Novel Biomarkers in Metastatic Renal Cell Carcinoma

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“Let us now pause for a moment of science”
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Original Publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:


## Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AE</td>
<td>Adverse event</td>
</tr>
<tr>
<td>Ang-1 &amp; -2</td>
<td>Angiopoietin 1 and -2</td>
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<tr>
<td>AS</td>
<td>Active surveillance</td>
</tr>
<tr>
<td>ASI</td>
<td>Angiotensin system inhibitor</td>
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<tr>
<td>BHD</td>
<td>Birt-Hogg-Dubé</td>
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<tr>
<td>c-Met</td>
<td>Tyrosine protein kinase Met</td>
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<tr>
<td>CAFs</td>
<td>Cytokine and angiogenic factors</td>
</tr>
<tr>
<td>CAIX</td>
<td>Carbonic anhydrase IX</td>
</tr>
<tr>
<td>ccRCC</td>
<td>Clear-cell renal cell carcinoma</td>
</tr>
<tr>
<td>chRCC</td>
<td>Chromophobe renal cell carcinoma</td>
</tr>
<tr>
<td>ColIV</td>
<td>Collagen IV</td>
</tr>
<tr>
<td>CR</td>
<td>Complete response</td>
</tr>
<tr>
<td>ctDNA</td>
<td>Circulating tumor DNA</td>
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<tr>
<td>DFS</td>
<td>Disease-free survival</td>
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<tr>
<td>EC</td>
<td>Endothelial cells</td>
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<tr>
<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
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<tr>
<td>ESMO</td>
<td>European Society for Medical Oncology</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FH</td>
<td>Fumarate hydratase</td>
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<tr>
<td>FLCN</td>
<td>Folliculin</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-induced factor</td>
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<tr>
<td>HLRCC</td>
<td>Hereditary leiomyomatosis and renal cell cancer</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>HPRC</td>
<td>Hereditary papillary renal cancer</td>
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<tr>
<td>HTN</td>
<td>Hypertension</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMDC</td>
<td>International Metastatic Renal Cell Carcinoma Database Consortium</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid suppressor cells</td>
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<tr>
<td>MiRNA</td>
<td>MicroRNA</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRCC</td>
<td>Metastatic renal-cell carcinoma</td>
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<tr>
<td>MSKCC</td>
<td>Memorial Sloan Kettering Cancer Center</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>ORR</td>
<td>Objective response rate</td>
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<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PD</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death 1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death ligand-1</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PLGF</td>
<td>Placenta growth factor</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>PR</td>
<td>Partial response</td>
</tr>
<tr>
<td>pRCC</td>
<td>Papillary renal cell carcinoma</td>
</tr>
<tr>
<td>PTP1B</td>
<td>Protein tyrosine phosphatase 1B</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
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<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SD</td>
<td>Stable disease</td>
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<td>SDHB</td>
<td>Succinate dehydrogenase B</td>
</tr>
<tr>
<td>SDHD</td>
<td>Succinate dehydrogenase D</td>
</tr>
<tr>
<td>SMGs</td>
<td>Significantly mutated genes</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SRM</td>
<td>Small renal mass</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor-node-metastasis</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin 1</td>
</tr>
<tr>
<td>T-regps</td>
<td>Regulatory T-cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
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ABSTRACT

During the past decade, basic and translational cancer research has provided new information regarding the mechanisms underlying tumor growth, proliferation, and metastasis in renal cell carcinoma (RCC). Understanding of the molecular pathogenesis of RCC has identified new targets for therapeutic intervention.

Angiogenesis, involving complex signaling pathways such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, and angiopoietins, plays a pivotal role in tumor growth and progression, and therapies targeting angiogenic factors from the VEGF family have become an effective strategy in the treatment of metastatic renal-cell carcinoma (mRCC) since 2006.

Sunitinib and pazopanib are tyrosine kinase inhibitors (TKI) and they are globally used as first-line treatments for mRCC. Although they primarily act through inhibiting angiogenesis, they might also have an effect on the function of immune cells such as T-reg. Despite the initial enthusiasm for these modern-era targeted therapies, however, some patients only receive moderate benefit, and resistance to these therapies eventually develops in all patients. At present, no biomarkers predicting treatment efficacy are known, despite intensive translational research during the last decade.

While the main component of the angiogenic process in RCC is VEGF, targeting of the mammalian target of the rapamycin (mTOR) pathway is important, because activation of the upstream PI3K/Akt/mTOR signaling pathways is one method by which constitutive HIF-1α activation or upregulation occurs. Everolimus is an orally available mTOR inhibitor and is commonly used after TKI treatment failure/intolerance. As with TKI inhibitors, however, there are wide differences in
responses to everolimus treatment, and it remains unclear which patients most benefit from the treatment.

Our results confirm TKI-induced hypertension (HTN) as a strong predictor of a good prognosis and response among patients treated with sunitinib/pazopanib. We additionally demonstrated that the use angiotensin system inhibitors (ASIs) in the treatment of TKI-induced HTN may have synergistic beneficial effects when used in conjunction with sunitinib or pazopanib.

In our investigation of tumor tyrosine protein kinase Met (c-Met) expression and the outcome, we demonstrated that high levels of c-Met expression associate with a worse prognosis among mRCC patients treated with sunitinib. We also demonstrated that high c-Met expression is associated with a poor outcome among patients without bone metastases at baseline, suggesting that the prognostic role may vary depending on the location of the metastases.

In our two studies conducted on everolimus-treated patients, we showed that everolimus-induced pneumonitis associates with an improved outcome, suggesting a role as a surrogate marker of the efficacy of everolimus treatment. We additionally confirmed the prognostic value of hyponatremia in a large cohort of everolimus-treated patients.
Review of the Literature

1. Renal Cell Carcinoma

1.1 Epidemiology

Kidney cancer accounts for 5% and 3% of all adult malignancies in men and women, respectively, making it the sixth most common cancer in men and tenth most common in women. The reported figures, however, also include urothelial cancer of the renal pelvis; renal cell carcinoma (RCC) accounts for ~80% of all kidney cancers. (1)

In Finland, the reported incidence of RCC in 2003–2007 was 9.1 per 100,000 men and 5.8 per 100,000 women (2). Worldwide, as well as in Finland, RCC incidence has shown signs of plateauing, with a simultaneous decrease in mortality rates (3, 4). This phenomenon is at least partly explained by earlier detection, allowing earlier intervention when the disease is potentially curable; currently, more than 50% of RCCs are detected incidentally (5, 6)

Well-known risk factors for RCC include cigarette smoking, hypertension, and obesity (7-14). Evidence also suggests that patients with end-stage renal failure, acquired renal cystic disease, tuberous sclerosis syndrome, and patients who undergo kidney transplantation are at increased risk of developing RCC (15-18). Additionally, epidemiologic studies have demonstrated that a family history of RCC is a risk factor for the disease (19, 20), and indeed, several hereditary syndromes predisposing patients to RCC have been identified.
In the United States, the overall 5-year cancer-specific survival (CSS) for patients with histologically confirmed kidney cancer of all stages between 1975 and 2009 was 60.4%. The stage-specific 5-year CSS rates were 87.0% for the localized stage, 62.9% for the regional stage, and 9.3% for the distant stage. (21)

1.2. Pathology

RCC is an extremely heterogeneous disease and comprises several different subclasses, the most common being clear-cell (75%), papillary (7–15%), and chromophobe (3–5%) carcinoma (22, 23). In addition to these common histological variants, several relatively uncommon subclasses such as collecting duct carcinoma, renal medullary carcinoma, tubulocystic renal cell carcinoma, and acquired cystic disease-associated renal cell carcinoma have been identified (23). Recent important changes from existing tumor types also include classification according to molecular alterations and familial predisposition syndromes. These subclasses, however, only account for less than one percent of all RCCs (23).

1.2.1 Clear-cell Renal Cell Carcinoma

Clear-cell renal cell carcinoma (ccRCC) is the most common variant of all RCCs and it originates from the epithelium of the proximal convoluted tubules (23). Histologically, ccRCCs are characterized by cells with a lipid- and glycogen-rich cytoplasmic content and frequently also present with an eosinophilic granular cytoplasm. Microscopically, an alveolar, acinar, or solid architectural pattern is commonly detected (24). Most cases of ccRCC are sporadic, with only 5% being
associated with hereditary syndromes such as von Hippel-Lindau disease and tuberous sclerosis. Both sporadic and hereditary forms of the disease are strongly associated with deletion in chromosome 3p, the site of the von Hippel-Lindau gene, with deletions at this site found in up to 96% of ccRCCs (25, 26).

1.2.2 Papillary Renal Cell Carcinoma

Papillary renal cell carcinoma (pRCC) is histologically characterized by a papillary or tubulopapillary architecture. The proportion of papillae varies and they often present with hemorrhage, necrosis, and cystic degeneration (27). Like ccRCC, pRCC may occur sporadically or in association with hereditary syndromes, and it is typically associated with trisomies of chromosomes 7, 16, and 17, renal cortical adenomas, and multifocal tumors (22).

Based on histologic appearance and biological behavior, two subtypes of pRCC have been identified: type 1 and type 2. It has been demonstrated that type 1 pRCC is associated with prolonged survival as compared to type 2 pRCC, most likely due to the fact that type 1 pRCC is typically detected at an earlier stage (28, 29).

Clinical features differentiating pRCCs from ccRCCs and chromophobe renal cell carcinomas (chRCCs) include spontaneous hemorrhaging as a presenting feature in 8% of cases, as well as multifocal presentation and calcification on imaging in 30% of cases (30).
1.2.3 Chromophobe Renal Cell Carcinoma

Less aggressive than other malignant renal tumors, chRCC accounts for 3–5% of all RCCs (22). It is characterized by huge pale cells with a reticulated cytoplasm, prominent cell membrane, and perinuclear halos (23, 31). A very close relationship between chRCCs and oncocytomas has been suggested, with both arising from intercalated cells of the collecting duct and showing similar alterations in mitochondria and mitochondrial DNA (32). Although this relationship is still under discussion, it has been hypothesized that chRCCs might represent a more aggressive counterpart for oncocytomas (33). ChRCC is associated with the loss of several chromosomes, as well a hereditary syndrome, Birt-Hogg-Dubé syndrome (34, 35).

1.2.4 Other Defined Subtypes

In addition to the above-mentioned subtypes, the WHO 2016 classification recognizes several other less common renal cell tumor subtypes. These include collecting duct carcinoma, renal medullary carcinoma, tubulocystic RCC, acquired cystic disease-associated RCC, MiT family translocation RCC, succinate dehydrogenase-deficient renal carcinoma, mucinous tubular and spindle cell carcinoma, clear cell papillary RCC, hereditary leiomyomatosis and renal cell carcinoma-associated RCC, and papillary adenoma (23). As these subclasses only account for a small proportion of RCCs, they will not be discussed in further detail.
1.2.5 Sarcomatoid Differentiation in RCC

Sarcomatoid RCC was first described as a tumor with pronounced cytologic atypia containing enlarged pleomorphic or malignant spindle cells (36). It used to be considered a histologic entity of its own, but it has since been demonstrated that sarcomatoid differentiation instead represents a transformation to a higher-grade malignancy and has been documented in numerous recognized histologic subtypes of RCC (23, 37). It occurs in approximately 8% of RCCs, but it remains unknown whether any specific histologic subtype has a predilection for sarcomatoid change (38). Furthermore, research has demonstrated that sarcomatoid RCC is associated with a worse prognosis, regardless of the underlying RCC subtype (38, 39).

1.3 Genetic Environment

The genetic environment and genetic changes associated with RCC are heterogeneous. Several hereditary syndromes predisposing patients to RCC, among other malignancies, have been described; von Hippel-Lindau disease (VHL), hereditary leiomyomatosis and renal cell cancer (HLRCC), hereditary papillary renal cancer (HPRC), and Birt-Hogg-Dubé syndrome (BHD) are the four most common autosomal dominantly inherited syndromes with distinct histologic features and genetic alterations (40). Hereditary RCC may account for 4–10% of all RCCs, but many of the chromosomal changes seen in the hereditary forms of the disease are also frequent in the sporadic forms (41, 42).
1.3.1 VHL Gene Alteration

VHL gene alteration is a broad concept of genetic abnormality that includes VHL gene mutation, promoter hypermethylation, and loss of heterozygosity. The VHL gene is a tumor suppressor gene and its alteration is present in 50–70% of clear cell RCCs (25, 43). Through its important role in the regulation of the hypoxia pathway, VHL gene alteration and subsequent functional loss of VHL protein is thought to play a significant part in tumorigenesis and tumor angiogenesis (44). In addition to RCC, the VHL gene has also been implicated in the development of other tumors, such as hemangioblastomas, pancreatic cysts, and pheochromocytomas (45). Although understanding of the underlying role of VHL gene alteration in the pathogenesis of RCC has led to the development of several novel therapeutic approaches, most importantly vascular endothelial growth factor (VEGF)-targeted agents, the clinical significance of VHL gene alteration in RCC is yet to be established.

1.3.2 Met Proto-oncogene and HPRCC

Contrary to the VHL gene, the MET gene, located on chromosome 7, is a proto-oncogene rather than a tumor suppressor gene (46). Activating mutations of the Met proto-oncogene have been associated with hereditary papillary renal cell carcinoma (HPRCC) (46-49) and to a lesser extent with sporadic forms of RCC. HPRCC is characterized by multifocal, bilateral type I papillary renal cell carcinomas, but in comparison to VHL disease, it is quite rare (50, 51). The Met proto-oncogene and its product, c-Met, a tyrosine kinase receptor, have been implicated as promoters of malignant transformation and tumorigenesis, and will be discussed in detail later in this thesis.
1.3.3 Other Hereditary Syndromes

Other hereditary syndromes associated with RCC are Birt-Hogg-Dubé (BHD) syndrome and hereditary leiomyomatosis and renal cell cancer (HLRCC). BHD syndrome is an autosomal, dominantly inherited condition characterized by the development of benign skin tumors, pulmonary cysts, and pneumothorax (52). The BHD-associated gene, folliculin (FLCN), is located on chromosome 17 and it acts as a tumor suppressor gene. In BHD syndrome, however, this gene is inactivated (35). Patients with BHD syndrome have a predisposition for RCCs, and unlike other hereditary syndromes with this predisposition, RCCs associated with BHD are histologically diverse. Over two-thirds of the RCCs associated with BHD are of chromophobe or hybrid chromophobe/oncocytoma histology, however (53)

Fumarate hydratase, the gene product of the fumarate hydratase (FH) gene, is an enzyme involved in the Krebs cycle. Mutations in the FH gene cause HLRCC. It acts as a tumor suppressor gene, and the impaired function of the FH gene may lead to chronic hypoxia, encouraging increased expression of VEGF and thus promoting tumor formation. HLRCC is associated with type 2 papillary RCCs. (54, 55)

2. Tumorigenesis and the Tumor Microenvironment

2.1 Tumorigenesis

Most of our understanding of tumorigenesis in RCC is based on the work of Dr Linehan et al. through the study of familial kidney cancer and VHL disease at the
National Institutes of Health, Bethesda, US (56-58). At least twelve different genes are known to promote the development of RCC: VHL, MET, folliculin (FLCN), tuberous sclerosis complexes-1 and -2, (TSC-1 and -2), TFE3, TFEB, MITF, FH, succinate dehydrogenase B (SDHB), succinate dehydrogenase D (SDHD), and phosphatase and tensin homologue (PTEN) genes (58). All these genes are associated with the regulation of essential mechanisms involved in tumor development. However, to emphasize the genetic heterogeneity of RCC, an analysis of more than 500 primary ccRCC tumor samples identified 19 significantly mutated genes (SMGs), including VHL, PBRM1, SETD2, KDM5C, PTEN, BAP1, MTOR, and TP53. Furthermore, in approximately 20% of cases, none of these SMGs were present (59). Probably the most thoroughly investigated and well-described pathways in RCC are the hypoxia-induced pathway, the mammalian target of rapamycin (mTOR) pathway, and the c-Met signaling pathway, all promoting angiogenesis and cell survival, which are essential events in the tumorigenesis of RCC. These three pathways are described in more detail below.

2.1.1 Hypoxia-induced Pathway

As described earlier, the VHL gene is a tumor suppressor gene. It encodes the VHL protein, which under normal circumstances targets hypoxia-inducible transcription factors (HIF-1α, HIF-2α) for ubiquitin-mediated proteolysis (60). However, under hypoxic conditions, as well as when the VHL complex is defective due to genetic alterations, this cascade is interrupted, leading to the accumulation of HIFs. Guo et al. demonstrated that alterations in genes involved in the ubiquitin-mediated proteolysis pathway (VHL, BAP1, CUL7, BTRC) are frequent in ccRCC and are significantly associated with overexpression of HIF-1α and HIF-2α (61). The overexpression of the transcription factors, in turn, leads to the upregulation of several important downstream promoters of angiogenesis such as vascular
endothelial growth factor (VEGF), epidermal growth factor, transforming growth factor α (TGF-α), platelet-derived growth factor (PDGF), erythropoietin, and glucose transporter 1 (62). Important pathways involved in RCC biology are presented in Figure 1.

2.1.1.1 HIF-1α and HIF-2α in RCC Tumorigenesis

Both HIF-1α and HIF-2α normally function as transcription factors mediating the activation of target genes in response to hypoxic conditions. With the inactivation of the VHL protein, VHL-associated degradation of HIF-1α and HIF-2α is disturbed, leading to the maintained activity of these transcription factors irrespective of the oxygen levels (63, 64). The mTOR pathway also leads to the accumulation of HIF-1α through the stimulation of ribosomal translation of mRNAs, including the translation of HIF-1α message, as demonstrated in Figure 1 (65).

HIFs promote tumorigenesis in a variety of ways, including angiogenesis, metabolism, proliferation, metastasis, and differentiation. The overexpression of HIF-1α has been implicated as a critical factor in renal carcinogenesis in several studies (66-70). The tumorigenic potential of HIF-2α, on the other hand, is more controversial, with contradicting evidence making its role in the development of RCC less clear (71, 72). Current understanding depicts HIFα proteins as factors clearly influencing tumor progression by direct regulation of both unique and shared target genes, as well as by exerting distinct effects on critical oncoproteins and tumor suppressors.
2.1.2 mTOR Pathway

The mTOR pathway regulates cell growth, proliferation, survival, and metabolism (73). Research has shown that in addition to its role in RCC, it is involved in the development and carcinogenesis of several different malignancies (74, 75).

mTOR is a serine/threonine kinase that in response to growth factors, hormones, and nutrients activates protein synthesis and contributes to many different cell functions, including protein degradation and angiogenesis (76). This response is controlled and activated by the phosphatidylinositol-3-kinase/Akt (PI3K/Akt) pathway and opposed by the activity of PTEN and TSC-1 and -2 (77, 78). Mutations of PTEN and TSC-1 and -2 leading to the loss of their activity and abnormal activation of the PI3K/AKT pathway have been demonstrated to play a role in RCC progression (78-80).

The mTOR protein forms two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 is involved in the regulation of several oncogenic proteins, such as HIF-1α, VEGF, FGFs, and c-Myc, and mTORC2 is important for cell survival (73). Genetic alterations upstream of or at mTOR leading to abnormal activation of the mTOR pathway promote the upregulation of these oncogenic proteins through key downstream effectors, such as the ribosomal S6 kinase and the eukaryotic translation initiation factor 4E binding protein (74, 81, 82). In an analysis of over 500 primary ccRCC tumor samples, the authors demonstrated that alterations targeting multiple components of the PI3K/AKT/mTOR pathway were present in 28% of the tumor samples (59). Furthermore, these alterations were associated with a worse or better outcome, depending on their respective action on the PI3K/AKT pathway, suggesting a role as a therapeutic target in ccRCC (59).
2.1.3 c-Met Signaling Pathway

The c-Met receptor tyrosine kinase is a product of the Met proto-oncogene located on chromosome 7q21-31. The ligand for c-Met, hepatocyte growth factor (HGF), promotes cell proliferation, survival, motility, differentiation, and morphogenesis. Under normal physiologic conditions, the c-Met signaling pathway regulates tissue homeostasis, but it has been found to play a role in the tumorigenesis of various human malignancies (83-85).

c-Met signaling is a complex entity involving several molecular events. The major downstream responses of c-Met activation include activation of the RAS/RAF/MAPK cascade and the PI3K/Akt signaling axis (86). The mitogen activated protein kinase (MAPK) activates a large number of genes, which in turn results in increased cell proliferation and cell motility (87). The PI3K/Akt signaling axis, on the other hand, is responsible for cell survival in response to c-Met activation (88).

In rodent models, activating mutations of Met have been shown to cause a variety of tumors, including sarcomas, lymphomas, and carcinomas, suggesting that aberrant Met signaling can also cause human cancer (89). In RCC, activating mutations in the c-Met kinase domain were first discovered in both sporadic and inherited forms of papillary RCC (48, 90).

c-Met/HGF signaling has been shown to promote tumor angiogenesis by directly acting on endothelial cells (ECs), inducing proliferation and migration (91, 92). In a more indirect manner, c-Met/HGF plays a part in activating the angiogenic switch by inducing VEGF-A expression and by suppressing thrombospondin 1 (TSP-1), a negative regulator of angiogenesis (93). Indeed, evidence suggests that there is considerable crosstalk between c-Met/HGF signaling and various other signaling pathways involved in cancer progression, as well as resistance to therapy.
Although c-Met/HGF and VEGF/VEGFR do not directly associate with each other, they share synergistic angiogenesis-activating intermediates such as the PI3K/Akt and MAPK cascades, described earlier. Furthermore, HIF-dependent expression of c-Met has been documented in several types of carcinoma cells (94-96). These studies suggest that anti-angiogenic therapy, which leads to hypoxic conditions, induces the accumulation of HIFs, further inducing c-Met expression. This, in turn, might promote the MET-dependent spread of cancer cells, making it an interesting target for therapeutic intervention.

2.1.4 Angiogenesis

The supply of oxygen and nutrients is crucial for the survival of tumor cells, and indeed, neovascularization is considered a key event for tumor progression in RCC. Through a complex process called the ‘angiogenic switch’, tumor cells induce angiogenesis and ensure the further supply of oxygen and nutrients, as well as the disposal of metabolic waste products and carbon dioxide by new sprouting vessels (97). The regulation of tumor angiogenesis is depicted in Figure 2.

HIFs activate the expression of several proangiogenic factors such as VEGF, VEGF receptors, angiopoietins (Ang-1 and -2), fibroblast growth factor 2 (FGF-2), platelet-derived growth factor B (PDGF-B), the Tie-2 receptor, and matrix metalloproteinases (MMP-2 and -9) (98). The role of VEGF, particularly VEGF-A, and its receptor, VEGFR-2, is well established regarding angiogenesis in RCC. VEGF-A and VEGFR-2 regulate the proliferation of ECs and the development of vasculature by their concentration and gradient, respectively (99). While VEGF-A and VEGFR-2 represent a key signaling system in the early stages of blood vessel growth (100), secondary stages of the process require a high level of activity of several other growth factors and their receptors.
Angiopoietins, Ang-1 and Ang-2, are endothelial cell-derived growth factors that act by binding to their receptors, Tie-1 and Tie-2. While the role of Tie-1 in angiopoietin signaling remains unclear, studies in Tie-2 knockout mice have demonstrated that the initial phases of angiogenesis are able to proceed normally, but the remodeling and hierarchical reorganization of the sprouting vessels are disturbed (101, 102). Current understanding depicts the Ang-Tie pathway as a mediator of vessel growth, remodeling, and maturation through the regulation of EC permeability, EC–extracellular matrix interaction, and inflammation (103). Ang-1 stabilizes vessels and decreases vascular permeability (104), whereas Ang-2 is involved in vessel regression and destabilization (105). Since tumor vasculature often appears as primitive, hemorrhagic, and disorganized, it is not surprising that elevated levels of Ang-2 have been found in several cancers and appear to be associated with a worse prognosis (106-108). Thus, blocking of Ang-2 and targeting of the Ang–Tie system has been of great interest in anti-angiogenic drug development.

PDGF-B and FGF-2 are angiogenic factors frequently expressed in tumors, and their expression has been linked to cancer progression and metastasis (109-111). PDGF-B is involved in the recruitment of pericytes and vascular smooth muscle cells, while FGF-2 displays a broad spectrum of biological functions. Several studies have reported FGF-2 and PDGF-B to have angiogenic synergism (112-114). Interestingly, murine models have shown that whereas FGF-2 and PDGF-B work in concert to promote vessel maturation and stabilization under physiological conditions, tumor vasculature induced by these factors is primitive and disorganized (114). It has been suggested that this might represent cross-communication with VEGF, counteracting the recruitment activity of PDGF-B (115), but the specific molecular mechanisms by which this happens remain to be elucidated.

As depicted in Figure 2, tumor neovascularization is a process induced by multiple individual growth factors, cytokines, and their complex interactions. While not all of
these interactions are thoroughly understood, increasing understanding of the underlying mechanisms of neovascularization has led to the development of several novel anti-angiogenic therapies.

Figure 2. Regulation of tumor angiogenesis (Adapted from Cao et al. Regulation of tumor angiogenesis and metastasis by FGF and PDGF signaling pathways, Journal of Molecular Medicine, 2008) (115).

2.2 Tumor Microenvironment

The tumor microenvironment is a complex mixture of proliferating tumor cells, tumor stroma, blood vessels, tumor infiltrating immune cells, and a variety of associated tissue cells. The tumor controls this unique environment through molecular and cellular events taking place in the surrounding tissues, thus promoting
tumor survival, progression, and metastasis. As a tumor progresses, the changes seen in the tumor microenvironment resemble those seen in the process of chronic inflammation. This ‘host reaction to the tumor’ is thought to be strongly involved in shaping the tumor microenvironment. Although the initial goal of this response is to wipe out the tumor, like any invader, evidence suggests that the tumor actively downregulates these anti-tumor responses through a variety of strategies. (116, 117)

Major components of the tumor microenvironment are tumor-infiltrating lymphocytes (TILs). TILs include B lymphocytes and T lymphocytes, which can be further categorized into CD4+ T-cells, CD8+ cytotoxic T cells, and natural killer (NK) cells. CD4+ T-cells differentiate into CD4+ helper and regulatory T-cells (T-reg). While CD4+ helper T-cells coordinate immune responses through the secretion of various cytokines, regulatory T-cells serve to limit adaptive immune responses by cytokine- and cell-contact-dependent mechanisms. CD8+ T-cells normally function to promote the lysis of infected, neoplastic, or otherwise targeted host cells. For T-cells to work effectively, the antigen/antigens must be presented to T-lymphocytes by major histocompatibility complex (MHC) molecules on the surface of an antigen-presenting cell. NK cells, on the other hand, do not require prior antigen exposure and they display lytic activity against cells that do not express MHC molecules. Thus, they form an important safeguard against neoplastic cells lacking the means to present self-antigens to T-lymphocytes. (118-120)

Other immune cells present in tumors include dendritic cells (DCs), macrophages, and myeloid suppressor cells (MDSCs). The main function of DCs is to process and present antigens to T-lymphocytes. Macrophages present in tumors are known as tumor-associated macrophages (TAMs). Under physiologic conditions, macrophages play a part in both the innate and the adaptive immune responses through phagocytosis, antigen presentation, and the excretion of cytokines. TAMs, however, are re-programmed to inhibit lymphocyte functions and may also possess
tumor growth promoting angiogenic potential (121, 122). Similarly, MDSCs are normal host cells of bone marrow origin with immunosuppressive and angiogenic properties. Under pathologic conditions, systemic signals result in MDSCs prematurely leaving the bone marrow and engaging in T-cell suppression at extramedullary locations (123, 124). Murine models have additionally demonstrated that MDSCs may promote tumor angiogenesis (125).

### 2.2.1 Tumor Escape

The tumor microenvironment forms an effective barrier against immune cell functions. The process by which a tumor escapes immune recognition, “immunoediting”, was first described by Dunn et al. (116). Although during the early phases, lymphocytes responsible for immune surveillance are able to recognize and eliminate malignant cells, tumor cells begin to undergo progressive selection favoring the proliferation of malignant cells more likely to evade immune recognition. The cancer cells additionally directly inhibit immune cells through overexpression of immune-checkpoint molecules (116). Several mechanisms leading to the dysfunction of immune cells and downregulation of the immune response by tumors have been identified. The four main pathways by which this happens are interference with the induction of anti-tumor responses, inadequate effector cell function, insufficient recognition signals, and the development of immunoresistance by the tumor (118). Given the pivotal role of immunoediting and chronic inflammation in tumorigenesis, it is not surprising that research suggests that the amount and presence of different immune cell populations in the tumor microenvironment is associated with the disease course.

As described earlier, effector T-cells are responsible for the lysis of neoplastic cells. The developing microenvironment of a growing tumor, however, prevents these
immunological reactions by secreting suppressive factors expressing inhibitory molecules and by attracting T-regs, TAMs, and MDSCs (126-128). T-regs downregulate the proliferation and anti-tumor functions of both CD4+ and CD8+ effector T-lymphocytes (129, 130). This is achieved by the expression of various molecules on T-regs, such as CTLA-4, preventing T-cell activation and DC functions (131, 132). Activated T-regs also induce cell death in effector T-cells by a granzyme-dependent pathway (133). The proliferation of T-regs is induced by interleukin-10 (IL-10), transforming growth factor β (TGF-β), and prostaglandin E2, all of which are abundant in the tumor microenvironment (134). Indeed, T-regs appear to be present in higher numbers in more aggressive and advanced cancers (135). In RCC, higher levels of T-regs are correlated with a poorer prognosis (136-139). Similarly, TAMs inhibit cytotoxic T-cell responses through several mechanisms. For example, they produce IL-10, which in turn induces the expression of programmed death ligand 1 (PD-L1). PD-L1 is a ligand for an inhibitory cell-surface receptor, programmed death 1 (PD-1), which negatively regulates T-cell activation (140). TAMs additionally promote tumor-associated angiogenesis and tumor cell invasion (141, 142). In ccRCC, higher numbers of TAMs are associated with a poor prognosis (143-145). In pRCC, however, an opposite relationship was noted (146).

MDSCs are another immunosuppressive immune cell population that, like T-regs and TAMs, promote tumor evasion by suppressing T-cell immunity. Among other effects, MDSCs deplete nutrients necessary for appropriate T-cell function, block MHC-bound peptides from being presented to T-cells, and induce the conversion of naive T-cells to a T-reg phenotype (147-149). Research also implicates MDSCs as a part of STAT3-VEGF-dependent angiogenesis (125). In RCC, elevated levels of MDSCs are associated with a poor prognosis (150).
Growing knowledge and understanding of immune responses and immune cells as a part of tumorigenesis and tumor progression have sparked interest in the development of several promising immunotherapeutic agents, such as vaccines, T-cell modulators, and immune checkpoint inhibitors.

2.2.2 Tumor Heterogeneity

Previous work on solid cancers has demonstrated that extensive genetic heterogeneity exists among individual tumors (151-153). This intratumor heterogeneity may contribute to treatment failure and drug resistance (154-156), and can manifest as spatial heterogeneity, with an uneven distribution of genetically diverse tumor subpopulations, and temporal heterogeneity, with dynamic variations in the genetic diversity of an individual tumor over time.

Gerlinger et al. investigated gene-expression signatures in RCC from spatially separated tumor samples obtained from primary renal carcinomas and associated metastatic sites. Up to 69% of all somatic mutations were not detectable across every tumor region. Additionally, gene-expression signatures of both a good and poor prognosis were detected in different regions of the same tumor. (157) As personalized medicine approaches traditionally rely on single tumor biopsy samples, intratumor heterogeneity may have significant consequences regarding antineoplastic treatment strategies. It is thought that tumor heterogeneity underlies resistance to therapy, thus complicating the selection of globally effective therapies (158). These observations emphasize the importance of multidimensional therapeutic approaches, as well as the need to develop ways to dynamically monitor patients during treatment.
3 Diagnosis and Management of Renal Cell Carcinoma

Since renal cell carcinoma often develops without any early warning signs, a rather high proportion of patients present with metastases at diagnosis. The most common clinical presentations of the disease are hematuria, abdominal pain, and a palpable mass in the flank or abdomen. This “classic triad”, however, is only present in less than 10% of patients (159). Research has shown that in 25–30% of patients, the disease has metastasized at the time of diagnosis (160, 161).

While surgical resection remains the golden standard of care for localized RCCs, relapse occurs in almost a third of the patients (162).

3.1 Diagnosis

The widespread application of computed tomography (CT) and ultrasonography for other indications has led to the increased detection of RCCs. More than 50% of RCCs are detected incidentally (163). RCC is sometimes referred to as the “internist’s cancer”, since in addition to the “classic triad” of symptoms, the disease is sometimes accompanied by hypercalcemia, unexplained fever, erythrocytosis, and Stauffer’s syndrome (signs of cholestasis unrelated to tumor infiltration of the liver or intrinsic liver disease, which typically resolves after kidney tumor resection). Laboratory examinations of serum creatinine, hemoglobin, leukocyte and platelet counts, lactate dehydrogenase (LDH), C-reactive protein (CRP), and albumin-corrected calcium are recommended if suspicion of RCC arises. (164)
3.1.1 Imaging

RCC is usually suspected based on ultrasonography findings, prompting further imaging with computed tomography. According to the ESMO 2016 guidelines, contrast-enhanced chest, abdominal, and pelvic CT are mandatory for accurate staging. The use of bone scanning and CT or magnetic resonance imaging (MRI) of the brain is recommended only when suggestive clinical symptoms or laboratory findings are present and not as a part of routine clinical practice. (164)
In most cases, a single examination using CT provides sufficient information for accurate staging and surgical planning with no need for additional imaging (165). MRI is mainly an alternative in patients requiring further imaging and in cases of allergies, pregnancy, or surveillance. MRI is also an excellent alternative in patients with renal insufficiency. Positron emission tomography/computed tomography (PET/CT) scanning is not a standard investigation in the diagnosis and staging of RCC and its use is not recommended. (164)

3.1.2 Staging and Pathology Assessment

For staging, the tumor-node-metastasis (TNM) staging system should be used. The TNM staging system is presented in Table 1.

A core needle biopsy is recommended before treatment with ablative therapies, as well as in patients with an unresectable solid renal mass and metastatic disease before starting systemic therapy. It is additionally practical in patients with significant comorbidities where the risk of biopsy is outweighed by the risk of surgery. (166, 167). The role of core needle biopsy is less clear regarding solitary renal masses <4 cm in diameter, as the probability of malignancy within a small renal mass has been found to be inversely related to tumor size (168). In a study by Halverson et al.
in which patients underwent both percutaneous renal mass biopsy and subsequent partial or radical nephrectomy, there was complete concordance between the histology rendered from core needle biopsy and that rendered by surgery (169). Given that core needle biopsy has a high sensitivity and specificity in confirming the histopathological nature of a tumor, it probably has a role when deciding on the active surveillance and operative management of a small renal mass (167). A specimen provided by nephrectomy, however, provides the final histopathological diagnosis, classification, and grading. Regarding the assessment of pathology, the ESMO 2016 guidelines recommend reporting the tumor histological subtype, the International Society of Urological Pathology (ISUP) nucleolar grading, sarcomatoid and/or rhabdoid differentiation, the presence of necrosis and presence of microscopic vascular invasion, in addition to TNM staging in routine clinical practice. (164)
Table 1. TNM classification System

<table>
<thead>
<tr>
<th>Primary Tumor (T)</th>
<th>Secondary Tumor (T)</th>
<th>Regional Lymph Nodes (N)</th>
<th>Distant Metastases (M)</th>
<th>Anatomic stage/prognostic groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>TX</td>
<td>NX</td>
<td>cM0</td>
<td>Stage I</td>
</tr>
<tr>
<td>T0</td>
<td>T0</td>
<td>N0</td>
<td>cM1</td>
<td>T1</td>
</tr>
<tr>
<td>T1</td>
<td>T1</td>
<td>N1</td>
<td>pM1</td>
<td>N0</td>
</tr>
<tr>
<td>T1</td>
<td>T1</td>
<td>N2</td>
<td></td>
<td>N0</td>
</tr>
<tr>
<td>T2</td>
<td>T2</td>
<td>T2a</td>
<td></td>
<td>N0</td>
</tr>
<tr>
<td>T3</td>
<td>T3</td>
<td>T2b</td>
<td></td>
<td>N1</td>
</tr>
<tr>
<td>T4</td>
<td>T4</td>
<td>T3a</td>
<td></td>
<td>N1</td>
</tr>
<tr>
<td>T4</td>
<td>T4</td>
<td>T3b</td>
<td></td>
<td>N1</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>T3c</td>
<td></td>
<td>N2</td>
</tr>
</tbody>
</table>

Adapted from the AJCC Cancer Staging Handbook, 7th edition (2010)
3.1.3 Small Renal Masses

An increasingly common and problematic clinical entity encountered by clinicians is small renal masses (SRMs) less than 4 cm in size. Based on epidemiological studies, SRMs account for nearly one-half of all newly diagnosed renal masses (170). Therapeutic options regarding SRMs include extirpative surgery (radical or partial nephrectomy), ablative therapies, and active surveillance (AS). Kutikov et al. demonstrated that 20–30% of solitary renal masses presumed to be renal cell carcinoma on preoperative imaging had benign pathologic findings on resection (171). Furthermore, in those lesions that are RCC, the majority of tumors are of low grade and unlikely to develop metastases (172, 173).

There are no definitive clinical guidelines for the management of SRMs. General recommendations for patient selection favoring AS include increased age, decreased life expectancy, suitability for surgery, and a decreased risk of metastatic disease. Several studies have shown that the risk of metastatic progression while on AS is <2%, making it a viable alternative to surgery (174-178). The main trigger for operative intervention, on the other hand, is believed to be the tumor growth rate (GR). Evidence regarding tumor GR is somewhat controversial, as some studies have demonstrated a high GR among AS patients developing metastases (174, 179), while a number of studies have demonstrated a low or zero GR for tumors of malignant pathology (176). Although the malignant potential of an SRM is likely to be defined by several important factors, progression to metastatic disease is exceptionally low in tumors that demonstrate a low or zero GR and remain <3 cm in diameter (177, 180).

In a multi-institutional prospective clinical trial investigating AS for the management of SMRs, the authors demonstrated non-inferiority of AS versus primary intervention. At five years, cancer-specific survival was 99% and 100% for primary intervention and AS, respectively, suggesting that AS should become part of the
standard discussion for the management of SRMs (180). An informed decision by the patient and physician, including a discussion of the small but real risk of cancer progression, loss of a window of opportunity for nephron-sparing surgery, and the lack of curative treatments for mRCC should, however, precede any treatment decisions.

3.2 Prognostication

For localized RCC, several multivariable models such as the University of California Los Angeles Integrated Staging System (UISS) and SSIGN score have been developed to predict cancer-specific survival after nephrectomy. In these models, prognostication is mainly based on clinical and histological parameters of the tumor, as presented in Table 2. Although these models can predict the natural history of RCC, they are not designed to account for the effect of targeted therapies in patients with mRCC. (181, 182)

**Table 2.** The UISS and SSIGN prognostic models assessing postoperative cancer-specific mortality

<table>
<thead>
<tr>
<th>Model</th>
<th>Sample Size</th>
<th>Target population</th>
<th>Predictors</th>
<th>C-index</th>
</tr>
</thead>
<tbody>
<tr>
<td>UISS</td>
<td>661</td>
<td>RCC of all stages</td>
<td>- AJCC</td>
<td>82–86%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Fuhrman grade</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- ECOG-PS</td>
<td></td>
</tr>
<tr>
<td>SSIGN</td>
<td>1801</td>
<td>Localized clear cell RCC</td>
<td>- TNM (1997)</td>
<td>81–82%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Tumor size</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Nuclear grade</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Tumor necrosis</td>
<td></td>
</tr>
</tbody>
</table>

AJCC = American Joint Committee on Cancer; ECOG-PS = Eastern Cooperative Oncology Group Performance Status
Prognostication of metastatic RCC relies heavily on traditional markers of risk. In addition to pathological and histological staging and grading, several clinical and biological prognostic markers have been identified.

While it seems that age, gender, and race are not associated with the disease course or outcome, the association between the performance status and prognosis is well established. Two different scales are used to evaluate the patient’s performance status, namely the Eastern Cooperative Oncology Group (ECOG) scale and Karnofsky performance status (KPS) score; both have demonstrated good reliability and validity.

Other validated prognostic factors for mRCC include serum hemoglobin lower than the lower limit of normal, platelets greater than the upper limit of normal, serum LDH greater than the upper limit of normal, corrected serum calcium greater than the upper limit of normal, neutrophils greater than the upper limit of normal, and a time from diagnosis to treatment of <1 year.

The two most widely used prognostic models combining these markers into risk classifications are the Memorial Sloan Kettering Cancer Center (MSKCC) risk classification (183) and the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk classification (184). These models apply exclusively to patients with mRCC.

In addition to the more traditional markers of risk, gene signatures are known to detect risk groups in RCC (185, 186). However, in current clinical practice, no specific molecular markers can be recommended for routine use, as is discussed later.
3.3 Treatment

Surgical resection of the tumor is the golden standard of care in localized RCC. While radical nephrectomy remains the most frequently used alternative, several novel minimally invasive techniques have emerged, mostly for the treatment of T1 tumors. Current evidence suggests that localized RCCs are best managed by partial nephrectomy (or nephron-sparing surgery) when feasible. The role of techniques such as radio-frequency ablation and cryoablation remains to be further elucidated. Disease recurrence occurs in 20–30% of patients after partial or radical nephrectomy. (187). The role of adjuvant therapies in localized RCC is discussed separately in conjunction with systemic therapies.

The treatment of mRCC mainly relies on systemic therapy, with surgery having a less well-defined role than in localized disease.

3.3.1 Cytoreductive Nephrectomy

A combined analysis by Flanigan et al. investigating the role of cytoreductive nephrectomy in the era of immunotherapy demonstrated that nephrectomy plus interferon was associated with longer median overall survival than interferon alone (13.6 vs. 7.8 months), representing a 31% decrease in the risk of death ($P = 0.002$) (188). Since then, cytoreductive nephrectomy has been a part of routine clinical practice in patients with a good performance status and large primary tumors with limited volumes of metastatic disease. It is not recommended, however, in patients with a poor performance status (164).

A recent phase III multicenter trial, CARMENA, investigated the role of nephrectomy among 450 mRCC patients with an intermediate or poor MSKCC risk classification. In this study, patients were randomized to undergo nephrectomy and then receive sunitinib or receive sunitinib alone. The median OS was 18.4 months in
the sunitinib-alone group and 13.9 months in the nephrectomy–sunitinib group, demonstrating the non-inferiority of sunitinib alone versus nephrectomy followed by sunitinib. (189) However, as Motzer and Russo pointed out in their editorial, the interpretation of the results from the CARMENA trial is complicated by several factors, including slow enrollment, a lack of information regarding the selection factors for performing nephrectomy, and the high percentage of poor-risk patients (43%). As such, these data should emphasize the importance of patient selection rather than lead to the abandonment of nephrectomy. (190)

3.3.2 Metastasectomy and Other Local Therapeutic Options for Metastases

Common metastatic sites in RCC include the lung, bone, liver, and brain, but metastases can be found at any anatomical site (191, 192). In addition to traditional surgery, options for local therapy of metastases include whole brain radiotherapy, conventional radiotherapy, stereotactic radiosurgery, stereotactic body radiotherapy, cyberknife radiotherapy, and hypofractionated radiotherapy.

The benefits of metastasectomy as well as other local therapeutic options have been under fervent discussion, with no general guidelines existing as to whether a patient should be referred for local treatment of metastases. Dabestani et al. systematically reviewed the potential benefits of these treatments, suggesting a survival benefit with complete metastasectomy vs. either incomplete or no metastasectomy (193). Complete metastasectomy was additionally associated with improved symptom control. The role of other local therapies is less clear, with consensus mainly existing on the palliative benefits in selected patients.
The ESMO 2016 guidelines do not recommend metastasectomy or other local therapies in routine practice, but rather after multidisciplinary review for selected patients (164).

3.3.3 Systemic Therapy

The low response rates of mRCC to classical cytotoxic agents such as fluoropyrimidines or vinblastine, as well as hormonal treatment and radiotherapy, has inevitably led to the development of alternative therapies. As ccRCC is considered an immunogenic tumor, immunotherapy comprised the first wave of systemic therapies. As our knowledge of the underlying mechanisms and the pathogenesis of RCC has evolved, an array of new therapies has emerged from anti-VEGF and tyrosine kinase inhibition to modern approaches of dual inhibition and immune checkpoint inhibition.

3.3.3.1 Immunotherapy

Before the advent of targeted therapies, systemic therapy of mRCC comprised interferon-α (IFNα) and interleukin-2 (IL-2).

IL-2 is a cytokine that enhances the proliferation and function of T-cell lymphocytes. Although high-dose IL-2 therapy only produces modest response rates of 15–16% in the treatment of mRCC, the responses that do occur seem durable (194). Additionally, research has shown that approximately 5–7% of patients achieve complete remission (195, 196). High-dose IL-2 treatment, however, is associated with severe adverse events, such as capillary leak syndrome, resembling the clinical manifestations of septic shock and producing major morbidity in patients receiving
IL-2 (197). The significant toxicity profile has limited the use of high-dose IL-2 in the treatment of mRCC.

IFNα is a cytokine with antiviral, immunomodulatory, and antiproliferative activities. IFNα alone has only shown minimal antitumor activity in the treatment of mRCC patients (198, 199). Negrier et al. demonstrated that the combination of IFNα and low-dose IL-2 resulted in prolonged progression-free survival and improved response rates as compared to either agent alone (200). No benefit was seen regarding overall survival, however.

Current guidelines suggest high-dose IL-2 and the combination of IFNα and bevacizumab as alternatives for the standard options among patients with a good or intermediate prognosis (164). In the era of targeted therapies, however, the role and use of these agents has become limited.

3.3.3.2 Tyrosine Kinase Inhibitors (TKIs)

The introduction of molecularly targeted therapies revolutionized the treatment of advanced RCC. Inhibition of the VEGF pathway has emerged as the primary therapeutic intervention for most patients with advanced disease. TKIs are multi-targeted kinase inhibitors that inhibit signaling in a variety of key receptors such as VEGFR-1, -2, and -3, PDGFR-α and -β, c-RET, macrophage colony-stimulating factor 1 (CSF-1R), FMS-like tyrosine kinase 3 receptor (FLT3), and c-KIT (201). Among the first TKIs approved for the treatment of mRCC were sunitinib and sorafenib. Sorafenib was approved after showing prolonged PFS and increased ORR when compared to placebo among mRCC patients resistant to standard therapy (202). It is currently recommended as one of several options in the second line setting. Sunitinib is the current standard of care for treatment-naïve mRCC patients.
after having demonstrated significantly longer median PFS and a higher objective response rate (RR) when compared with IFNα (201).

Pazopanib, a second-generation TKI, has since been approved after demonstrating non-inferiority when compared to sunitinib, providing yet another option for first-line therapy (203).

Other current second-line options include axitinib, a highly potent and selective inhibitor of the various kinase domains of VEGFRs, distinguishing it from previous multi-targeted TKIs. In a phase III randomized clinical trial (AXIS), axitinib demonstrated an improved PFS when compared to sorafenib in the second-line treatment setting (204). Axitinib has also demonstrated clinical activity and safety in the first-line setting, but no significant PFS benefit was noted in a phase III randomized comparison between axitinib and sorafenib (205).

3.3.3 mTOR Inhibitors

The mammalian target of rapamycin (mTOR) is a downstream effector of the PI3-K/Akt pathway that exerts its biological functions as two distinct complexes, mTORC1 and mTORC2, as depicted earlier (206, 207). Everolimus and temsirolimus are allosteric inhibitors of mTOR that have shown clinical activity in mRCC. A phase III trial enrolling 626 mRCC patients with poor prognostic features comparing temsirolimus, IFNα, and the combination of both showed that temsirolimus alone resulted in longer OS and PFS in this patient population (208). Based on these results, temsirolimus is still recommended as a first-line therapy for poor-risk mRCC patients (164). However, the use of temsirolimus in first-line treatment for patients with a poor risk classification has been called into question by
the results from the RECORD-3 trial, in which sunitinib showed superiority over everolimus, even among patients with a poor prognosis (209).

Everolimus is the standard of care for mRCC patients whose disease progresses after initial anti-VEGFR therapy. It was approved based on the results from Renal Cell Cancer Treatment With Oral RAD001 Given Daily (RECORD-1), a phase III trial, in which everolimus exhibited superior efficacy to placebo (210).

Although mTOR inhibitors initially showed promising clinical activity in mRCC, their effects have proven to be far from durable and only a subset of patients experience significant clinical benefit from these agents.

The cellular action mechanisms of TKIs and mTOR inhibitors are depicted in Figure 3.
Figure 3. Cellular action mechanisms of TKIs and mTOR inhibitors (Adapted from Rini et al. Resistance to targeted therapy in renal-cell carcinoma, Lancet 2009) (211).

3.3.3.4 Novel Approaches

Most patients treated with either VEGF- or mTOR-targeted therapy ultimately develop resistance to these drugs. Whether, and more importantly how, this phenomenon is embedded in the intricate network of complex regulation and feedback loops these different pathways possess remains to be elucidated. With first-line sunitinib or pazopanib, the median PFS ranges from 8 to 11 months for all
patients (201, 203). Additionally, only a few treatments have shown a survival benefit in previously treated mRCC patients.

The upregulation of prometastatic MET and AXL as a result of VHL protein dysfunction in ccRCC has been implicated as a potential mediator of resistance to anti-VEGFR therapy (212). Cabozantinib is an inhibitor of tyrosine kinases, including MET, VEGFR, and AXL (213). In the fairly recent phase III METEOR trial comparing cabozantinib and everolimus among patients who progressed after initial VEGFR tyrosine-kinase treatment, cabozantinib became the first agent to show a survival benefit in all efficacy endpoints (PFS, OS, and ORR) in previously treated mRCC patients (214). Given the dim estimations of a PFS of only 5.6 months for first-line VEGFR-targeted therapy among patients with an intermediate–poor risk classification (215), cabozantinib has also been investigated in the first-line setting in the CABOSUN trial. In this setting, cabozantinib significantly increased median PFS (8.2 vs. 5.6 months) and was additionally associated with an increased reduction in the rate of progression when compared to sunitinib (216). As these results demonstrate a possible role for this dual inhibitor in the first-line setting, cabozantinib is currently recommended as an alternative after prior to anti-VEGFR therapy (164).

As Dunn et al. described, one of the mechanisms by which tumors evade host immune reactions is by directly inhibiting immune cells through overexpression of immune checkpoint molecules (116). Recently, immune checkpoint blockade has become a new avenue of immunotherapy in several tumor types.

PD-L1 is a ligand for an inhibitory cell-surface receptor, programmed death 1 (PD-1), which negatively regulates T-cell activation (140). In RCC, studies have shown that PD-L1 expression is associated with a poor prognosis (217-219). Nivolumab is a PD-1 immune checkpoint inhibitor that selectively blocks the interaction between PD-1 and PD-1L. Nivolumab has shown consistent efficacy in the treatment of
advanced melanoma, both alone and in combination with a CTLA-4 inhibitor, ipilimumab (220). In addition to melanoma, nivolumab has been approved for the treatment of squamous and nonsquamous non-small-cell lung cancer and Hodgkin’s disease (221, 222). In advanced RCC, nivolumab has been granted approval based on the results from a phase III study comparing nivolumab with everolimus in previously treated mRCC patients. In this setting, nivolumab was associated with improved OS and ORR, as well as fewer grade 3 or 4 adverse events (223). According to the ESMO 2016 guidelines, nivolumab is currently recommended in second- and third-line settings, depending on the patient’s risk classification (164). Several ongoing studies are evaluating nivolumab as well as other anti-PD-1/PD-L1 agents in combination with VEGF-targeted therapies.

3.3.3.5 Optimal Sequencing

With several new agents available for the treatment of advanced RCC, sequencing of these therapies is of great relevance. The treatment strategies according to the ESMO 2016 guidelines portrayed in Figure 4 are on the verge of a major change. When choosing the first-line treatment, the options have traditionally been relatively limited. Current data support the use of VEGF tyrosine kinase therapies, sunitinib and pazopanib, in this setting. In some institutions, high-dose interleukin-2 therapy is used for young and healthy patients with a good performance status, as it is the only therapy associated with durable long-term responses (194-196). Recent data from the CheckMate-214 and CABOSUN studies are, however, likely to change the first-line treatment paradigm of mRCC. Results from the phase II CABOSUN trial showed a median PFS of 8.6 months compared with 5.3 months for previously untreated mRCC patients taking cabozantinib or sunitinib, respectively ($P = 0.0008$).
Similarly, results from the CheckMate-214 study demonstrated significantly improved OS with combination immunotherapy (nivolumab+ipilimumab) in comparison to standard of care sunitinib among 1082 treatment-naïve mRCC patients (225).

Optimal sequencing of drug classes beyond first-line treatment remains unknown. Options include TKIs, cabozantinib, nivolumab, and the combination of lenvatinib, a receptor tyrosine kinase inhibitor, and everolimus. In the AXIS trial, which led to the approval of axitinib, patients with first-line immunotherapy demonstrated a median PFS of 12 months on axitinib as compared to 6.5 months on sorafenib, supporting its use among patients with prior immunotherapy (226). As approval for the combination of lenvatinib and everolimus was based on a phase II study with only 100 patients (227) as compared to the large randomized phase III trials investigating cabozantinib and nivolumab (214, 228), the level of evidence favors the use of cabozantinib and nivolumab among post-TKI patients. Ongoing trials investigating these agents alone and/or in combinations in first- and later-line settings may change current clinical guidelines regarding the optimal sequencing of varied therapies.
Figure 4. ESMO Guidelines 2016 (Adapted from ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Escudier et al. Ann. Oncol. 2016) (164).

1 Patients categorized into good, intermediate, and poor risk groups based on IMDC risk classification.
3.3.3.6 Adjuvant Therapy in RCC

The only curative treatment for patients with stage I–III RCC is surgery. However, the 5-year relapse rates after surgical treatment in patients with stage II–III disease are 30% to 40% (162). With an increasing variety of novel agents now available for systemic therapy, the search for effective adjuvant therapy, i.e. systemic therapy following surgery, is underway. The first positive phase III adjuvant therapy trial (S-TRAC) investigated the use of sunitinib in an adjuvant setting. With disease-free survival (DFS) as the primary endpoint, the study demonstrated a median DFS duration of 6.8 years versus 5.6 years in the placebo arm. However, the largest trial to date, the ASSURE trial, in which nearly 2000 patients with completely resected RCC were randomly assigned to sunitinib, sorafenib, or placebo, showed no significant differences between treatment arms in DFS or OS (229). Important differences between S-TRAC and ASSURE are that whereas S-TRAC recruited patients with pT3-4 disease, ASSURE also enrolled patients with PT1b and pT2 disease. Additionally, S-TRAC only included patients with ccRCC histology, and the final dropout rates due to treatment toxicity were significantly different in the two studies. A recent phase III trial, PROTECT, evaluated the efficacy and safety of pazopanib versus placebo among 1538 post-nephrectomy patients with localized or locally advanced RCC. Results from the primary analyses of DFS showed no benefit of pazopanib over placebo (230). However, a relationship between dose exposure and an improved clinical outcome was noted, suggesting that patients achieving higher levels of pazopanib exposure derived more clinical benefit from the treatment (231).

As promising as the results from S-TRAC are, there are several questions surrounding these findings. It has been hypothesized that adjuvant sunitinib may simply delay the time to recurrence with no effect on the cure rate. In addition,
concern over possible resistance to therapies for metastatic RCC after adjuvant sunitinib has been raised. (232)

Specific reasons as to why sunitinib improved DFS in S-TRAC and not in ASSURE remain unanswered. Similarly, it has been hypothesized that the lack of significant benefit in the PROTECT trial could be due to the lack of efficacy with 600 mg pazopanib. In addition to the three completed adjuvant trials, there are several ongoing phase III trials (depicted in Table 3), which may shed light on these questions. A closer examination of whether the differences between trials reflect differences in patient selection, dosing, and/or study design is of critical importance.

Several trials investigating immunotherapy agents in an adjuvant setting are additionally recruiting patients. These trials are targeting high-risk populations, and they include PROSPER investigating nivolumab, IMMotion investigating atezolizumab, KEYNOTE investigating pembrolizumab, and CheckMate investigating the combination of nivolumab and ipilimumab. As these agents have shown impressive clinical activity in patients with mRCC, the results are eagerly anticipated.
## Table 3. Completed and ongoing adjuvant trials

<table>
<thead>
<tr>
<th>Trial</th>
<th>Randomization</th>
<th>Treatment Details</th>
<th>N</th>
<th>Inclusion Criteria</th>
<th>Inclusion Criteria (Histology)</th>
<th>Results</th>
</tr>
</thead>
</table>
| ASSURE | Sorafenib      | 400 mg daily, 54 wks | 1943 | pT1b N0 M0 (grade 3-4) pT2-pT4 N0 M0 pT (any) N1 M0 | Any histology | **DFS:** HR 0.97 (CI 0.80-1.17) vs. placebo  
**DFS:** HR 1.02 (CI 0.85-1.23) vs. placebo   |
|        | Sunitinib      | 37.5 mg daily (4 wks on/2 wks off), 54 wks | | | | |
| S-TRAC | Sunitinib      | 50 mg daily (4 wks on/2 wks off), 9 cycles | 615 | pT3 N0 M0 (grades 2-4) pT4 N0 M0 pT (any) N1 M0 | ccRCC only | **DFS:** HR 0.76 (CI 0.59-0.98; P = 0.03) |
|        |                 |                  | | | | |
| PROTECT | Pazopanib      | 600 mg daily, 1 year | 1500 | pT2 N0 M0 (grades 3-4) pT3-4 N0 M0 pT (any) N1 M0 | ccRCC only | **DFS:** HR 0.86 (CI 0.70-1.06) |
|        |                 |                  | | | | |
| ATLAS  | Axitinib       | 5 mg twice per day, 3 years | 724 | pT2 N0 M0 pT (any) N1 M0 | ccRCC only | Ongoing |
| SORCE  | Sorafenib      | 400 mg daily, 1 year or 400 mg daily, 3 years | 1420 | pT1a N0 M0 (grade 4) pT1b N0 M0 (grades 3-4) pT2-4 N0 M0 pT1b-4 N1 M0 | Any histology | Ongoing |
|        |                 |                  | | | | |
| EVEREST | Everolimus     | 10 mg daily | 1545 | pT1b N0 M0 (grades 3-4) pT2-4 N0 M0 pT (any) N1 M0 | Any histology | Ongoing |

DFS = disease-free survival
4 Biomarkers in mRCC

The term ‘biomarker’ refers to a broad subcategory of medical signs, which can be measured accurately and reproducibly. They stand in contrast to medical symptoms perceived by patients themselves and are by definition objective, quantifiable characteristics of biological processes and are commonly used in basic and clinical research. Examples of biomarkers include everything from blood pressure to more complex tests of blood and other tissues. (233)

The ever-growing understanding of the biology and the molecular mechanisms underlying the pathogenesis of RCC have offered novel means to predict tumor behavior. Indeed, since the discovery of the inactivation of the VHL gene in the majority of ccRCCs, several new and promising tissue- and blood-based biomarkers have been identified. The complexity of the molecular pathways and the heterogeneous nature and wide diversity of RCCs at the molecular level have, however, made the incorporation of these biomarkers into clinical practice challenging. Presently, the prognostication of RCC relies heavily on clinical factors and, even more importantly, despite molecularly-targeted therapies comprising the foundation of treatment strategies in mRCC, treatment decisions are solely based on clinical factors. Current biomarkers mainly provide information regarding the outcome independent of treatment, and no validated predictive biomarkers are available. The search for predictive biomarkers identifying patients for more personalized treatment of advanced RCC is rigorous and ongoing.
4.1 Prognostic and Predictive Biomarkers

Prognostic biomarkers are biomarkers used to identify the likelihood of a clinical event such as disease progression or recurrence, whereas predictive biomarkers identify individuals more likely to experience a favorable or unfavorable effect from exposure to a medical product or agent than similar individuals without the biomarker. As discussed earlier, the current prediction of a patient’s clinical outcome mainly relies on clinical and pathological variables in both localized and metastatic RCC; these variables, however, poorly reflect the individual tumor biology seen in RCC. Biomarkers improving prognostication are urgently needed to allow for more accurate prognostic models.

Most of the identified biomarkers in RCC are directly associated with the VHL defect. The VHL protein (pVHL) regulates hypoxia inducible factor-1α (HIF-1α), which is a transcription factor inducing the transcription of several hypoxia-regulated genes (234). Under normal oxygen tension, pVHL binds to HIF-1α and is subsequently destroyed by the cell through ubiquitinization. With genetic alterations rendering the VHL gene inactive, however, the loss of pVHL leads to dysregulation of this cascade, resulting in overexpression of various angiogenic proteins. (235)

Enhanced understanding of this molecular pathway played a key role in identifying new approaches in drug development for mRCC and has since also led to the discovery of several potential biomarkers. Several molecular markers have indeed been investigated, but so far none have proven reliable in predicting the treatment outcome. Their use is therefore not recommended in routine clinical practice. However, the success of targeted therapies relies on appropriate patient selection to identify patients likely to respond to treatment and to avoid unnecessary toxicity.
4.1.1 Clinical-related Biomarkers

Motzer et al. were the first to investigate pretreatment clinical features and survival. In a study among 670 mRCC patients, a low Karnofsky performance status, high serum lactate dehydrogenase, low hemoglobin, high corrected calcium, and a time from diagnosis to treatment of <1 year were identified to associate with shorter survival. Based on the presence or absence of these risk factors, a risk classification, the Memorial Sloan Kettering Cancer Center (MSKCC) prognostic model, was constructed, categorizing patients into favorable, intermediate, and poor risk groups for which the median survival times were separated by 6 months or more. In the favorable risk group with zero risk factors, the median time to death was 20 months. In the intermediate and poor risk groups, median survival was 10 and 4 months, respectively. (236)

Later, in 2009, Heng et al. validated the use of components of the MSKCC prognostic model in a landmark retrospective study among 645 mRCC patients treated with VEGF pathway inhibitors. In addition to the previously described risk factors, Heng et al. demonstrated that high pretreatment platelet and neutrophil counts were associated with shorter survival. Based on these findings, a prognostic model, the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC), was constructed, demonstrating a median OS of 27 and 8.8 months in intermediate and poor-risk patients, respectively. Among patients with a favorable risk profile, the median OS was not reached, and the 2-year OS was 75%. (237) These results were later externally validated among 1028 mRCC patients treated with sunitinib, sorafenib, pazopanib, bevacizumab, or axitinib (184).

The MSKCC and IMDC prognostic models are still used for the prognostication of mRCC patients, as well as the stratification of patients for clinical trials. As these models have mainly been used in the era of cytokines and anti-VEGF therapies, their
role with modern immunotherapy agents remains to be elucidated. The MSKCC and IMDC prognostic models are depicted in Table 4.

The further search for new predictive and prognostic markers has additionally provided preliminary evidence of hyponatremia as a prognostic factor for mRCC patients. Jeppesen et al. were the first to demonstrate that baseline sodium levels below the normal range were associated with shorter survival among mRCC patients treated with IL-2 and IFN-α (238). Since then, several studies have shown a similar association between hyponatremia and a poor outcome among mRCC patients treated with tyrosine kinase inhibitors (239-241). Sodium values are not, however, routinely used in clinical practice to improve the prognostication of mRCC patients.
### Table 4. Comparison between the MSKCC and the IMDC Prognostic Risk Criteria for RCC

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>MSKCC Criteria</th>
<th>IMDC Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karnofsky performance status &lt; 80%</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Time from diagnosis to treatment &lt; 1 year</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>LDH level ≥ 1.5xULN</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin level below LLN</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Corrected serum calcium level above ULN</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Platelet count above ULN</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Neutrophil count above ULN</td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

**Entry Population Criteria**

- Patients with metastatic RCC
- Treated with interferon alfa as initial systemic therapy
- Treated with first-line TKI therapy

**Distribution of Risk Groups**

- 0 criteria (favorable) 168 pts (25%) 133 pts (21%)
- 1–2 criteria (intermediate) 355 pts (53%) 301 pts (47%)
- ≥3 criteria (poor) 147 pts (22%) 152 pts (32%)

**Median OS, by Risk Group**

- 0 criteria (favorable) 20.0 mo Not reached
- 1–2 criteria (intermediate) 10.0 mo 27.0 mo
- ≥3 criteria (poor) 4.0 mo 8.8 mo

LDH = lactate dehydrogenase; LLN = lower limit of normal; ULN = upper limit of normal; pts = patients; mo = months
4.1.2 Vascular Endothelial-derived Growth Factor

The VEGF family comprises five different mammalian ligands: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PLGF). These growth factors function as regulators of angiogenesis and lymphangiogenesis, and they bind to three different but structurally related receptor tyrosine kinases: VEGFR-1, VEGFR-2, and VEGFR-3 (242).

In RCC, where angiogenesis is an essential event of tumorigenesis, upregulation of VEGF due to the loss of pVHL is well documented (243, 244). VEGF-A, which is the most widely studied member of the family, has been shown to promote tumorigenesis through a variety of ways. It acts on tumor endothelial cells to increase their proliferation, migration, and permeability and it inhibits vessel maturation (245). An immunomodulatory role has also been suggested (246). Since many tumor cells express VEGFRs, VEGF-A may also possess more direct effects in supporting tumor growth and invasion (247).

Previous research has demonstrated that a low baseline concentration of VEGF-A is prognostic for overall survival in mRCC in univariate analyses (248, 249). High serum VEGF-A levels have additionally been associated with the tumor stage and grade and a poor prognosis (250). Rini et al. reported that low VEGF-C and soluble VEGFR-3 concentrations were associated with longer PFS and higher response rates in bevacizumab-refractory patients treated with sunitinib (251). These results are supported by a phase III multicenter trial demonstrating an association with low baseline levels of VEGF-C and prolonged PFS among a total of 750 mRCC patients (248). Additionally, soluble VEGFR-3 concentrations correlated with the outcome among patients treated with sunitinib, but not among patients on the IFNα treatment.
arm, suggesting a possible role for soluble VEGFR-3 in predicting sunitinib treatment efficacy (248).

Evidence regarding soluble VEGFR-2 as a biomarker remains controversial. Gruenwald et al. noticed decreased soluble VEGFR-2 concentrations during sunitinib treatment, but this kinetic modulation was insufficient to predict the tumor response (252). On the other hand, Terakawa et al. showed that increased VEGFR-2 expression in a specimen obtained from radical nephrectomy correlated significantly with longer PFS (253).

4.1.3 Carbonic Anhydrase IX

Carbonic anhydrase IX (CAIX) is a HIF-1a-regulated, transmembrane protein that regulates intracellular pH in response to hypoxia. In ccRCC, CAIX is overexpressed due to inactivation of the VHL gene product, resulting in overexpression of HIF-1a, a transcription factor for CAIX (25).

CAIX is present in more than 80% of primary and metastatic RCCs, whereas it is detected in only 9% of normal kidneys (254). Several studies have reported increased CAIX expression as an independent predictor of longer disease-specific survival in mRCC (255-257), but recent studies have been unable to confirm this finding (186, 258). Considering that 94–100% of ccRCCs stain positively for CAIX (254, 257), it may be helpful in establishing a diagnosis, but its value as a biomarker improving prognostication or predicting the treatment response is unclear.
4.1.4 Cytokine and Angiogenic Factors

Several cytokines as well as other proteins of the angiogenic cascade have been evaluated as biomarkers for the response in RCC. A recent retrospective analysis of phase II and phase III trials evaluated 17 different cytokine and angiogenic factors (CAFs) among patients with mRCC treated with pazopanib, identifying seven promising biomarkers: interleukin 6 (IL-6), interleukin 8 (IL-8), VEGF, osteopontin, E-selectin, hepatocyte growth factor (HGF), and tissue inhibitor of metalloproteinases (TIMP)-1. In the study, low concentrations of these factors correlated with increased tumor shrinkage, PFS, or both. IL-6, IL-8, and osteopontin were additionally shown to be stronger prognostic markers than any single clinical classification when stratified by the ECOG performance status, MSKCC risk group, or HENG risk group. Based on the results, a CAF signature was built demonstrating a significantly shorter PFS and OS for pazopanib-treated patients in the high CAF group (259).

Another study investigating CAFs in sorafenib-treated mRCC patients identified six baseline markers that significantly correlated with PFS. Higher concentrations of osteopontin, CAIX, VEGF, collagen IV (ColIV), and soluble VEGFR-2 were associated with shorter PFS, while the opposite relationship was seen for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In a dichotomized CAF signature for these six biomarkers, ‘signature-negative’ patients demonstrated improved PFS with a hazard ratio of 0.2 ($P = 0.00002$). Furthermore, a significant interaction was noted between the CAF signature and treatment arm (sorafenib alone or sorafenib + IFNα), suggesting a possible predictive role for this biomarker panel (260).
While several studies have yielded promising results for CAFs as prognostic, and even predictive, biomarkers in mRCC, some issues have limited their clinical usefulness. First, CAFs have mainly been investigated among mRCC patients treated with TKIs. Little or no data exist regarding CAFs in mRCC patients treated with other therapies such as mTOR inhibitors. Given the significant number of therapies available for mRCC, prospective studies comparing CAFs between different treatment arms are needed. Second, methodological problems regarding measurement and cut-off values for these biomarkers exist, limiting their more widespread application in clinical settings.

4.1.5 Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) are variations occurring in a single nucleotide at a specific position in the genome. They are common mutations that can alter the function of genes in any chromosome. Several studies have investigated the possible association of various SNPs and the outcome in mRCC. The main focus has been on identifying prognostic and predictive SNPs for VEGF-targeted therapy.

Two studies investigating SNPs involved in the pharmacokinetic and pharmacodynamic pathways of sunitinib suggested that polymorphisms in VEGFR1, VEGFR3, CYP3A5, NR1/3, and ABCB1 might be able to define a subset of patients with a decreased sunitinib response and tolerance (261, 262). Additionally, three separate studies investigated two SNPs of VEGFR1 in sunitinib-treated patients, demonstrating a favorable association between these mutations and the outcome (263-265). However, a recent meta-analysis by Liu et al. was unable to verify this association, thus questioning their clinical use as biomarkers of the sunitinib response (266).
Escudier et al. investigated 15 different SNPs among patients in the phase III AXIS trial, a study comparing axitinib and sorafenib in a second-line setting. Although the authors were able to identify a polymorphism in VEGFR2 predicting improved OS and PFS for sorafenib-treated patients, the authors concluded that sensitivity/specificity limitations preclude its use for selecting patients for sorafenib treatment (267).

For pazopanib-treated patients, three polymorphisms in IL-8 and HIF-1α and five polymorphisms in HIF-1α, NR1/2, and VEGF-A were shown to be significantly associated with PFS and the response rate in a study by Xu et al. (268). The same authors pursued these suggestive associations among patients from the phase III trial COMPARZ comparing pazopanib and sunitinib, and from an observational study of sunitinib-treated patients. In a combined analysis, IL-8 polymorphisms were associated with shorter OS in both pazopanib- and sunitinib-treated mRCC patients, raising the possibility that IL-8 polymorphisms may be associated with the outcome, irrespective of the treatment (269).

4.1.6 Immune Markers

Several clinical and laboratory factors have been investigated and identified as being prognostic and predictive for mRCC patients treated with IFNα and IL-2. These include the performance status, clear cell histology, MSKCC risk classification, C-reactive protein (CRP), neutrophil levels, and the number of metastatic sites. For modern immunotherapy agents, the search for predictive and prognostic biomarkers is ongoing and some preliminary results have already shown promise.
PD-1 is an immune checkpoint molecule expressed in activated T and B cells. It binds to two ligands, PD-L1 and PD-L2. The interaction of PD-1 and PD-L1 leads to immune suppression through negative regulation of activated T cell effector functions. In RCC, PD-L1 is overexpressed in 30% of tumors and correlates with a more advanced tumor stage, higher Fuhrman grade, sarcomatoid differentiation, and poor survival (270, 271). With the emerging role of anti-PD-1/PD-L1 agents in the treatment of mRCC, PD-L1 expression is currently being investigated as a potential predictive biomarker for patients treated with these agents. A phase I trial using a 5% cut-off value for PD-L1 expression among previously treated mRCC patients reported higher response rates for nivolumab among PD-L1-positive patients as compared to PD-L1-negative patients (22% vs. 8%) (219). Research has revealed similar associations among patients with metastatic melanoma and non-small-cell lung cancer (272). In the recent phase III CheckMate 214 study comparing the combination of nivolumab and ipilimumab with sunitinib, the ORR significantly favored the combination over sunitinib in intermediate/poor-risk patients with baseline PD-L1 expression of ≥1% (58% vs. 25%; P = 0.0002) (225). Additionally, in a phase I study of atezolizumab, an anti-PD-L1 agent, exploratory subanalyses demonstrated that upregulation of PD-L1 in on-treatment biopsies as compared to baseline expression was associated with an improved response rate, suggesting a possible role as an on-treatment predictive biomarker (273).

Several issues, however, limit the use of PD-L1 expression as a predictive biomarker. First and foremost, it fails to identify all responders and not all PD-L1-positive tumors respond to treatment. Furthermore, there are currently no standardized cut-off points to define the positivity of a sample.

Another promising immune-related biomarker is tumor T-cell infiltration. In a phase I study investigating nivolumab, baseline tumor CD3+ and CD8+ T-cell infiltrates correlated with a decrease in the tumor burden (272). A prospectively designed
exploratory analysis from the S-TRAC trial also identified tumor CD8+ T-cell density as predicting longer DFS for sunitinib but not placebo (274).

Other biomarkers under investigation include PD-L2 expression, the presence of CD20+ B-cells, gene expression profiling, and the T-cell receptor repertoire, but none of them have so far been validated.

### 4.1.7 Mechanism-based Adverse Events

Current understanding depicts treatment-related toxicity events as on-target effects resulting from the inhibition of a given pathway. It therefore stands to reason that these on-target effects are associated with treatment efficacy and could serve as surrogate markers of the pharmacodynamic effect. Research has indeed shown that a class-specific adverse event of VEGFR inhibitors, hypertension (HTN), associates with an improved outcome. Following the preliminary results of Bono et al., demonstrating a favorable association of bevacizumab-induced HTN and the outcome (275), a phase III trial investigating the combination of bevacizumab and interferon-α vs. interferon-α alone later confirmed these findings. In the study, patients on combination therapy who developed grade 2 HTN had significantly longer PFS (13.2 vs. 8.0 months; \( P = 0.001 \)) and OS (41.6 vs. 16.2 months; \( P = 0.001 \)) as compared to patients who did not develop HTN (276). A retrospective analysis among 544 mRCC patients by Rini et al. revealed a similar association of sunitinib-induced HTN and the outcome (277). Supporting evidence exists for axitinib (278), sorafenib (226), and tivozanib (279).
In addition to hypertension, several other class-specific adverse events have been examined as potential biomarkers of anti-VEGFR treatment efficacy. Donskov et al. examined hand-foot syndrome, fatigue, neutropenia, and thrombocytopenia among sunitinib-treated mRCC patients. In the analyses, neutropenia was significantly associated with longer PFS and OS, and hand-foot syndrome with longer OS (280). Similarly, Rautiola et al. demonstrated that in addition to HTN, neutropenia and thrombocytopenia were predictors of improved treatment efficacy. A biomarker profile categorizing patients into favorable, intermediate, and poor risk profiles according to the presence of these AEs was constructed, demonstrating a clear difference in OS to the benefit of patients with all three AEs vs. patients with none of the AEs (OS; not reached vs. 5.3 months; $P < 0.001$) (281). Evidence regarding treatment-induced hypothyroidism is less clear. While many smaller trials have suggested a possible association of hypothyroidism and the treatment outcome, a large meta-analysis comprising several retrospective and prospective trials found no significant association of acquired hypothyroidism and the outcome for mRCC patients treated with sunitinib (282).

For mTOR inhibitors, previous research has shown that non-infectious pneumonitis, a class effect of mTOR inhibitors, may associate with an improved outcome. In the largest study to date, Atkinson et al. demonstrated that pneumonitis independently predicted improved OS (HR 0.32; $P < 0.001$) for mRCC patients treated with everolimus. Other class-specific AEs, including serum cholesterol, triglyceride, and glucose levels, have also been investigated. In a phase III trial among intermediate and poor-risk temsirolimus-treated mRCC patients, increases in on-treatment serum cholesterol levels above baseline values were associated with longer PFS (HR 0.81, $P < 0.0001$) and OS (HR 0.77, $P < 0.0001$). However, no such association was noted for hypertriglyceridemia or hyperglycemia (283).
4.2 Incorporation of Biomarkers into Clinical Practice and Future Directions

Despite the widespread use of targeted therapies in mRCC, reliable predictive biomarkers are still lacking, and the accepted biomarkers are mainly prognostic and clinical-related. Even though mechanism-based toxicities, such as hypertension, seem to serve as surrogate markers of treatment efficacy, they do not help in patient selection. As discussed earlier, several promising results exist regarding modern immunotherapy agents. However, most of the published evidence remains at an immature stage with inconsistencies for many of the investigated biomarkers. In addition to problems arising from the lack of cut-off points and standardization, tumor heterogeneity and the use of archival tissue samples remain unresolved issues.

Promising avenues that have recently emerged are the sampling of circulating tumor DNA (ctDNA) and microRNA (miRNA) profiling. ctDNA is obtained from peripheral blood and is therefore non-invasive and allows for dynamic evaluation of tumor genomics and the response to treatment. A few studies have already shown that ctDNA may be a useful tool in monitoring the tumor burden and response to treatment (284, 285). Similarly, miRNA from blood plasma is easily obtained and recent studies have demonstrated that specific miRNA signatures may predict the disease outcome and recurrence in ccRCC (286-288).

With the renaissance of immunotherapy agents in the treatment of mRCC, the integration and optimization of these therapies into new treatment algorithms is of vital importance. While decades of research have demonstrated the immunological and molecular heterogeneity of RCC, novel technologies such as real-time PCR, microarrays, and next-generation sequencing are bringing us closer to identifying robust and reliable prognostic and predictive biomarkers, allowing for a molecularly stratified biomarker-guided treatment approach.
AIMS OF THE STUDY

The aim of the study was to discover markers predicting treatment efficacy for targeted therapies used in the treatment of mRCC and to gain knowledge of those deeper factors through which we could enhance treatment efficacy.

Specific aims of the study were:

1) To identify clinical prognostic and predictive biomarkers for mRCC patients treated with targeted therapy;
2) To identify molecular prognostic and predictive biomarkers for mRCC patients treated with targeted therapy;
3) To investigate the mechanism-based treatment-related adverse events of targeted therapies and their correlation with clinical efficacy.
PATIENTS AND METHODS

1. Patients and Treatment

The patient cohorts are described in detail in the original publications I–IV. The first study included 303 consecutive mRCC patients treated with a first-line anti-VEGF agent, either sunitinib or pazopanib, at the Comprehensive Cancer Center, Helsinki University Hospital, between October 18, 2006 and December 31, 2014. None of the patients had received prior TKI therapy. Eighteen patients (5.9%) had received prior interferon alfa. Both sunitinib and pazopanib were administered according to standard care until disease progression or unacceptable toxicity. Altogether, 23% (N = 59) of the patients started sunitinib with intermittent dosing (4 weeks on-treatment, 2 weeks off-treatment) and 77% (N = 208) with continuous dosing. Pazopanib was administered continuously.

The second study comprised mRCC patients treated with first-line sunitinib at the Comprehensive Cancer Center, Helsinki University Hospital, between October 18, 2006 and May 31, 2012. We were able to identify 137 patients for whom sufficient clinical data and sufficient histologic specimens were available.

Studies III and IV were performed in collaboration with Aarhus University Hospital, Denmark. We identified a total of 85 patients who were treated with everolimus after prior anti-VEGFR therapy failure at the Comprehensive Cancer Center, Helsinki University Hospital, between October 18, 2006 and December 31, 2014. A similar cohort comprising 148 patients treated between January 25, 2010 and June 6, 2016 at Aarhus University Hospital, Denmark, was collected. In both cohorts, everolimus was administered according to standard care until disease progression or unacceptable toxicity.
1.1 Assessment of the Tumor Response and Adverse Events

In all studies, the response to treatment was assessed by computed tomography at 8- to 12-week intervals. Treatment efficacy was reported according to the Response Evaluation Criteria in Solid Tumors (RECIST) versions 1.0 and 1.1. Adverse events were captured every 4–6 weeks and were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) versions 3.0 and 4.0.

1.2 Assessment of Pneumonitis

To correctly assess whether a patient developed pneumonitis during everolimus treatment, medical files, including CT scan reports, were retrospectively reviewed for pneumonitis. The radiographic studies were subjected to a blinded review by an experienced radiologist for findings indicative of pneumonitis. A more detailed description is provided in the original publication (III). Figure 5 depicts typical radiographic findings indicative of pneumonitis.

Figure 5. CT scans at the onset of pneumonitis showing peripheral, bilateral ground glass opacities.
1.3 Immunohistochemistry

In the second study, the expression of c-Met was analyzed from formalin-fixed paraffin-embedded samples by using an anti-c-Met rabbit monoclonal ready-to-use antibody by Roche, and BenchMark XT immunostainer by Ventana Medical Systems. The staining was carried out according to the manufacturer’s protocol. c-Met staining was scored by two independent evaluators with good concordance (kappa value 0.7) according to intensity. A 4-tier system was used with 0 as no staining, 1+ weak, 2+ strong, and 3+ as very strong staining intensity. The c-Met immunohistochemistry score was also determined by assessing the proportion of stained cells as the intensity of the staining. Negative (0): fewer than 50% of tumor cells with membrane and/or cytoplasmic staining (any intensity); weak (1): ≥50% of tumor cells with weak or higher membrane and/or cytoplasmic staining but <50% of tumor cells with a moderate or strong staining intensity; strong (2): ≥50% of tumor cells with moderate or strong membrane and/or cytoplasmic staining but <50% of tumor cells with a strong staining intensity; very strong (3): ≥50% of tumor cells with a strong membrane and/or cytoplasmic staining intensity. Figure 6 displays examples of each category.

Figure 6. Examples of negative, moderate, strong, and very strong staining (A–D) of anti-c-Met antibody with 400x magnification. (Reprinted from Peltola KJ et al. Correlation of c-Met Expression and Outcome in Patients With Renal Cell Carcinoma Treated With Sunitinib. Clin Genitourin Cancer. 2017 Aug;15(4):487-494 with the permission of Elsevier)
2. Statistical Analysis

All the statistical analyses were performed using IBM SPSS Statistics for Windows (versions 22.0–24.0, Armonk, NY, USA, IBM Corp.). Tests used to assess the association of various clinicopathological factors were the Mann-Whitney U-test for continuous data and the chi-squared test for categorical data. In all original publications, OS was defined as the time from treatment initiation to death from any cause, and (ii) PFS as the time from treatment initiation to the first event (tumor progression or death from any cause). The Kaplan-Meyer method was used to estimate the median survival times with 95% confidence intervals for both OS and PFS, censoring the patients who were alive or had no disease progression at the last follow-up visit.

Uni- and multivariate Cox proportional hazard models were used to assess the association of OS and PFS with various clinicopathological factors. Interactions between the factors of interest were tested with inclusion of the interaction terms in the models. The proportional hazards assumptions were assessed graphically, obtaining plots of (log(-logS(t))) versus time and Schoenfeld residuals versus time.

Uni- and multivariate logistic regression models were used to investigate the effects of various clinicopathological factors and categorical end-point data. The results are expressed as odds ratios (ORs) with 95% CI.

To control for time bias when investigating potentially time-biased parameters, time-dependent Cox regression models and landmark survival analyses were applied. According to the landmark method, PFS and OS were defined as the time from the landmark to progression or death from any cause.

All statistical tests were two-sided and P-values of <0.05 were considered as statistically significant.
3. Ethical Considerations

The Hospital District of Helsinki and Uusimaa (HUS) accepted the research plan §36/28.4.2014 and the Ethics Committee of the Department of Surgery of HUS accepted the research plan §190/23.10.2013. The National Authority for Welfare and Health licensed the use of pathological archive material.
RESULTS AND DISCUSSION

Ever since the introduction of targeted therapies in the treatment of metastatic RCC in the mid-2000s, the search for reliable biomarkers has been of great interest. Even though there are presently no such biomarkers that could identify patients for specific treatments, the ever-growing knowledge of the intricate molecular and immunogenic mechanisms of tumor biology in RCC has brought us closer to the moment of finding clinically applicable predictive biomarkers. This is even more important now that the first-line options to treat advanced RCC are on the verge of a major change.

1. The use of angiotensin system inhibitors (ASIs) in the treatment of TKI-induced hypertension (I)

Hypertension (HTN) is a class effect of VEGF receptor tyrosine kinases and it has been reported as an adverse event of all agents in this class (289-292). This class effect also seems to function as a surrogate marker of treatment efficacy in patients with mRCC (277, 278, 281, 293). As antihypertensive medication, angiotensin system inhibitors have been of interest in the treatment of TKI-induced HTN, as xenograft models have demonstrated that ASIs may possess antiangiogenic potential (294, 295)

We investigated treatment-induced hypertension and the use of ASIs among 303 consecutive mRCC patients treated with a first-line anti-VEGFR agent. Of these 303 patients, 197 (65%) patients had baseline hypertension and 126 (64%) of these
patients had ASI as the baseline antihypertensive medication, either as monotherapy or in combination with other antihypertensive medication. Treatment-induced HTN, defined as a recurrent, persistent, or symptomatic rise in diastolic BP of >20 mmHg or a systolic BP rise to >150 mmHg, if previously within the normal range (CTCAE v. 3.0, grade ≥2), developed among 110 patients, of whom the majority were ASI users (82.7%).

We found no difference in the outcome between patients with and without baseline HTN (OS: 20.3 months (95%CI 16.4–24.2 months) vs. 20.1 months (95%CI 15.5–24.7 months); PFS: 8.2 months (95%CI 6.6–9.7 months) vs. 8.2 months (95%CI 5.4–11.0 months); P = 0.54 and P = 0.72, respectively). Similarly, we found no significant differences in outcomes between patients with and without baseline ASI use. On the other hand, patients who developed HTN during treatment had significantly longer OS (unadjusted HR 0.41, 95%CI 0.30–0.55, P < 0.001) and PFS (HR 0.43, 95%CI 0.33–0.56, P < 0.001). The same was true for patients receiving ASI as a new drug or dose escalation (N = 91) when compared to the remaining patients (OS: 37.7 months (95%CI 26.0–49.5) vs. 16.5 months (95%CI 12.9–20.2), HR 0.38, 95%CI 0.26–0.54, P < 0.001; PFS: 16.7 months (95%CI 6.1–27.2) vs. 6.6 months (95%CI 5.8–7.5), HR 0.41, 95% CI 0.30–0.57, P < 0.001).

Due to the strong multicollinearity of HTN and ASI use, they could not be fitted in the same multivariate analyses. We therefore conducted subgroup analyses among patients with TKI-induced HTN. In these analyses, ASI users (N = 91) had longer OS (37.5 vs. 18.1 months; P = 0.001) and PFS (17.1 vs. 7.2 months; P = 0.004) than ASI nonusers. These results were supported by multivariate analyses conducted in the same subset of patients, demonstrating superior OS (adjusted HR, 0.35; 95%CI
0.18–0.66; \( P = 0.001 \)) and PFS (adjusted HR, 0.42; 95%CI 0.23–0.77; \( P = 0.005 \)) for ASI users when adjusted for the baseline HENG risk classification.

Regarding TKI-induced HTN, our results are well in line with previously published findings. In our patient cohort, treatment-induced hypertension was detected in 36.3% of patients. The previously reported incidence of TKI-induced all-grade HTN has varied between 17% and 49.6% (296). Several studies have depicted TKI-induced HTN as a predictive biomarker of treatment efficacy (278, 280, 281, 293, 297, 298). We found a similar association in our data, with TKI-induced HTN having a strong association with prolonged OS and PFS, even when corrected for the possible time bias from longer treatment. Due to disproportionate group sizes, patients treated with sunitinib (88%) and pazopanib (22%) were not separately analyzed.

Several retrospective studies have investigated the use of ASIs in cancer patients. In a meta-analysis comparing ASIs in a variety of cancer patients, their use was associated with a significant improvement in DFS and OS. Benefits were noted among patients with urinary tract cancer, colorectal cancer, pancreatic cancer, and prostate cancer (299). In RCC, a few studies have reported a significant association between ASI use and an improved clinical outcome among mRCC patients receiving VEGF-targeted therapy. All of the previous studies have investigated ASI use at baseline or within 30 days of therapy initiation (300-302). The largest study to date, investigating this association among 4736 patients, demonstrated a significant improvement in OS (HR 0.84, \( P = 0.0105 \)) for ASI users vs. users of other antihypertensive medication. Additionally, this association was more prominent among patients on VEGF-targeted therapy (302). Contradicting evidence also exists, however, as Sorich et al. recently noted no difference in the outcome for baseline ASI users vs. nonusers among 1545 mRCC patients treated with sunitinib and
pazopanib. In this study, however, for patients treated with sunitinib there was also a trend towards improved survival and baseline ASI use. (303) There are no obvious differences between these studies explaining the contrasting results, but classifying baseline ASI use within the first 30 days of VEGF-targeted therapy may contribute to the disparity, as TKI-induced HTN, a validated mechanism-based adverse event, often manifests within the first weeks of therapy. Furthermore, the concomitant use of other antihypertensive medication was in most studies unaccounted for.

To the best of our knowledge, our study is the first to investigate ASI use in the treatment of TKI-induced HTN. Within this subset of patients, we demonstrated a significant association between the use of ASIs and an improved outcome. There are currently no guidelines for cancer patients suffering from HTN as a result VEGF-targeted therapies. The pathogenesis of TKI-induced hypertension is also not completely understood. Key hypotheses include the inhibition of endothelial nitric oxide (NO) synthase, increased vascular stiffness, inhibition of the renin-angiotensin system, and a decrease in the density of microvessels leading to an increase in systemic vascular resistance (304-307). Regarding the role of the renin-angiotensin system in cancer development, preclinical data have shown that angiotensin II may promote cancer development, as it is involved in the regulation of apoptotic mechanisms and angiogenesis through the up-regulation of VEGF expression and neovascularization (308, 309). It additionally facilitates cellular growth and proliferation through several growth factors (310). In RCC, angiotensin II has been demonstrated to correlate with tumor aggressiveness and decreased survival (311). In murine models, ASIs have been shown to prevent the development of metastatic lung nodules, as well as potentiate the antiangiogenic effects of sunitinib (294, 295).
McKay et al. hypothesized that the improved outcome noted among ASI users could represent a synergistic interaction of TKIs and ASIs (302). The mechanisms by which this happens are yet to be elucidated, but it has been suggested that ASIs may reduce sarcopenia (312). Earlier research has shown that sarcopenia combined with a body mass index of <25 kg m\(^{-2}\) predicts sunitinib-induced early dose-limiting toxicities and sorafenib-induced dose-limiting toxicities in mRCC patients (313, 314). The use of ASIs could thus improve the therapeutic index of VEGF-targeted therapies, resulting in improved efficacy.

Our results support the hypothesis of possible synergistic interactions between TKIs and ASIs. Based on our study, conclusions on whether the possible underlying interactions are the result of combinatory effects on angiogenesis, tumor cell proliferation, reduced blood flow to the tumor, or improvement in the therapeutic index cannot be drawn. In the absence of published guidelines for the treatment of HTN in this setting, ASIs do, however, present an interesting option as antihypertensive medication, as they might offer a survival benefit as well as feasible management of TKI-induced HTN in patients with mRCC. We do recognize that for these results to be properly validated, further studies, preferably in a randomized prospective setting, are warranted.

2. **c-Met expression is associated with the outcome among mRCC patients treated with sunitinib (II)**

c-Met, a receptor tyrosine kinase, is involved in cell growth/differentiation, neovascularization, and tissue repair in normal tissues. Interfering and directly
activating mutations leading to dysregulation of c-Met and its ligand, hepatocyte growth factor (HGF), have been implicated in tumor development, invasion, and angiogenesis in several malignancies (83-85). In RCC, both clear cell and papillary subtypes have demonstrated excessive activation of the c-Met/HGF pathway (315, 316). Furthermore, overexpression of c-Met is associated with poor pathologic features and prognosis in RCC (317). In our study, we sought to analyze the possible association of c-Met expression and the outcome among mRCC patients treated with first-line sunitinib.

c-Met expression was analyzed from 137 formalin-fixed paraffin-embedded tumor samples collected at diagnosis. The samples consisted of resected primary RCC tumors (N = 134) and core needle biopsies (N = 3) originally retrieved for diagnostic purposes. Clinical data were collected, including patient characteristics, treatments, adverse events, hospitalizations, and outcomes. c-Met expression was divided into two groups, with low expression consisting of expression levels 0 to 1 and high expression consisting of levels 2 and 3.

Among the 137 patients, 78 (56.9%) patients had low c-Met expression and 59 (43.1%) high c-Met expression. Patients with low c-Met were significantly older than patients with high c-Met (P = 0.007). Additionally, patients with low c-Met were significantly more likely to belong to the favorable HENG criteria risk group (P < 0.001) and have a clear cell histology (P = 0.024).

In survival analyses, patients with low c-Met expression had significantly longer PFS (14.3 months vs. 6.5 months; P < 0.001) and OS (32.1 months vs. 20.1; P = 0.049) than patients with high c-Met expression. In multivariate analyses, low c-MET
expression remained significantly associated with improved PFS when adjusted for the HENG risk classification (adjusted HR 0.61; \( P = 0.016 \)). For OS, we found no statistically significant association (adjusted HR 0.75; \( P = 0.34 \)). In a logistic regression model investigating c-Met expression and the response to treatment, patients with high c-Met expression were more than four times more likely to have PD as their best response to sunitinib therapy (unadjusted OR 4.19; \( P = 0.004 \)).

Based on previous hypotheses regarding an association between c-Met and the presence of bone metastases, we conducted further subgroup analyses among patients with \((N = 31)\) and without \((N = 106)\) bone metastases present at the onset of treatment. Even though the level of c-Met expression and presence of bone metastases were not significantly associated with each other \((P = 0.47)\), in survival analyses, low c-Met expression was associated with improved OS (unadjusted HR 0.63; \( P = 0.034 \)) and PFS (unadjusted HR 0.47; \( P < 0.001 \)) in patients without bone metastases, but not among patients with bone metastases.

To the best of our knowledge, this is the first study to establish an association between c-Met expression and the outcome among patients treated with first-line anti-VEGFR therapy. Our results are similar to previously published data in the sense that higher c-Met expression was associated with a poor prognosis and response rate. We hypothesize that c-Met expression could thus serve as a biomarker defining patients who are more likely to benefit from alternative therapy.

Regarding the reasons why c-Met expression was associated with PFS but not OS in multivariate analyses, we hypothesize that this difference may be associated with the availability of active therapies beyond first-line sunitinib. Additionally, the number
of registered events was higher when using PFS as the endpoint (129 events for PFS vs. 113 events for OS), possibly leading to a lack of statistical power in OS analysis. In the oncology literature, there is a general trend for the HR for OS to be closer to 1.00 than the HR for PFS. Due to the longer median duration of OS, a greater number of events is required to reach statistical significance. (318)

Preclinical evidence suggests that inhibition of VEGF activity leads to the induction of compensatory mechanisms upregulating growth factors and cytokines. The developing resistance is thought to be largely indirect, with angiogenic tumors adapting to the presence of angiogenesis inhibitors. While the specific therapeutic target remains inhibited, several adaptive mechanisms, including the activation of alternative pro-angiogenic pathways, recruitment of bone marrow-derived proangiogenic-cells, and increased pericyte coverage of the tumor vasculature, lead to tumor evasion and ultimately resistance to antiangiogenic therapy (319). In a xenograft study investigating sunitinib efficacy, the investigators demonstrated increased levels of HGF in the stroma of sunitinib-resistant tumors, suggesting that HGF-stimulated c-Met signaling may enable tumor cells to bypass the desired effects of anti-VEGFR therapy (320). Regulation of c-Met activity during anti-VEGF therapy is illustrated in Figure 7. Given the possible role of the c-Met/HGF pathway in the development of resistance to anti-VEGFR therapy, our results stand to reason, as patients with high c-Met expression achieved less benefit from sunitinib treatment.

Previous research in breast cancer has depicted c-Met as a mediator of the interactions between tumor cells and the bone microenvironment, suggesting that it is involved in the bone metastatic process (321). D’Amico et al. demonstrated that c-Met expression on RCC stem cells drives renal cancer progression to bone, since RCC stem cells expressing c-Met directly formed bone lesions. Furthermore, in the same study, treatment with a selective c-Met inhibitor hindered the development of
bone metastases, indicating that HGF/c-Met signaling is relevant in the metastatic process induced by RCC stem cells (322). These findings are supported by clinical data from the METEOR phase III trial, in which patients with bone metastases treated with the dual inhibitor cabozantinib had a 46% risk reduction for OS and 67% risk reduction for PFS when compared to patients treated with everolimus. Additionally, the objective response rate in bone scans was 17% vs 0%, for cabozantinib and everolimus, respectively (323). In our analyses, we found that in the subgroup of patients without bone metastases, low c-Met expression predicted an improved outcome. The implications of this finding are presently unclear, but it could be hypothesized that low c-Met expression among patients without bone metastases represents a subset of patients in whom resistance to antiangiogenic therapy has not yet developed or is less likely to develop.

In the METEOR phase III trial comparing cabozantinib and everolimus among mRCC patients after previous VEGFR tyrosine-kinase treatment, the authors additionally investigated MET expression levels and the treatment outcome. While the study did demonstrate relevant clinical activity for cabozantinib vs. everolimus, it did not provide evidence for a significant relationship between the treatment modality, c-Met expression, and the outcome. However, in as many as 41.5% of patients, c-Met expression could not be evaluated. Additionally, archival tumor tissue rather than fresh biopsies was used in the analyses (324). A recent phase II trial investigating crizotinib, a small-molecule TKI inhibiting MET, anaplastic lymphoma kinase (ALK), and ROS proto-oncogene 1 receptor tyrosine kinase (ROS1), demonstrated that crizotinib induced long-lasting disease control in metastatic papillary renal cell carcinoma type 1 with MET mutation and long-term stable disease in a case with MET amplification (325). These results are of special interest now that the CABOSUN trial has demonstrated that cabozantinib may have
a role as first-line therapy and has indeed already been approved by the Food and Drug Administration (FDA) to be used as such in the United States (326). Our results, along with the growing evidence implicating the c-Met/HGF pathway as one of the key regulators of tumorigenesis and angiogenic escape, warrant further studies investigating c-Met as biomarker among patients treated with dual c-Met/VEGFR inhibitors.
Figure 7. Regulation of c-Met activity during anti-VEGF therapy. PTP1B = protein tyrosine phosphatase 1B. (Adapted from McCarty J., Glioblastoma Resistance to Anti-VEGF Therapy: Has the Challenge been MET? Clinical Cancer Research 2013) (327).
3. Everolimus-induced pneumonitis predicts the treatment outcome among mRCC patients treated with everolimus (III)

Several studies have depicted the treatment-related adverse events associated with targeted therapies as possible predictive markers of treatment efficacy. Treatment-induced hypertension was one of the first biomarkers shown to be favorably associated with the efficacy of sunitinib treatment (277). Since then, multiple different adverse events have been associated with the outcome. In addition to hypertension, Donskov et al. demonstrated that on-treatment neutropenia was significantly associated with longer OS and PFS and hand-foot syndrome with OS (280). Similar results were recorded by Rautiola et al., who demonstrated that the development of hypertension, neutropenia, and thrombocytopenia was significantly associated with OS and PFS (281). For mTOR inhibitors, such on-treatment biomarkers predicting treatment efficacy do not presently exist. We sought to investigate the possible association between everolimus-induced pneumonitis and the treatment outcome.

Our study population comprised two patient cohorts: cohort A (N = 85) and a validation cohort B (N = 148), which were analyzed separately. In cohort A, 29 (34.1%) patients had CT-verified pneumonitis during everolimus treatment. Eight cases were grade 1 (27.6%), 18 cases grade 2 (62.1%), and three cases were grade 3 (10.3%). No grade 4 (life-threatening) pneumonitis was recorded. In cohort B, 29 (19.6%) patients had CT-verified pneumonitis. Among patients with pneumonitis, the median time to onset was 2.4 months (range 0.4–7.5 months) in cohort A and 2.8 months (range 0.1–14.1 months) in cohort B.
In survival analyses, everolimus-induced pneumonitis was significantly associated with OS (cohort A: 24.7 vs. 8.5 months; \( P < 0.001 \); cohort B: 12.9 vs. 6.0 months; \( P = 0.02 \)) and PFS (cohort A: 5.5 vs. 3.2 months; \( P = 0.002 \); cohort B: 6.0 vs. 2.8 months; \( P = 0.02 \)) in both cohorts. Additionally, pneumonitis was associated with an improved clinical benefit rate (CBR): 57.1% vs. 24.1% and 79.3% vs. 24.1% for cohorts A and B, respectively. In multivariate analyses, pneumonitis remained significantly associated with both OS (cohort A: HR, 0.22; 95%CI 0.12–0.44; \( P < 0.001 \); cohort B: HR 0.58; 95%CI 0.36–0.94; \( P = 0.03 \)) and PFS (cohort A: HR 0.37; 95%CI 0.21–0.66; \( P = 0.001 \); cohort B: HR 0.61; 95%CI 0.39–0.95; \( P = 0.03 \)) when adjusted for age, the number of prior treatment lines, and the MSKCC classification for previously treated patients.

Pneumonitis, like all treatment-related adverse events, is dependent on the time spent on the study treatment. To account for this possible time bias resulting from longer treatment, we performed a multivariate analysis with pneumonitis as a time-dependent covariate. To provide sufficient statistical power, the two patients cohorts were combined for this analysis after it was verified that the association between pneumonitis and OS was not significantly different in the two cohorts. In a Cox regression model adjusted for age, gender, cohort, the number of previous treatment lines, and the MSKCC risk classification for previously treated patients, CT-verified pneumonitis was independently associated with longer OS (HR, 0.67; 95%CI 0.46–0.97; \( P = 0.03 \)). A landmark analysis demonstrated similar results with a significant association between pneumonitis and improved OS (17.4 vs. 7.8 months; \( P = 0.01 \)).
In cohort B, data had also been collected on patients who had clinical symptoms of pneumonitis/pneumonia but no radiological evidence of either. We therefore divided patients in cohort B into three groups: patients with pneumonitis verified by CT \((N = 29)\), patients with clinical symptoms of pneumonitis/pneumonia \((N = 25)\), and patients with no clinical or radiological signs of pneumonitis/pneumonia \((N = 94)\). In a subgroup analysis comparing OS, PFS, and the CBR between these groups, there was no statistically significant difference in any of the endpoints between patients with CT-verified pneumonitis and patients with clinical symptoms of pneumonitis/pneumonia.

In this study, we demonstrated that everolimus-induced pneumonitis significantly associated with all efficacy endpoints. Furthermore, we validated these findings in an independent patient cohort. Previous work on this subject has revealed that mTOR-inhibitor-induced pneumonitis associates with OS among mRCC patients treated with everolimus/temsirolimus (328). Research has additionally shown that the mean tumor shrinkage and SD according to the RECIST were significantly higher in patients with pneumonitis (329). However, neither study controlled for bias resulting from longer treatment, an important aspect when investigating adverse events caused by a given treatment. We accounted for the possible time bias by performing a landmark analysis, as well as multivariate analyses with pneumonitis as a time-dependent covariable. Both methods demonstrated a significant association between pneumonitis and longer OS, suggesting that our results are unlikely to be impacted by time bias.

In our study, the incidence of CT-verified pneumonitis was 34.1% and 19.6% in cohorts A and B, respectively. In the previous literature, the incidence of pneumonitis has varied widely, with reported figures between 13.5% and 48.7% among mRCC
patients treated with mTOR inhibitors (210, 329-332). In a large meta-analysis comprising 2233 patients with breast cancer, neuroendocrine tumors, or mRCC, the reported pulmonary toxicity was only 10.4% (333). Prior work has, however, demonstrated that mTOR-inhibitor-related adverse events are more common among patients with compromised renal function (334, 335), a phenomenon more prevalent among patients with mRCC. Another possible explanation for the discrepancy in the reported incidence of mTOR-inhibitor-induced pneumonitis may be due to its non-specific clinical and radiological nature, leading to inaccurate reporting of this adverse event. Clinically, pneumonitis manifests heterogeneously and symptoms may include fever, fatigue, coughing, and dyspnea, nonspecific signs and symptoms that do not facilitate diagnosis. Moreover, the traditional chest X-ray may not show any abnormalities (335). As the diagnosis of pneumonitis is difficult and is often made by excluding other causes such as infections, it is of relevance that clinicians recognize pneumonitis as a possible, and a rather common, adverse event of mTOR-inhibitor treatment.

The precise pathogenesis of mTOR-inhibitor-induced pneumonitis remains unclear. Hypothesized mechanisms include both direct toxic effects and immunological toxicity, and the combination of both. In a study by Morelon et al. investigating sirolimus-associated interstitial pneumonitis, patients displayed signs of lymphocytic alveolitis in bronco-alveolar lavage fluid analyses (336). The authors suggested that this might represent an autoimmune response to exposed cryptic antigens, leading to lymphocytic alveolitis and interstitial pneumonitis. The rapid response of pneumonitis to treatment withdrawal, however, is in support of more direct toxic effects (337, 338). A dose-dependent effect has also been suggested. Weiner et al. investigated the relationship between plasma concentration levels of sirolimus and pneumonitis. While the risk of pneumonitis was indeed higher among
patients with increased levels of sirolimus, pneumonitis also occurred at relatively low concentration levels (335). With regard to our finding of pneumonitis being associated with a favorable outcome, no clear evidence exists on whether this represents an on-target dose-dependent effect or whether there are more intricate immunological pathways at work.

The management of mTOR-inhibitor-induced pneumonitis comprises treatment withdrawal or dose reduction and corticosteroid administration, as shown by the RECORD-1 trial, in which among the 18 patients with grade 2 pneumonitis, complete resolution was obtained in 11 patients and 1 patient improved to grade 1 as a result of corticosteroid therapy (10/18), dose adjustment (12/18), and/or discontinuation (3/18) (332). Similarly, in an overview among organ transplant patients receiving mTOR inhibitors, treatment withdrawal resulted in complete resolution in 94.3% of pneumonitis cases (339). Even though most cases of pneumonitis are relatively unaggressive and respond well to the therapeutic measures described above, life-threatening lung toxicity may occasionally occur (337, 340). Current guidelines suggest that everolimus treatment can be continued, or temporarily interrupted, among patients with mild symptoms (grades 1 to 2). In more severe cases of pneumonitis (grade 3), reintroduction of everolimus may also be attempted (341, 342). While we did not investigate how patients with pneumonitis were managed after the diagnosis of this adverse event, our results do prompt careful consideration regarding permanent treatment withdrawal, as these patients seem to draw clear benefit from everolimus treatment.

Our results add to the growing field of on-treatment biomarker research in mRCC. They demonstrate pneumonitis to be a strong biomarker of everolimus efficacy. As pneumonitis occurs relatively early after treatment initiation, with a median onset
after 2.4 and 2.8 months in cohorts A and B, respectively, it may also be clinically applicable in reassuring the patient and the treating physician of clinical benefit during therapy.

4. Hyponatremia associates with a poor outcome among mRCC patients treated with everolimus (IV)

Hyponatremia is commonly encountered among cancer patients. Most frequently, it occurs with small cell lung cancer (SCLC) as a result of the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) (343). It is also relatively common among other tumor types, although the distribution of causes is different. In mRCC, the reported incidence of hyponatremia varies between 14–25% (238-241).

Previous research has shown that hyponatremia is a predictor of a poor outcome in several different medical conditions, such as liver cirrhosis (344), congestive heart failure (345), and infectious diseases such as pneumonia (346), childhood meningitis (347), and necrotizing soft-tissue infection (348). Furthermore, several studies have reported a similar association between hyponatremia and a poor outcome in cancer patients (349-351).

Vasudev et al. were the first to show that hyponatremia is associated with shorter OS and DFS among pre-nephrectomy patients in localized RCC (352). Since then, evidence has accumulated on the possible prognostic role of hyponatremia in mRCC. Indeed, hyponatremia has been shown to associate with a poor outcome among mRCC patients treated with cytokines (238) and TKIs (239-241). Evidence
regarding the prognostic value of hyponatremias among everolimus-treated mRCC patients is lacking, however.

The mechanisms underlying hyponatremia are not entirely understood. Of the several mechanisms that have been suggested, an intriguing hypothesis is the possible association of chronic inflammation and hyponatremia through elevated interleukin-6 levels (240).

Our aim was to evaluate baseline and on-treatment sodium as prognostic biomarkers in mRCC patients treated with everolimus. We additionally evaluated the association between baseline sodium and baseline neutrophils or thrombocytes, markers that are often elevated in chronic inflammation.

Our study population comprised 233 mRCC patients treated with everolimus at Helsinki University Central Hospital, Finland (N = 85) and Aarhus University Hospital, Denmark (N = 148). Among these 233 patients, 65 (27.9%) had sodium below the lower limit of normal (LLN) at baseline. Within 12 weeks of treatment initiation, 41 (18.4%) patients had sodium <LLN. Of the 65 patients with baseline sodium <LLN, 35 (58.3%) had reversal to values ≥LLN during therapy. Baseline sodium <LLN was significantly associated with elevated baseline neutrophils above the upper limit of normal (ULN) (P = 0.002), and a similar tendency was noted for baseline sodium <LLN and thrombocytes >ULN (P = 0.08). Additionally, baseline sodium correlated inversely with baseline neutrophils (Spearman’s r = -0.23; P = 0.001) and baseline thrombocytes (Spearman’s r = -0.25; P < 0.001) when evaluated as continuous variables.

In univariate survival analyses, baseline sodium <LLN was significantly associated with shorter OS (6.1 months vs. 10.3 months; P < 0.001) and PFS (2.8 months vs. 3.5 months; P = 0.04). Patients with on-treatment hyponatremia had significantly
shorter OS (5.4 months vs. 9.9 months; \( P < 0.001 \)) and PFS (2.8 months vs. 4.0 months; \( P < 0.001 \)), as well as a significantly worse CBR (17.5\% vs. 50.0\%; \( P < 0.001 \)). In multivariate analyses adjusted for the IMDC risk classification and factors not included in the IMDC classification that were significantly associated with the outcome in univariate analysis (at \( P < 0.1 \)), baseline sodium <LLN remained significantly associated with OS (adjusted HR 1.46; \( P = 0.02 \)) and on-treatment sodium <LLN with OS (adjusted HR 1.80; \( P = 0.002 \)) and PFS (adjusted HR 1.71; \( P = 0.004 \)).

We additionally conducted subgroup analyses among patients with and without baseline sodium <LLN and on-treatment sodium <LLN, demonstrating that shifts from baseline sodium values <LLN to \( \geq \)LLN, and vice versa, were associated with the outcome. Results from the subgroup analyses are depicted in Table 5.

Since baseline neutrophil and sodium values were inversely correlated, we additionally analyzed the association between baseline hyponatremia and the outcome among patients with (\( N = 40 \)) and without baseline neutrophilia (\( N = 193 \)). Among patients with normal baseline neutrophil values, baseline sodium <LLN was associated with shorter OS (6.9 vs. 11.4 months; unadjusted HR 1.96; \( P < 0.001 \)). Among patients with baseline neutrophils >ULN, baseline sodium <LLN was not predictive of shorter OS, but survival was equally modest in both groups (5.2 vs. 4.2 months; unadjusted HR 0.83; \( P = 0.60 \)). Similarly, among patients with normal baseline thrombocytes (\( N = 189 \)), baseline sodium <LLN was associated with shorter OS (5.9 vs. 11.0 months; unadjusted HR 2.29; \( P < 0.001 \)), whereas in patients with baseline thrombocytes >ULN (\( N = 44 \)), baseline hyponatremia did not significantly affect OS (7.3 vs 5.8 months; unadjusted HR 1.04; \( P = 0.91 \)).
### Table 5. Shifts in baseline/on-treatment sodium values and OS

**A.** Baseline sodium $\geq$LLN and on-treatment sodium $\geq$LLN as a reference group

<table>
<thead>
<tr>
<th>Baseline Sodium</th>
<th>Sodium during treatment</th>
<th>$N$</th>
<th>Median OS</th>
<th>HR</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\geq$LLN</td>
<td>$\geq$LLN</td>
<td>147</td>
<td>11.1</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>$\geq$LLN</td>
<td>$&lt;$LLN</td>
<td>11</td>
<td>5.6</td>
<td>2.00</td>
<td>0.037</td>
</tr>
<tr>
<td>$&lt;$LLN</td>
<td>$\geq$LLN</td>
<td>35</td>
<td>8.3</td>
<td>1.68</td>
<td>0.012</td>
</tr>
<tr>
<td>$&lt;$LLN</td>
<td>$&lt;$LLN</td>
<td>30</td>
<td>5.4</td>
<td>2.60</td>
<td>$&lt;$0.001</td>
</tr>
</tbody>
</table>

**B.** Two different reference groups demonstrating the dynamic association of sodium values and OS

<table>
<thead>
<tr>
<th>Baseline Sodium</th>
<th>Sodium during treatment</th>
<th>$N$</th>
<th>Median OS</th>
<th>HR</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\geq$LLN</td>
<td>$\geq$LLN</td>
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<td>Ref.</td>
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</tr>
<tr>
<td>$\geq$LLN</td>
<td>$&lt;$LLN</td>
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</tr>
<tr>
<td>$&lt;$LLN</td>
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<td>30</td>
<td>5.4</td>
<td>1.60</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Our results demonstrate that both baseline and on-treatment hyponatremia are associated with shorter OS and on-treatment hyponatremia additionally with shorter PFS and a worse CBR. To the best of our knowledge, this is the largest study to confirm the prognostic role of hyponatremia among everolimus-treated patients. Our subgroup analyses additionally suggest that sodium is probably a dynamic biomarker, as shifts from baseline sodium $\geq$LLN to $<$LLN during treatment, and vice versa, were associated with OS among this patient cohort. This dynamic nature of the prognostic value of hyponatremia has not previously been investigated.

As the negative prognostic impact of hyponatremia has been demonstrated in mRCC patients treated with cytokines (238) and TKIs (239-241), possible future applications of these results in clinical practice may include the incorporation of sodium in prognostic models. The assessment of hyponatremia among mRCC patients treated with novel immunotherapy agents is therefore warranted. Additionally, the normalization of sodium values during treatment may reassure the treating physician of clinical benefit and thus encourage the continuation of everolimus therapy.

The underlying mechanisms of hyponatremia are unclear. In addition to SIADH, other possibilities include renal dysfunction, poor adrenal gland function, existing comorbidities, concomitant medications (such as diuretics and steroids), cancer therapy, and/or its adverse effects, such as diarrhea and vomiting (353-357). One intriguing hypothesis is the association of chronic inflammation and hyponatremia. Chronic inflammation has a well-established role in tumorigenesis (358). Experimental and clinical studies suggest that chronic inflammation may lead to the overproduction of interleukin-6 (IL-6), which induces neutrophilia and the secretion of ADH, resulting in hyponatremia (359). In our analyses, there was a significant association between baseline sodium and baseline neutrophils/thrombocytes. Furthermore, our subgroup analyses demonstrated that baseline hyponatremia was
associated with worse OS and PFS among patients with baseline neutrophils/thrombocytes $\leq$ ULN, but not in patients with baseline neutrophils/thrombocytes >ULN, where OS and PFS were modest. We find these results in support of the notion that hyponatremia may be associated with chronic inflammation in mRCC. More research is needed in this area, however.

Another interesting question is whether the treatment of hyponatremia associates with the treatment outcome. Several recommendations and treatment algorithms for hyponatremia exist, but none specifically address patients with cancer (360). In a retrospective analysis among 57 hyponatremic patients with cancer of various origins, the median OS for the 32 patients among whom sodium levels returned to normal was significantly longer (13.6 vs. 5.1 months; $P < 0.001$) (361). It is, however, difficult to establish whether the improvement in OS reflects the treatment of hyponatremia, the continuation of anticancer therapy, an improvement in the patients’ clinical condition, or bias resulting from the longer follow-up. An opposite view depicts the negative prognostic impact of hyponatremia to result from the underlying pathology rather than hyponatremia itself (362). Considering our results, further studies examining hyponatremia among cancer patients are warranted.

In conclusion, we demonstrated that sodium independently associated with the outcome among mRCC patients treated with everolimus. As it is a readily available, inexpensive, and reproducible laboratory parameter, it has great potential as a clinically applicable prognostic biomarker. Additionally, monitoring on-treatment sodium values may in the future aid in clinical decision making. The association of baseline neutrophilia/thrombocytosis and hyponatremia is an intriguing finding, supporting previous evidence of chronic inflammation as a driver of disease progression and metastasis and warranting further investigations among mRCC patients and cancer patients in general.
Limitations of the Study Materials and Methods

In study I, the methods used to assess treatment-induced hypertension as well as the use of antihypertensive medication were based on data obtained from patient case records. This may have led to underestimation of the incidence of hypertension if it was not properly documented. Similarly, this may have led to overestimation of the true use of antihypertensive medication, as patient case records rather than prescription records were used. Furthermore, data regarding why some patients were treated with ASIs and some not were lacking, possibly representing confounding factors, e.g. renal dysfunction.

In study II, archival tumor samples rather than fresh tumor biopsies were used. This may have affected our results in a few ways. Firstly, from a technical standpoint, the detected c-Met expression may vary based on the age of the sample. Secondly, c-Met expression seen in an older sample may not represent the true c-Met expression of the tumor at the onset of systemic therapy due to tumor biology and behavior. However, there was no significant difference in the median age of tumor samples for low and high c-Met expression (5.1 vs. 5.0 years; \( P = 0.62 \)). In further analyses, we did note a statistically significant association between the frequency of low and high c-Met expression and the age of the tissue samples, with older samples having a higher percentage of low c-Met expression as compared to the less aged samples (65.2% vs. 48.5%, \( P = 0.049 \)). As this might call into question the fidelity of the c-Met assays for the older samples, we hypothesize that the difference might rather be explained by the more aggressive tumor behavior in patients with high c-Met expression. As described earlier, all patients in this study were identified from hospital case records based on the initiation of sunitinib treatment. Patients with older tissue samples therefore had a longer time between the collection of the sample (nephrectomy) and initiation of sunitinib therapy, most likely due to less aggressive
tumor behavior. Although this might explain the association between the tissue sample age and c-Met expression, no definitive conclusions on the fidelity of the c-Met assays can be drawn, and we recognize this as a potential limitation of this study. Additionally, the number of patients with bone metastases was relatively low ($N = 31$) and this could be the reason why c-Met expression did not reach statistical significance among this patient subgroup.

In study III, a potential limitation in addition to its retrospective nature arises from the assessment of pneumonitis. Due to overlapping symptoms and similar radiologic findings, pneumonitis can be difficult to differentiate from other diseases of the lung parenchyma such as pneumonia, and we recognize this as a potential limitation. The study did, however, comprise two independent patient cohorts and the radiographs were subjected to a blinded radiologic review. Additionally, when comparing the results of the subgroup analysis performed in cohort B, there was no significant difference in the outcome between patients with CT-verified pneumonitis and patients with clinical symptoms of pneumonitis/pneumonia.

In study IV, we assessed on-treatment hyponatremia based on the highest sodium value within 12 weeks of treatment initiation. This may have led to underestimation of the true incidence of hyponatremia, as a patient would be categorized as normonatremic with only one on-treatment normonatremic sodium value. This stricter assessment of hyponatremia may therefore limit the clinical applicability of the results concerning on-treatment hyponatremia.
Conclusions

As the past decade has seen marked advances in the treatment of metastatic RCC in the form of targeted agents, including sorafenib, sunitinib, bevacizumab, pazopanib, axitinib, everolimus, and temsirolimus, a number of additional targets aside from VEGF and mTOR have been identified. Recent phase III trials have demonstrated that cabozantinib and the combination of nivolumab and ipilimumab have relevant clinical activity in first-line therapy when compared with sunitinib. Recommendations regarding first-line therapy are likely to be updated in the near future based on the results from CABOSUN and Checkmate-214. (224, 225)

As the number of treatment options for mRCC has increased, it has become increasingly important to identify predictive biomarkers that would allow the clinician to identify patients who are more likely to benefit from the differing treatments. At present, there are no biomarkers in clinical use. A few have shown promise in this regard, including the tumor PD-L1 expression level for nivolumab-treated patients.

In our study, we demonstrated that high levels of c-Met expression associate with a worse outcome among mRCC patients treated with sunitinib. We also found that high c-Met expression is associated with a poor outcome among patients without bone metastases at baseline, suggesting that the prognostic role may vary based on the location of the metastases. These results are of special interest given the results of the CABOSUN trial and should be further investigated among patients treated with cabozantinib. In the future, the evaluation of tumor c-Met expression may be incorporated into clinical practice to improve the prognostication of mRCC. The role of c-Met expression as a possible predictive biomarker, however, remains to be determined.
We additionally demonstrated that the use of angiotensin system inhibitors may have beneficial effects in conjunction with sunitinib and pazopanib in the treatment of TKI-induced hypertension. In the future, these results may guide the choice of antihypertensive medication for patients being treated with angiogenesis inhibitors. However, for these results to be properly validated, the use of ASIs in the treatment of TKI-induced HTN needs to be investigated in a randomized prospective setting.

Finally, we identified on-treatment biomarkers for everolimus-treated patients. To the best of our knowledge, we showed for the first time that treatment-related pneumonitis is significantly associated with longer overall survival and progression-free survival, as well as a higher clinical benefit rate, in two independent patient cohorts. These results verified pneumonitis as a strong marker of everolimus efficacy. Furthermore, our results demonstrated that both baseline and on-treatment hyponatremia independently associate with shorter overall survival and that on-treatment shifts in sodium values from baseline ≥LLN to <LLN, and vice versa, are associated with the outcome. These findings add to the growing field of on-treatment biomarker research in metastatic renal cell carcinoma.

The routine use and incorporation of biomarkers into clinical practice, whether to aid in treatment selection or to reassure clinicians of the clinical benefit during treatment, is slowly developing. Future directions in mRCC biomarker research should include further investigation of promising leads such as tumor PD-L1 expression and c-Met expression.
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