosis to start of systemic treatment, performance status, a hemoglobin level < 120 g/l, calcium-level, platelet and leukocyte count above normal range. A favourable HENG score (≤ 1p) is associated with a median survival of 43.2 months compared with only 7.8 months in the poor risk group (≥ 3p) [20].

Yet other studies that have shown impact on prognosis have included treatment, performance status, time passing from diagnosis to start of treatment, number of metastatic lesions, hemoglobin level, white blood count, lactate dehydrogenase, alkaline phosphatase and serum calcium [21]. Another recent study from Saroufim et al 2014 proposed CD105 as an independent prognostic factor in curative resected clear cell RCC [22].

The most robust single prognostic factor is thus still the T-stage. According to a survey published 2014, patients with localized disease carries an excellent prognosis, with a

relative survival of 91.7% [23]. For patients with regional disease, the 5-year survival is 64,2%, which decreases to 12.3% if distant metastasis is evident [23]. However, many previous reports include patients that were treated before modern targeted therapy was introduced on a broad scale. Wahlgren and colleagues have reported increased overall survival for mRCC patients in Sweden treated after introduction of targeted therapy. Patients with metastatic disease diagnosed 2009–2012 and 2006–2008 had a median-OS of 18 and 13 months, respectively, whereas mRCC patients diagnosed 2002-2005 had a median OS of 10 months [24].

1.6 Animal Models of RCC

Few robust animal models for RCC have been presented, but Harlander and collegues introduced an autochtonous mice model mimicking ccRCC by a combined deletion of VHL, Trp53 and Rb1 in renal epithelial cells. The mouse tumors shared several similarities with human ccRCC such as activation of the mTOR pathway detected by high expression of P-4E-BP1. On the other hand, high expression of phospho-ERK indicative of activated MAP-kinase pathway was not detected. In this study, VHL-deletion alone was not sufficient to cause tumor in mice, [25]. In this model, the authors tested sequential therapy with first sunitinib followed by everolimus and the HIF-inhibitor acriflavine upon progression. In general, there was mainly mixed responses, reflecting the heterogeneity of tumor clones within the same individual. This approach offers a possibility to test response-predictive hypothesis in the future.

Another model is described by Murphy et al from University of Minnesota. They have developed a technique of injecting mouse renal adenocarcinoma (RENCA) cells directly into the kidney. This primary tumor has then spread to the lungs, which enables study of metastasized disease [26]. How well this model mimics human RCC regarding genes expressed and activated pathways is not described.

2 TREATMENT

2.1 Surgery

Surgery remains the cornerstone in the treatment of RCC, despite advances in targeted treatment in recent years. Nowadays when possible laparoscopic, nephron-sparing surgery is the standard surgical procedure. When it is not feasible radical nephrectomy en bloc including perirenal fat, adrenal glands, lymphnodes in renal hilus and Gerotas fascia is the method of choice. In the interferon era, nephrectomy in patients with disseminated disease was generally considered superior to only interferon. This approach was supported by a study published by Flanigan and colleagues showing that survival after nephrectomy followed by interferon was 11.1 compared 8.1 months for interferon alone [27]. Similar results were reported by Mickisch et al. in Lancet 2001 [28], where nephrectomy before interferon-treatment significantly prolonged progression-free survival of patients with mRCC.

2.2 Anti-angiogenic treatment

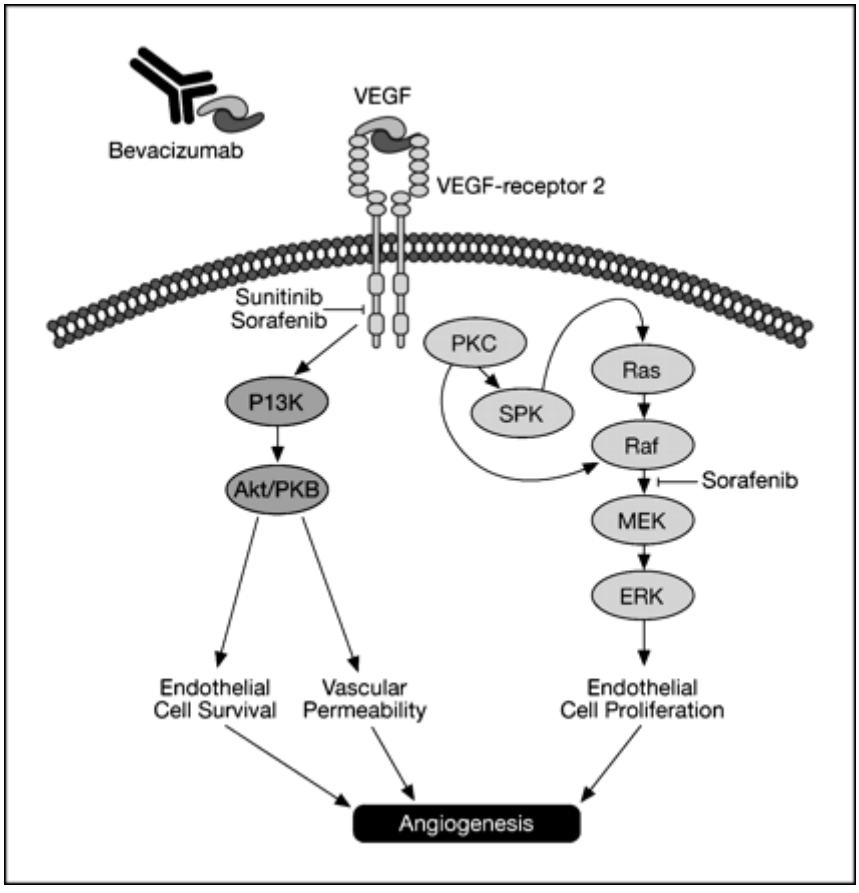
Refractory to conventional chemotherapy, little was to offer patients with metastatic disease prior to introduction of interferon treatment in the early 1980s. The response rate for interferon-treatment have been reported to be from 3,3-31% in selected materials [29]. The therapeutic arsenal was broadened when anti-angiogenic treatment with VEGFR targeting drugs sorafenib and sunitinib were introduced in 2006-2007 [30, 31] followed by the VEGF targeted monoclonal antibody bevacizumab in 2008[32]. These drugs have then been followed by pazopanib [33], axitinib [34] and cabozantinib [35]. Another class of drugs that target angiogenesis are the mTOR-inhibitors (temsirolimus/everolimus), which were introduced around 2010 and is often used in second line or third line, when TKI-treatment has failed [36]. They inhibit the mTOR-serine/threonine-kinase which is a part of the PI3K/Akt-pathway leading to upregulation of HIF, PDGF and cyclinD1 [37].

Sunitinib: This is a small molecule TKI that compete with ATP at the active site on the intracellular portion of tyrosine kinase receptors VEGF, PDGF, c-Kit and Flt-3 and thus prevents the phosphorylation of the substrate which inhibit further downstream signalling pathways [38]. It is administered orally at a dose of 50 mg per day, in a treatment cycle of four weeks on medication followed by two weeks off. In the pivotal phase III-study comparing sunitinib with interferon alpha in the first line setting, 750 patients were enrolled. PFS was 11 months for the sunitinib group vs 5 months for the interferon-alpha

group [31]. Besides RCC, sunitinib has been tried in studied in many tumor types, for example gastrointestinal stromal tumors and hepatocellular cancer [39-42].

Sorafenib: is a small molecule multi-kinase receptor inhibitor targeting VEGF, PDGF- and FGF-receptors as well as kinases KIT, RAF and RET [43]. Sorafenib is reported to have less severe adverse effects [44]. In the pivotal 2007 phase III-study comparing sorafenib with placebo, 903 patients were enrolled to either placebo or 400 mg sorafenib twice a day. The median PFS in the sorafenib group was 5.5 months versus 2.8 months in the placebo group [30].

Bevacizumab: is an anti-VEGF antibody approved for a variety of tumors where it often is used in combination with cytotoxic drugs or interferons. In the pivotal phase-III-trial from 2008, 732 mRCC patients were enrolled and received either IFN + bevacizumab or only IFN. Median PFS in the IFN/bevacizumab group was 8.5 months compared with 5.2 months for IFN alone [45]. Positive results from phase III studies, with bevacizumab combined with chemotherapy, have been reported for colorectal, lung, ovarian and cervical cancer [46-52].



2.3 Immunotherapy

The newest contribution to the pharmacological armamentarium for RCC are the immune-modulators that acts through the receptor of programmed cell death (PD1) and CTLA-4 pathways which activate anti-tumoral cytotoxic T-cells [53].

*Nivolumab***:** is a human IgG4 monoclonal antibody that bind to the PD-1 receptor and prevent its interaction with its ligands PDL-1 and 2 leading to activation of cytotoxic T-cells which in turn attack the tumor cells [53]. Clinical trials with PD-1 inhibitors in patients with unresectable melanoma resistant to other targeted therapies showed objective responses rates in 26–40%, with acceptable toxicity profiles [54]. In a phase III study comparing nivolumab to everolimus in mRCC patients which had progressed on prior treatment with TKIs, nivolumab showed improved survival. Based on this study nivolumab has received approval for therapy in mRCC.

*Ipilimumab***:** is a human monoclonal antibody that bind to cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and inhibit the interaction between CTLA-4 and its ligands, CD80 and CD86. CTLA-4 serves as downregulator of anti-tumor immune-response by inhibiting cytotoxic T-cells from attacking tumor cells, and by blocking CTLA-4, this mechanism is reversed [55]. Ipilimumab is not yet approved for RCC, but at ESMO 2017 Congress in Vienna, early data from a phase III trial comparing a combination of nivolumab and ipilimumab vs sunitininib in first line. In the subgroup of patients with intermediate- or high-risk advanced mRCC, the overall response rate was 42% with immunotherapy vs 27% for sunitinib ($p < .0001$). Complete responses were seen in 9.4% of patients who received the combination of nivolumab and ipilimumab, compared to only 1% with sunitinib (dr Schmindinger, Abstract LBA-6, September 10, 2017). This data might challenge sunitinib in the first line setting, but further studies are required, and it is important to keep in mind that these treatments are very expensive and combination therapy carries high risk for adverse events.

2.4 Adjuvant studies in RCC

Hitherto, adjuvant treatment for RCC has been carried out within clinical trials but is so far not introduced in routine clinical practice. However, long-term follow-up data from several adjuvant studies is now beginning to emerge. One study reported in NEJM 2016 showed prolonged time to recurrence for patients receiving sunitinib for 12 months after

nephrectomy vs placebo [56]. Whether this also confers a survival benefit is debated. Another study published in Lancet 2016 showed no benefit of sunitinib or sorafenib vs placebo for patients with high risk of recurrence post-nephrectomy [57].

3 TUMOR MICROENVIRONMENT IN RCC

3.1 Cancer-associated fibroblasts in RCC

3.1.1 General biology of CAFs

Fibroblasts constitute the major component of the tumor stroma, and are often referred to as cancer-associated fibroblasts, or CAFs. They show specific phenotypic characteristics that differs from normal fibroblasts. For example, CAFs cultured in vitro show more rapid proliferation compared with normal fibroblasts [58]. The origin of CAFs is subject for debate. Suggestions include hematopoietic stem cells [59], adipose-derived stem cells [60], pericytes [61], endothelial cells [62], bone marrow -derived mesenchymal stem cells [63] and residing fibroblasts, whose transformation is induced by TGF-β [64]. TGF-β can be secreted by the tumor cells but also by CAFs themselves in an autocrine fashion creating a positive feedback loop [65]. Other growth factors secreted by CAFs include FGF, IGF, HGF, EGF [66].

Several studies using mice models have demonstrated an ability of CAFs to support tumor growth. Overexpression of HGF and TGF-β in mouse breast stroma induces malignant transformation of epithelial cells [67] and TGF-β expression by fibroblasts induced intra-epithelial neoplasia in the prostate gland and invasive squamous-cell gastric carcinoma in mice [68]. Further, CAFs, but not normal fibroblasts, could induce growth of tumors in mice grafted with simian virus 40 (SV40)-transformed 'normal' prostate epithelial cells [69-71].

Common markers for CAFs include α-SMA, vimentin, endosialin, podoplanin, FSP-1, FAP, PDGFR-α and PDGFR-β [72-79].

*Metastasis***:** Metastasis development is a complex process still not fully understood. CAFs probably play a role both at the primary as well as the metastatic site. Pro-metastatic effects of CAFs at the primary site was originally proposed based on findings mesenchymal stem

cells, used as CAF models, when co-injected with cancer cells could promote metastasis in a breast cancer model [80]. Luga et al showed that fibroblasts can release exosomes, which induces autocrine Wnt-PCP signaling in tumor cells leading to metastasis [81]. In another study, combining tissue culture with animal experiments, Pena et al showed pro-migratory and invasive effects by PDGF-stimulated fibroblasts [82]. Similar findings have been made concerning TGF-beta activated fibroblasts [83]. Pre-clinical studies have also suggested that activation of fibroblasts is a critical component in the formation of a pre-metastatic niche [84, 85].

Cancer stem cell support: Malanchi and colleagues reported 2011 of how a small population of cancer stem cells was crucial for metastatic growth through the expression of the extracellular matrix protein periostin by CAFs. Invading tumor cells needed to induce periostin expression by stromal cells in the target organ to manage to establish metastatic growth. Periostin seems to be involved in Wnt-signaling in the cancer stem cells through recruiting Wnt-ligands [86]. In colon cancer, Wnt signaling has been implicated to maintain stem ness not only in normal colon stem cells but also in their malignant counterparts [87]. According to Medema et al, CAFs-derived factors activate Noch and Wnt pathways which eventually promote cancer stemness [88]. Identification of such factors, like HGF, opens new opportunities for targeted therapy [83].

CAFs and Immune cells**:** Inflammatory processes accompanies tumor growth and progression [89]. CAFs exhibit pro-inflammatory gene signature and recruit macrophages in mouse model of squamous cell carcinoma. Interestingly, normal skin fibroblasts can be "educated" by cancer cells to express pro-inflammatory genes [90]. Elimination of CAFs in a murine model of metastatic breast cancer induced a shift from Th2 to Th1 in tumor stroma, increased expression of IL-2 and IL-7, and reduced recruitment of macrophages and regulatory T-cells leading to improved effect of doxorubicin [91]. Depletion of FAP-positive CAFs can enhance the immune response, and lead to tumor regression in pancreatic cancer models [92]. Another model of pancreatic cancer showed better outcome with immune-modulatory drugs if CAFs were depleted [74]. Furthermore, melanoma-derived fibroblasts have in co-culture experiments been shown to interfere with NK-cell cytotoxicity and cytokine production [93].

*Drug uptake***:** The effect of chemotherapeutic drugs depends on the uptake of the compound by the cancer cells. One obstacle for drug delivery is the interstitial pressure within the

tumor, which counteracts the passage in to the tumor cell [94]. Reduction of the interstitial pressure by enzymatic degradation of fibroblast-derived hyaluronan was shown to re-expand the vasculature and improve drug delivery [95]. In another study, PDGFR-β-antagonists, targeting CAFs, reduced interstitial fluid pressure and improved transcapillary transport and thus uptake of cytotoxic drugs as well as radio-immunotherapeutic antibodies [96]. Pietras et al showed that inhibition of PDGFR-β signaling improved therapeutic effects of Taxol and 5-fluorouracil in animals [97, 98]. The observation that fibroblast-targeting can improve tumor drug-uptake has also been supported by studies using hedgehog-inhibitors targeting the stroma in models of pancreatic cancer [98, 99].

*Drug sensitivity***:** Beside the effects on drug uptake, CAFs can also regulate drug sensitivity through paracrine signaling which reduce sensitivity to chemotherapeutic agents [99]. Furthermore, CAF markers or derived factors, such as the PDGFR-family have demonstrated independent association with survival [100]. Microarray studies have created gene expression signatures that is indicative of an activated fibroblast state. These "stroma signatures" have been investigated with regard to specific CAF features that correlates with prognosis in independent data sets of breast and lung carcinoma cohorts. In breast carcinoma, fibroblast features have been shown to influence response to therapy [100]. IHC-based analyses from two randomized breast cancer cohorts exploring the role of stromal PDGFR-β showed that benefit of adjuvant tamoxifen was significantly higher in patients with low PDGFR-β [101].

3.1.2 Treatment targeting CAFs

Today, there are no anti-tumoral drugs that act exclusively on CAFs, but many of the TKIs previously described are inhibitors of PDGF receptors which are well-known regulators of CAFs. How this inhibition contributes to the therapeutic effect is not clear. Among other CAF-related targets, FAP and TGF-beta have been subjected to clinical investigation, but studies have so far been inconclusive with no marked tumor responses reported [102-104].

3.1.3 Mechanistic studies of CAFs in RCC

Mechanistic studies of CAFs in RCC are somewhat limited, but an increasing interest for the topic is seen. CAFs cocultured with RCC-cells showed increased proliferation and migration as well as reduced sensitivity to everolimus compared with RCC-cells grown without CAFs [105]. Another study showed that co-culture of mouse fibroblasts with human RCC-cells increased periostin transcription and accumulation leading increased

fibroblast cell proliferation and Akt-activation [106]. ERK-activation in fibroblasts was not inhibited to the same extent as in endothelial cells in RCC-cultures treated with sunitinib, implicating that fibroblasts are involved in resistance to ant-angiogenic drugs [107].

3.1.4 Prognostic and response-predictive studies of CAFs in RCC

CAF activation detected by FAP has been shown to correlate with prognosis in RCC [108], and another study indicated that accumulation of stromal paladin was associated with worse outcome [109]. To our knowledge, there is no fibroblast-derived response-predictive marker described in the literature.

3.2 Vessels and Pericytes in RCC

3.2.1 General tumor biology

During the embryogenesis vessels are created in two ways:

- vasculogenesis: the formation of new endothelial cells and their assembly into tubes.

- angiogenesis: new vessel sprouting from existing ones.

One situation where angiogenesis plays an important role is in the case of wound healing. Under normal conditions this process is strictly regulated and is turned off after playing its physiological role. Tumors exploit these programs for induction of new vessel formation [110]. Tumor vessels develop in response to angiogenic chemokines, produced by both stromal and cancer cells [111]. Multiple pro- and anti- angiogenic factors have been identified. Some of the most studied factors are the pro-angiogenic VEGF-A and the angiogenesis-suppressor TSP-1. VEGF-A is important during embryonic development but also in supporting physiological endothelial cell homeostasis. In cancer tissue VEGF-A can be activated by e.g. altered oncogene signaling or hypoxia [112].

Under normal circumstances, the vasculature is organized in a strict hierarchical manner including: arteries, arterioles, capillaries, venules, and veins. Tumor vasculature, however, is composed of vessels with bizarre and disorganized appearance. They are larger than normal vessels, irregularly shaped and leaky. Because of these abnormalities, nutrients and oxygen supplies are altered, as well as removal of waste products. Altogether, these changes lead to hypoxia and lower pH in tumors [113], a fact that is believed to be an

important step in tumor progression [114]. Tumor vasculature is related not only to primary tumor growth, but also to cancer cell intravasation and formation of distant metastases [115]. HIF-1/α has been shown to regulate nitric oxide turnover in endothelial cells, thus affecting tumor cell extravasation and migration in opposite ways. HIF-1α knockout leads to decreased ability to form metastasis, while deletion of HIF-2α has the opposite effect [116].

In 1923, K.W. Zimmermann introduced the term "pericytes" and postulated that these are advential cells located within the basement membrane of capillaries and postcapillary venules. Pericytes provide support to the endothelial cells by stabilizing the vessel wall and takes part in the regulation of blood flow [117]. Precapillary arterioles are surrounded by smooth muscle cells, which give them their contractile properties. Several different markers have been used to describe pericyte status.

One widely used marker is α-smooth muscle actin (α-SMA) which is expressed by tumor pericytes but is lacking in normal tissue [118]. Another pericyte marker is desmin, which is an intermediate filament protein expressed both in normal and tumor pericytes [118].

PDGF receptor β, and its ligand PDGF-B, are known to be involved in pericyte recruitment in mouse models [119]. Other molecular pathways related to pericyte function include TGF-β, S1P1 and EDG1, Ang1 and Tie2 [120].
In the mature vasculature, the blood vessels are usually covered by pericytes and recruitment of them is important for the formation and stabilization of blood vessels [121]. Based on this observation, pericyte coverage is regarded as an indicator of the grade of maturation.
Absence of pericytes in tumor vasculature is shown to be associated with metastasis and a shorter survival in patients with colorectal cancer [122]. A study by Qian et al. indicated that undifferentiated micro vessels are not covered with pericytes, which correlated with poor prognosis [123]. In this study, irregular coverage was seen in a fraction of the differentiated vessels. These findings in turn run counter to the findings by Cao et al., described in [124].

Tumor perivascular smooth muscle cells and pericytes (PVCs) have abnormal structural features, consistent with the features of tumor vasculature [125]. Tumor pericytes and smooth muscle cells are often detached from endotheliocytes, which has been suggested to

facilitate endotheliocyte motility as a part of vasculogenesis. Tumor PVCs are also characterized by irregular shape and abnormal cytoplasmic processes [118]. As outlined in the following section, a series of pre-clinical studies imply pericyte status as a determinant of tumor growth, metastasis and response to VEGF-targeting anti-angiogenic therapies. Altogether, this indicate that undifferentiated vessels are completely immature, whereas differentiated vessels could be sub-divided into mature differentiated vessels and immature differentiated vessels, depending on the grade of pericyte coverage.
Tumor growth: As mentioned above PDGF signaling is involved in pericyte recruitment. Preclinical 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 [126]. However, when overexpression of PDGF-BB ligand was induced in colorectal and pancreatic cancer models marked tumor growth inhibition was noted, together with increased pericyte coverage [127]. This study [127] also noted enhanced tumor growth following treatment with a PDGF-inhibitor, which together with reduced PVC coverage rate.

*Metastases***:** Both stimulatory and inhibitory effects of PDGF-dependent pericytes have been seen in animal models exploring links between pericytes and metastasis. Moreover, it was shown that in PDGF-BB-expressed tumors inhibition of PDGFβ was associated with increased metastasis which was coupled to tumor hypoxia and HGF-mediated EMT of tumor cells [128]. The level of hypoxia might thus be important for the role the pericytes play for metastatic ability. This was further supported by LeBleu et al. showing that pericyte depletion in primary mammary tumors in mice led to decreased ability to form lung metastasis in non-hypoxic, small tumors but the opposite in more advanced stages where hypoxia was evident [129].

However, contrasting effects were seen in other studies, where reduced pericyte coverage was linked to decreased formation of metastasis [119]. The latter study suggested that the net-effect on metastasis of reducing PDGF-dependent pericyte coverage would differ between tumors which displayed a high or low pericyte coverage. Experiments by Augustin et al. on endosialin-knockout and wild type mice showed that endosialin-expressing pericytes enhanced formation of distant metastasis without affecting the growth of the primary tumor, probably due to facilitation of tumor cell intravasation, since the number of circulating tumor cells was higher in mice endosialin-expressing mice [130]. In the same

study, the authors reported a correlation with high endosialin expression and increased metastasis rate in human mammary cancer. This group followed up with a study in Nat. Comm. 2017 where they showed that deletion of Tie2 in pericytes resulted in increased tumor growth indicating the importance of angiopoietin/Tie signaling in pericytes [131].

3.2.2 Pericytes and immune cell interactions

Besides the role pericytes play in the vascular integrity/metastasis setting, there is also reports of their influence on leucocyte migration. In a study by Genové et al using a mouse model with genetic pericyte deficiency, they found a defect vasculature leading to a more hypoxic environment and upregulation of Il-6 in tumor cells. Il-6 in turn recruited myeloid derived suppressor cells (MDSCs) myeloid derived suppressor cells (MDSC) which inhibits anti-tumor T-cell activity . Restoration of pericyte coverage was found to dampen infiltration of MDSCs [132].

Effects on sensitivity to VEGF-targeting drugs: Based on early studies in mouse models, Folkman suggested anti-angiogenic therapies as a novel approach for anti-cancer drugs [133]. Pivotal studies in animal models with VEGF-blockade provided experimental support for this notion [127]. This led to a series of clinical trials demonstrating benefit of anti-VEGF-agents, used in combination with chemotherapy, in colorectal, breast and lung cancer [52, 134-136]. Positive xenografts studies with mono-treatment with VEGF-inhibitors have also been reported for renal cancer, ovarian cancer and neuroendocrine tumors [137, 138]. Anti-VEGF-treatment is this now an established modality, although survival effects in metastatic settings are in general modest (reviewed in [111] and [139]).

Anti-VEGF inhibitors are generally believed to exert their anti-tumoral effects through vessel-targeting and a “starvation-effect” on tumors. In addition to this mechanism a “vascular normalization” hypothesis has been proposed. According to that hypothesis anti-VEGF therapy rather normalize vessels then reduce vessel number. 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 phenotype of more normal-like vessels with proper pericyte coverage [140-142]. These findings have also been used to argue that pericyte-coverage is a determinant of the “starvation-effect” of anti-VEGF-drugs, and as such represent candidate biomarkers for sensitivity and resistance to these drugs.

There is now emerging evidence that some tumors rather than inducing angiogenesis in

some circumstances instead take advantage of preexisting blood vessels from the surrounding normal tissue and incorporate them in the tumor. This process is described as vessel co-option or vascular co-option [143-145]. Several studies have shown that this is the case in non-small-cell lung cancer, where a “non-angiogenic” subtype show growth of cancer cells in the alveoli. Intact alveolar walls including its capillaries are here incorporated [146-150]. Similar results have been reported in cases of human lung metastasis [151, 152]. This finding has also been reproduced in preclinical models of lung metastasis [153].

3.2.3 Prognostic studies of vessels and pericytes

Mechanistic studies have implicated that pericyte depletion leads to different outcome depending on when in the tumor development it occurs as discussed above. In addition to the animal models, Viski et al. also studied a cohort of human mammary tumors and found that high endosialin expression was associated with shorter survival [129]. The same result was seen for angiopoietin-2 expression in human mammary cancer, with high expression correlating to shorter survival [129]. Other prognostic studies have shown that pericyte status also might be determined by expression of PDGFR-α and -β which have been reported for colorectal [154] and ovarian cancer [155].

3.2.4 Mechanistic studies of vessels and pericytes in RCC

Mechanistic studies of vessels and pericytes in RCC are scarce, due to few established animal models for RCC. In one study from 2001, Hemmerlein et al. [156] performed experiments including microspheres of cultured RCC-cells and suggested that high-proliferative RCCs outgrow their vascular supply and develop chronic hypoxia, which decreases proliferation rate.

3.2.5 Prognostic studies of vessels and pericytes in RCC

RCC is a hyper-vascularized tumor where data on the relation between tumor vascularity and prognosis are conflicting [157]. Yoshino et al [121] reported that patient survival was significantly improved if the tumors had lower micro vessel density (MVD), while a meta-analysis including 15 studies showed no correlation to overall survival [158]. Thus, the grade of maturation of vessels might be important, as well as the extent of pericyte coverage, which is proposed by Cao et al [124]. They found that a higher pericyte coverage (PC) was related with more aggressive disease, and that the MVD:PC ratio was a more reliable prognostic factor than MVD alone [124].

3.2.6 Response-predictive studies of vessels and pericytes in RCC

Much attention has been attributed to find response-predictive markers among the angiogenesis related factors in RCC, given the impact of these factors in the initiation and development of this disease. So far, no exclusively response-predictive factor has thus been identified and validated. One candidate marker is the microvessel area (MVA), and a study by Aziz et al report a correlation with high MVA and better response to sorafenib [159] in a cohort of 96 patients. In this study, they also evaluated the impact of VEGF, VEGF-R1, VEGF-R2, VEGF-R3, c-RAF, B-RAF, c-Kit, and PDGFR-β expression in primary tumor tissue without finding any correlation to outcome.

Other studies have focused on circulating angiogenic factors like VEGF. Analysis of baseline circulating VEGF-levels from patients included in the pivotal phase-III study for sorafenib showed that both high and low VEGF-groups benefited from sorafenib but those patients with highest VEGF-levels benefited more [160].

Yet another type of vessel-related factors that have been studied are the single-nucleotide polymorphisms (SNPs) in the VEGF-pathway. One study by Beuselinck et al showed that a variant in VEGFR1 (CC-genotype in VEGFR1 SNP rs9582036) predicted short PFS and OS on sunitinib-treatment [161].

3.3 B-cells in RCC

3.3.1 General tumor biology

B-cells plays an important role in the humoral part of the adaptive immune system and is vital for upholding tissue homeostasis in mammals. Their main task is to produce and secrete immunoglobulins after they have been presented for antigens by antigen-presenting cells. [162]. B-cell development starts with a hematopoietic stem-cell in the bone marrow through pro-B cell (CD19+ CD20- Ig-) via pre-B cells (CD19+ CD20+ Ig-) to immature B-cells (CD19+ CD20+ Ig+) The immature B-cells leaves the bone marrow and enter in to the blood stream to relocate to the peripheral lymphoid tissues, becoming naïve B-cells (CD19+ CD20+ Ig+ CD38+/-). After being presented for antigens, they become active naïve B-cells (CD19+ CD20+ Ig+ CD38+) [163].

When maturation is complete and the B-cells have migrated to their peripheral site, stochastic events determine further differentiation into subsets with specific functions. Examples of subsets are: B-1 cells which produce natural antibodies, B-2 marginal zone precursors, B-regulatory cells (Bregs) and plasma cells which secrete immunoglobulins

[164]. Bregs secrete IL-10, which is an anti-inflammatory cytokine which dampens immune response [165]. A majority of B-cell lineages express the cell surface receptor CD20 (except pro B-cells and plasma cells). CD20 is a Ca2+ ion channel [166] important for antibody-responses not involving T-cells [167]. CD20 can be targeted by monoclonal antibody therapy with rituximab which is successfully used in B-cell lymphomas [168]. Besides the important role in maintaining tissue homeostasis, B-cells have during the last decade become known as actively involved in initiation and formation of solid tumors. Gunderson and Coussens describe in review several ways in which B-cells contribute to tumorigenesis [169]. One is through deposition of immune complexes consisting of immunoglobulins and complement factors in the TME, leading to ligation of the immune complexes to either FcγR or C5aR expressed on myeloid cells infiltrating the tumor. These interactions lead in turn to expression of various cytokines and initiation of T-helper 2 cell expansion and inhibition of cytotoxic T-cells, as well as secretion of pro-angiogenic and pro-survival factors.

In a study published in Cancer Cell 2014 by Affara et al, B cells were found to enhance HPV16 induced squamous cell carcinoma in mice through deposition of immune complexes. Depletion of B-cells led to slower tumor progression and made the tumors more susceptible to chemotherapy. In a mouse model of orthotopic pancreatic cancer, Pylayeva-Gupta et al showed that depletion of B-cells in KRAS-mutated mice significantly reduced tumor volume and the pro-tumorigenic effect was exerted by IL-35 secretion from a subset of B-cells [170]. Another KRAS-mutated murine pancreatic cancer model is described by Lee at al [171]. They used an autochtonous cancer model to show how deletion of HIF-α increased tumor growth and was accompanied by infiltration of B-cells, an effect that could be reversed by using CD20-monoclonal antibodies.

3.3.2 Mechanistic studies of B-cells in RCC

Animal models of RCC where B-cells are studied are to our knowledge not described in the literature. Cell culture experiments focusing on B-cells in RCC is also scarce, but one article Cai et al report of IL-10-secreting B and T-cells in RCC [172]. Il-10 secreting cells had generally lower expression of CD19 and CD20 compared with non-IL-10 producing cells, and also lacked IgM and IgD. Further, Il-10 was found to suppress the immune system by recruiting Tregs [172].

3.3.3 Prognostic studies of B-cells in RCC

Prognostic studies of B-cells alone have not been previously described, but several studies

report of tumor infiltrating lymphocytes (TILs, which is both B- and T-cells) that have prognostic relevance. One study by Wang et al. compared RCC TILs to peripheral blood lymphocytes (PBL) in RCC-patients or melanoma TILs, and found that RCC had fewer CD27+ T-cells, and less naïve and central memory T-cells than melanoma, but instead more effector memory T-cells [173]. Their hypothesis was that the RCC tumor microenvironment were skewing the TIL phenotype toward effector memory T-cells. Another study by Liotta et al. showed that regulatory T-cells were significantly higher in TIL than in peripheral blood of patients with RCC. Regulatory T-cells showed in vitro an inhibitory activity on effector T cells isolated from kidney tumors. The increase in both peripheral and intra-tumoral regulatory T-cells was associated with worse prognosis [174]. Notably, also infiltrating CD8+ T-cells was associated with more aggressive disease and higher risk of recurrence if they expressed PD-1 and Tim-3, which shows that not only Tregs are suppressing anti-tumor immunity [175]

3.3.4 Response-predictive studies of B-cells in RCC

No studies presenting B-cells in the response-predictive setting have, to my knowledge, been published.

4 PRESENT INVESTIGATION

4.1 Aims

The aim of this thesis was to analyze the tumor micro environment of RCC and search for prognostic markers and response-predictive markers for tyrosine kinase inhibitors. Analyses of tumor vasculature and fibroblast-rich stroma were performed using a computerized algorithm. Selected immune cells were analyzed using conventional semi-quantitative scoring. Analyses were performed on primary tumors from different RCC cohorts.

4.2 Results

Article I

Perivascular PDGFR-β is an independent marker for prognosis in renal cell carcinoma.

In this study, a cohort of 314 untreated RCC-cases was investigated regarding perivascular and vessel characteristics using IHC-double stainings with CD34 together with either PDGFR-β or α-SMA. Initial analysis revealed a heterogeneous perivascular expression of these markers in RCC. For each of the two perivascular markers, two metrics were collected: fraction of covered vessels (FCV) and median intensity of staining in perivascular areas (PVI). A correlation study was performed showing both PDGFR-β and α-SMA FCV being negatively correlated to vessel density, but neither was correlated to vessel size. A significant positive correlation was also detected between the two perivascular markers.

Data on perivascular status, vessel density and vessel size were analysed regarding associations to clinic-pathological characteristics. Analyses showed that high perivascular PDGFR- β expression was associated with high tumour stage and high Fuhrman grade. High perivascular α-SMA was significantly associated with high Fuhrman grade but not to stage. For vessel density and size, there was an association with high Fuhrman grade. Low vessel size but not vessel density was associated with high T-stage. An association was also seen between male sex and low vessel density.

Analyses were expanded to include associations to clinical outcome, which revealed a significant correlation between high PDGFR-β FCV, as well as high α-SMA FCV, and shorter OS. For low PDGFR- β, OS was 48 months vs 26 months for high PDGFR- β. For α-SMA, median OS in the low-expression group was 52 months vs 29 months in the high-

expression group. Also vessel density was associated with worse outcome; the group with low vessel had a median survival of 29 months vs 43 months for the high vessel density group.

These results were then corrected for clinicopathological parameters in a multivariate analysis, including T-stage, nuclear grade, histology, metastatic disease upon presentation, patient age and sex. This analysis showed that all three metrics acted as independent predictors for overall survival in RCC.

An in-depth analysis was undertaken to investigate the impact of the three metrics in patient subgroups according to their clinico-pathological features. This showed a significant association of high PDGFR-β FCV with poor survival in patients with high T- stage of disease (T4), older patients, male patients and patients with clear cell type renal cancer. High α -SMA FCV was correlated to poor survival in T4-tumours and tumours of clear cell histology. Vessel density showed an association with survival in the T4 group, high age, clear cell histology, metastasis at diagnosis and in female patients.

The variation of perivascular expression of PDGFR-β of individual vessels enabled determination of an index related to intra-case heterogeneity of perivascular intensity, described as inter-quartile range of perivascular intensity (PVI IQR). Analyses showed that high heterogeneity was significantly associated with shorter survival in both uni- and multivariate analysis. This novel metric was introduced in an ovarian cancer cohort of 138 patients, showing similar results regarding overall survival in both uni- and multivariate analysis.

Article II

Multi-parametric profiling of renal cell, colorectal and ovarian cancer identifies tumor-type-specific stroma phenotypes and a novel vascular biomarker.

In this article, patient cohorts from three different tumor types were analysed and compared regarding stromal- and vessel phenotypes including intra-tumor heterogeneity.

Tumor stroma: Regarding stromal features, RCC showed the highest intensity of PDGFR-β staining in the tumor stroma, whereas the fraction of tumor area positive for PDGFR-β was highest in CRC.

Vessels: RCC cases showed higher vessel density as compared to OC and CRC. Regarding vessel size, analyses showed that both absolute values and intra-case variation of vessel size were larger in OC in comparison to CRC and RCC

Perivascular cells: CRC-cases displayed higher absolute values and higher intra-case variation of perivascular PDGFR-β -expression than OC and RCC.

Correlation analysis: Values for vessel density, vessel size, and perivascular status were correlated pairwise within the different tumour types.

Associations were in general low. In RCC, but not in the other two tumor types, cases with higher vessel density were also characterized by lower perivascular expression of PDGFR-β. Strong positive correlations were seen for all three tumor types regarding PDGFR-β positive stroma fraction and perivascular intensity of PDGFR-β.

Heterogeneity as a marker for poor prognosis

In article I, we demonstrated that high heterogeneity of perivascular expression of PDGFR-β is associated with poor prognosis in RCC and OC. In article II, we introduce a novel metric termed 'vessel distance IQR', which describes the variation in distance to the closest vessel for each vessel in the tumor sample. This metric was analysed regarding its association with clinico-pathological characteristics and overall survival. High heterogeneity in vessel density were found to be significantly associated with shorter overall survival in RCC and CRC but not in OC.

High vessel distance IQR correlated with female sex, advanced T- and M-stage and low differentiation in CRC. In RCC, this metric was correlated with male sex and high Fuhrman grade. In OC, no significant association was found between vessel distance IQR or any of the clinico-pathological parameters.

Article III

Identification of a CD20/ MS4A1-high minority-group of renal cell cancer associated with poor prognosis.

In paper III, two cohorts of RCC-patients were evaluated regarding infiltration of B-lymphocytes. A gene signature for B-cells were then analysed in a TCGA-cohort to validate the findings from the initial IHC-based findings. In the main cohort, 297 RCC patients were analysed regarding CD20+ lymphocyte infiltration. A large majority of cases displayed absence of, or only low infiltration of CD20+ lymphocytes. In a minority of patients (14%), a prominent infiltration of CD20+ cells were seen. This high CD20-group was found to have significantly shorter overall survival compared with patients with low or no infiltration of CD20+ cells. These findings remained significant in a multi-variable analyses correcting for sex, age, histology, T-stage, M-stage and Fuhrman-grade. The B-cell-high group was also associated with high Fuhrman grade and a trend towards association with metastasis (M-stage) was observed. Regarding B-cell-status and sex, age, tumor stage or histology, no associations were detected.

The findings from the main cohort were validated in a second cohort of 64 sunitinib-treated mRCC-cases. In this cohort, 28 % was identified as having high infiltration using the same cut-off level as in the main cohort. In this cohort B-cell-status was not found to be associated with any clinic-pathological parameters including sex, age, histology, MSKCC-grade, T- or M-stage. Survival analyses in this cohort confirmed a significant association between shorter overall survival and high B-cell infiltration.

To further test the hypothesis based on the IHC-based findings, a B-cell gene signature was created using a combination of MS4A1 (CD20-gene), CD19 and PAX5 (B-cell transcription factor) for each patient. Based on the dichotomization of the population-based cohort, a group of high B-cell signature score was defined composed of the 14% of cases with highest score. Analyses showed a significantly shorter survival in the B-cell-signature-high group in uni-variable analysis. However, this did not remain significant in multivariable analysis including sex, age, T-, N- and M-stage.

Article IV

Vessel diameter predicts response to sunitinib in mRRC.

In article IV, a cohort of 137 sunitinib-treated mRCC-patients were analyzed in order to analyze potential associations between vessel characteristics and response to sunitinib. When dividing the study-population into groups with low, intermediate or high median vessel diameter based on CD34-IHC (CD34D), we found that intermediate CD34D was associated with longer PFS and "sunitinib-OS" in uni-variable Cox-regression analysis in sunitinib-treated mRCC. The difference remained significant for both PFS and OS in multi-variable Cox-regression analyses including MSKCC-score, sex, histology or age. Intermediate CD34D was not associated with sex, age, histology and MSKCC-score. The CD34D-defined groups did not show differences regarding vessel density or perivascular intensity of PDGFRβ.

Additional analyses explored if CD34D acted as a true response-predictive factor, or if survival associations rather reflected a more aggressive phenotype. In this context time to development of metastasis was used as proxy for the intrinsic aggressiveness of the disease. CD34D was not associated with time to development of metastatic disease in the sunitnib-treated cohort. Furthermore, CD34D was not associated with survival in a larger cohort of sunitinib-untreated patients.

4.3 Conclusions and future perspectives

The aim of the first two studies was to characterize stromal and vascular features and their relevance for the disease course. The third study was aimed at investigating the importance if infiltrating B-cells and the fourth study was set up to detect possible vessel-related response-predictive markers for sunitinib.

Article I was focusing on perivascular status as determinants for prognosis in RCC, while article II was a comparative study regarding stromal and vascular features in RCC, CRC and OC.

Results from these two studies support the assumption that the tumor micro environment has an impact on tumor growth and prognosis in some but not all tumors and that TME-contribution to tumorigenesis is a complex and not yet fully understood process.

The differences in stromal and vascular features across tumor types might reflect differences in underlying driver mechanisms which are initiated at the earliest steps in tumor formation, and directs the tumor stroma and perivascular/vessel development in a context-dependent way. Tumor-type-specific features could also reflect differences in physiological “stroma-programs” of the tissues.

Both models might explain the apparently contradictive results from pericyte manipulation in various tumor models. It is also recognized that yet unknown biological effects of pericytes might contribute to the observed survival associations. The advent of novel RCC mouse models [25] should allow continued mechanistic studies on the role(s) of PDGFR-β-positive pericytes in RCC initiation, growth and progression. Further validation of the potential of these findings in independent cohorts and continued experimental studies is required for identification of underlying biological mechanism(s) between perivascular PDGFR-b and survival.

One limitation of study I and II is the small size of tumor samples of TMA-based cohorts, given the known intra-case heterogeneity of tumors.

In paper III, the significance of infiltrative B-lymphocytes is studied in the context of prognosis, where a high infiltration is associated with shorter survival in two independent cohorts and also supported by a B-cell gene signature in a TCGA-dataset. The interest for infiltrating immune cells has shown a surge in the last five years, and probably immune-modulating therapies will replace anti-angiogenic drugs in first line treatment for mRCC. The CD20+ lymphocytes found in article III may represent a subset of B-cells with regulatory function that possibly recruit Tregs which in turn hampers immune response. Continued research in this area should incorporate analyses of other immune cell types. As

above, the novel RCC mouse model appears as an interesting resource for studies where effects of B-cell-depletion can be analyzed. In summary, the observations in this study suggest validation studies on independent RCC-cohorts with known clinical, genetical and molecular data. This sort of studies could give information regarding what molecular and genetic features that are associated with CD20-infiltration, and also suggest mechanistic explanations.

In article IV, an association with median vessel size and prolonged PFS and OS was found in a cohort of 137 sunitinib-treated patients. This finding implies that vessel size determines the susceptibility to sunitinib, possibly due to more sensitive pericytes which when targeted destroy the tumor-supporting vasculature. One way to test this is to perform pre- and post-treatment biopsies from RCC-bearing mice subjected to sunitinib therapy. This approach would also allow for mechanistic studies and a more in-depth molecular profiling. Similar studies can be envisioned in biopsy-based clinical studies, where vessel size properties can be compared before and after treatment. As discussed above it is recognized that a limitation of the study is the reliance on primary tumors rather than the metastatic lesions that are subjected to treatment. The correlative findings of study IV should also be validated in independent patient cohorts with sunitinib-treated RCC-cases. Furthermore, future studies should investigate potential associations between vessel size and sensitivity to other vessel-targeting RCC drugs such as pazopanib. Ideally, these validation cohorts should come from randomized trials were RECIST-data for detection of true progression are included.

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