Predictive biomarkers for response to treatment with sunitinib in renal cancer patients

Martin Pilskog

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2020

UNIVERSITY OF BERGEN

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Date of defense: 23.01.2020

Year: 2020

Title: Predictive biomarkers for response to treatment with sunitinib in renal cancer patients

Name: Martin Pilskog

Print: Skipnes Kommunikasjon / University of Bergen

Scientific environment

The work presented in this thesis was carried out in the Anti Angiogenic Research Group, led by Professor Oddbjørn Straume, and the Cancer Biomarker Group, led by Professor Lars A. Akslen, at the Centre for Cancer Biomarker CCBIO, at the Department of Clinical Medicine, University of Bergen, from 2014 to 2019.

The laboratory work was done at CCBIO, Section for Pathology, Department of Clinical Medicine, University of Bergen.

The studies were carried out under the supervision of Professor Oddbjørn Straume (main supervisor), Professor Christian Beisland (co-supervisor) and Professor Lars A. Akslen (co-supervisor).

University of Bergen and the Norwegian Research Council through CCBIO funded the work.

Acknowledgements

I am very grateful to the University of Bergen, Department of Clinical Medicine and CCBIO for the opportunity to do this PhD. I am thankful for the sharing of workspace at Department of Pathology and Department of Oncology at the Haukeland University Hospital.

I would like to thank all the patients and relatives for their contribution, time and willingness to be a part of this translational project.

My warmest gratitude to Oddbjørn Straume, for critical questions, comments and sharing of great knowledge about cancer biology, kidney cancer and introduction to research. I would like to thank, Christian Beisland and Lars A. Akslen, my co-supervisors, for critical questions, support and sharing of your excellent knowledge in cancer treatment and biology. Great thanks to all three to let me be part of this translational project involving a multiple of disciplines in the hospital and the university environment, and the patience to let me finish.

I would like to thank all my co-authors for their contribution to the papers. A special thanks to Leif Bostad for performing the ISUP classification, discussion and comments, Karin Margrethe Hjelle, Åse Haug and Daniel Heinrich for performing the outpatient consultations and Gry-Hilde Nilsen for performing the ELISA tests.

I would like to give special thanks to Gerd Lillian Hallseth, now retired and former chief technician in the research laboratory, for great support and teaching at the lab. Bendik Nordanger for the excellent technical support with preparation of the tissue section used in immunohistochemistry. My best thanks to Jannicke Frugård and May Britt Kalvenes who organized, collected and handled the blood samples and tissue from the beginning. A great thank to Randi Lavik for your great support in technical and other practical issues.

I also would like to give a special thanks to Kristi Krüger, Sura Aziz and Maria Ramnefjell for sharing of the office, knowledge, support and smile. And a great thanks to other members and former members of the Cancer Biomarker Group at CCBIO (Cornelia Schuster, Emilia Signe Hugdahl, Ingeborg Winge, Reidunn J. Edelmann, Ole Johan Halvorsen, Elisabeth Wik, Kenneth Finne, Silje Kjølle, Even Birkeland, Jessica Furriol, Hanne Puntervoll, Monica Mannelqvist) for your support, comments, questions, and valuable discussions and assistance in different matters.

I would like to thank the Routine laboratory at the Department of Pathology, the Outpatient wards at the Department of Urology and Department of Oncology at Haukeland University Hospital for treatment of the patients and handling of blood samples and tissue collection.

Great thanks to my parents and parent's in-law for support and baby-sitting. To the rest of the family and all my friends for support and interest. To my two beautiful boys Benjamin and Theodor born into the thesis. Finally, to Maren-Sofie, my lovely wife and greatest supporter for taking care of family business, early wake ups with the boys, still smiling and the best cappuccino.

Bergen, August 2019 Martin Pilskog

Abstract

Aims:

Patients with metastatic or inoperable kidney cancer with positive biomarkers of vascular endothelial growth factor (VEGF) driven angiogenesis might have improved clinical benefit from treatment with an anti-VEGF-receptor kinase inhibitor. The primary goal in this study was to identify predictive markers of response in first line treatment with sunitinib against kidney cancer, and the secondary goal was to estimate response, proportion of patients with stable disease on treatment, time to progression, and survival.

Material and methods:

Forty-six patients with metastatic clear cell renal cell carcinoma (ccRCC) were enrolled in a prospective single-arm study of predictive markers for sunitinib response.

In **paper I**, response rates according to RECIST v.1.1. were used as primary end-point. Secondary objectives were to evaluate prognostic value of candidate markers with regard to progression free survival (PFS) and overall survival (OS). In addition, toxicity rates and quality of life were reported.

In **paper II**, we investigated the predictive value of immunohistochemical biomarkers associated with angiogenesis and systemic inflammation in metastatic ccRCC. Forty-six patients with metastatic or non-resectable ccRCC treated with sunitinib were included. Metastatic and/or primary tumor tissue were stained by immunohistochemistry for selected markers related to angiogenesis (VEGF-A, VEGFR2, PDGFRβ, HSP27) and immune responses (IL6, IL6Rα, JAG1). The predictive potential of the candidate markers was assessed by correlations with response rates. In addition, PFS and OS were analyzed.

In **paper III**, full blood samples were collected at baseline before start of sunitinib and after every second cycle of treatment during the study time. Markers of immune response (pIL6, pIL6Rα and pIL6ST) at baseline and week 12 were analyzed by

ELISA. The predictive potential of the candidate markers was assessed by correlation with response rates. In addition, PFS and OS were analyzed.

Results:

Paper I: Median PFS and OS were 9.1 months and 15.0 months, respectively. Of 38 patients evaluable for response, 1 patient had complete response (CR), 7 had partial response (PR), 18 had stable disease (SD) and 12 had progressive disease (PD). Normal CRP at baseline was significantly associated with objective response (CR + PR) (Fisher's exact test, $p = 0.01$). Normal CRP was also significantly associated with improved PFS and OS (Log rank, $p = 0.05$ and < 0.01, respectively). Early hypertension, neutrophil-to-lymphocyte ratio (NLR) and IMDC risk score were not significantly associated with response rates or survival.

Paper II: Low tumor cell expression of IL6Rα was significantly associated with improved response to sunitinib (Fisher's exact test, $p = 0.03$), but not with PFS or OS. Median/high expression of IL6Rα showed significant association with median/high expression of VEGF-A and HSP27. Furthermore, low tumor cell expression of IL6 was significantly associated with improved PFS, but not OS or response rates. High expression of IL6 was significantly associated with high expression of JAG1, VEGF-A, VEGFR2 and PDGFRβ.

Paper III: Low pIL6 at baseline was significantly associated with improved response to sunitinib (Fisher's exact test, $p < 0.01$). Furthermore, low pIL6 at baseline was significantly associated with improved PFS (Log rank, $p = 0.04$). In addition, patients with a decrease in concentration of pIL6Rα between baseline and week 12 showed significantly improved PFS (Log rank, $p = 0.04$) and patients with high pIL6ST at baseline showed significantly improved OS (Log rank, $p = 0.03$).

Conclusion:

Baseline CRP was a significant predictive factor of sunitinib response and a prognostic factor of survival. Baseline CRP might be a useful biomarker in the treatment planning of metastatic ccRCC. Loss of tumor cell expression of IL6Rα in patients with metastatic ccRCC patients treated with sunitinib predicts improved

treatment response. Low pIL6 at baseline in metastatic ccRCC patients treated with sunitinib predicts improved treatment response. Both might represent candidate predictive markers. Because of the relatively small sample size, the results need validation in larger studies.

List of publications

I. Pilskog, M., Beisland, C., Akslen, L.A., Bostad, L., Haug, Å., Heinrich, D., Hjelle, K.M., Straume, O. (2017): “Predictive value of C-reactive protein in patients treated with sunitinib for metastatic clear cell renal cell carcinoma.” BMC Urology 17:74.

II. Pilskog, M., Bostad, L., Edelmann, R.J., Akslen, L.A., Beisland, C., Straume, O. (2018): «Tumor cell expression of Interleukin 6 receptor α is associated with response rates in patients treated with sunitinib for metastatic clear cell renal cell carcinoma.” J Pathol Clin Res. 2018 Apr;4 (2):114-123.

III. Pilskog, M., Nilsen, G.H., Beisland, C. and Straume, O. (2019). "Elevated plasma interleukin 6 predicts poor response in patients treated with sunitinib for metastatic clear cell renal cell carcinoma." Cancer Treat Res Commun 19: 100127.

Abbreviations

BOR - Best overall response

CaIX - Carbonic anhydrase IX

CB – Clinical benefit

ccRCC - Clear cell renal cell carcinoma

chRCC - Chromophobe RCC

CN - Cytoreductive nephrectomy

DBP - Diastolic blood pressure

DFS - Disease free survival

ECOG - Eastern Cooperative Oncology Group

EGF - Epidermal growth factor

eHTN - Early hypertension

ELISA - Enzyme linked immunosorbent assay

EMT - Epithelial-mesenchymal transition

EORTC - European Organization for Research and Treatment of Cancer

FACT-G - Functional Assessment of Cancer Treatment General

FGF - Fibroblast growth factor

FKSI-15 - FACT-Kidney Symptom Index-15

HIF1α - Hypoxia-inducible factor 1 α

HRQoL - Health Related Quality of Life

HSP27 - Heat shock protein 27

HUNT - Helseundersøkelsene i Nord-Trøndelag

IFN- α - Interferon α

IL6 - Interleukin 6

IL6Rα - Interleukin-6 receptor α

IL6ST - Interleukin 6 Signal Transducer (Human gp130 transducer chain gene)

IMDC - International Metastatic renal cell carcinoma Database Consortium

ISUP - International Society of Urological Pathology

JAG1 - Jagged 1

KPS - Karnofsky performance scale

LDH - Lactate dehydrogenase

LLN - Lower level of normal

MDSC - Myeloid-derived suppressor cells

mRCC - metastatic Renal Cell Carcinoma

MRI - Magnetic Resonance Imaging

mTOR - Mammalian target of rapamycin

NF-kB - The Nuclear Factor κB

NLR - Neutrophil-to-lymphocyte ratio

OR – Objective response

OS – Overall survival

PD – Progressive disease

PD-1 - Programmed cell death protein 1

PD-L1 - Programmed death ligand 1

PFS - Progression Free Survival

PN - Partial nephrectomy

PR – Partial response

pRCC - Papillary renal cell carcinoma

QoL - Quality of Life

RCC - Renal Cell Carcinoma

RECIST - Response Evaluation Criteria in Solid Tumors

rTKI - Receptor tyrosine kinase inhibitor

SB – Stable disease

SBP - Systolic blood pressure

STAT-3 - Signal transducer and activator of transcription 3

TKI - Tyrosine Kinase Inhibitor

ULN - Upper limit of normal

VEGF - Vascular endothelial growth factor

VEGFR2 - VEGF receptor 2

VHL - von Hippel-Lindau

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1. Introduction

This thesis focuses on predictive biomarkers for treatment of metastatic clear cell renal cell carcinoma (ccRCC) with sunitinib.

1.1 Epidemiology of renal cell carcinoma

Kidney cancer represent three to four percent of malignant tumors among adults in Norway [1]. Renal cell carcinoma (RCC) arise from cells in the kidney and is a heterogeneous group of sporadic or hereditary cancers [2]. According to the Cancer Registry of Norway, during the last decade men have a higher and steeper increase of incidence than women [1]. The mortality rate has been moderately decreasing and survival rates have been increasing (Figure 1) [1]. Worldwide in 2012, kidney cancer was the ninth most common cancer in men and the 14th most common cancer in women. It was the 16th most common cause of death from cancer in 2012 [2]. It is more frequent in countries with high and very high levels of human development [2].

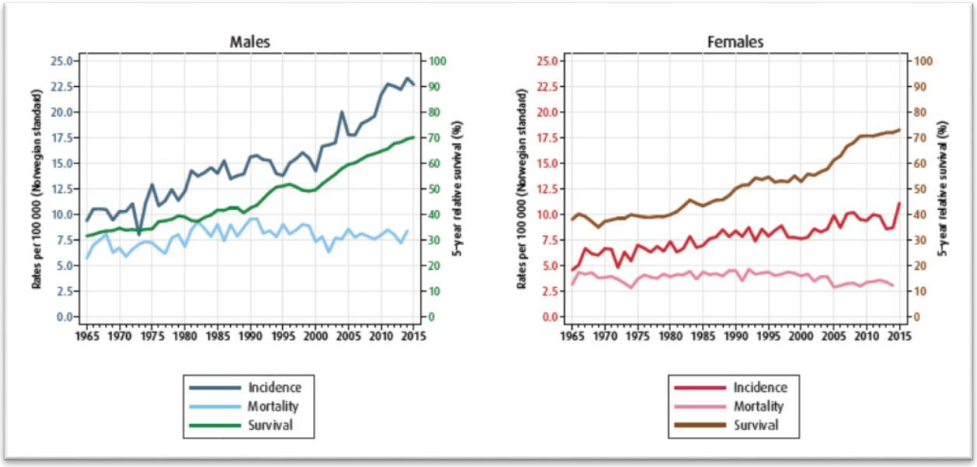


Figure 1. Kidney excluding renal pelvis (ICD-10 C64). Printed with permission by The Norwegian Cancer Registry [1].

1.2 Etiology and risk factors

1.2.1 Risk factors

Increasing age and higher incidence among men versus women at a ratio 1.5:1 are risk factors of RCC [3].

Smoking and obesity are the most common risk factors related to RCC [2,4,5]. The link between smoking and RCC is thought to be through induction of hypoxia over time [6]. There is a strong dose-dependent increase in risk associated with numbers of cigarettes smoked per day [2]. The risk was reduced among persons that quit smoking more than 10 years ago versus those less than 10 years [5].

Obesity increases the risk of RCC. In a meta-analysis the relative risk estimate was 1.07 (95% CI 1.05–1.09) per unit of increase in BMI [4]. RCC and obesity are interconnected via inflammation, tissue hypoxia, lipid peroxidation, increased production of insulin-like- and other growth factors [7]. Obesity may be considered a low-grade chronic infection through activation of acute phase proteins and systemic inflammatory response proteins [7]. The majority of inflammatory cytokines in obesity are from infiltrating macrophages in adipose tissue. Of these, interleukin 6 (IL6) is one of the most important [7].

Hypertension increased the risk of RCC among men and women in a cohort of 156,774 subjects, where increasing blood pressure was correlated to increased hazard ratio [8]. In the Norwegian HUNT study, the relative risk of RCC was increased in women with hypertension, but not among men [9].

Kidney disease as acquired cystic kidney disease, in end-stage kidney disease, has a six-fold higher incidence of RCC compared to the general population [10]. Kidney transplantation and immunosuppression increase the risk of RCC in the non-transplanted kidney and this might argue for a role of the immune system in repressing kidney cancer development [11].

Substances like asbestos, trichloroethylene, cadmium, lead and chronic exposure to arsenic in drinking water are linked to the risk of RCC [12-19], but more robust studies are needed to confirm this [3]. Moreover, modern diets with high protein and low fat have been linked to increased risk of RCC [10].

Physical activity may reduce the risk by reducing obesity, blood pressure, insulin resistance and lipid peroxidation, and it was shown to reduce risk of RCC in a meta-analysis (relative risk (RR) 0.88, 95% CI 0.79 - 0.97) [3,20].

1.2.2 **Genetic risk factors**

Only around 2% of RCC is hereditary; still the hereditary syndromes have given insight to mechanisms found in sporadic tumors [21]. Young age (under 40 years of age), bilateral or multifocal renal tumor and family history of renal cancer suggest an inherited predisposition [22]. Mutations in the von-Hippel-Lindau (VHL) gene are important in hereditary RCC [21]. The gene is located on chromosome 3 (3p25-p26) and was first identified and cloned in 1993 [21]. Inactivation of the VHL gene appears in 60-80 % of the sporadic ccRCC [23,24]. The two hit tumor suppressor gene, VHL, has one allele inactivated by mutation or promoter methylation and the other lost because of a large deletion [25]. Loss of the VHL-gene leads to high levels of hypoxia-inducible factor 1 α (HIF1α), a transcription factor for a number of stress response proteins [26]. In turn, this leads to upregulation of vascular endothelial growth factors (VEGF), which induces angiogenesis and thus represents the rationale behind sunitinib treatment [27].

In ccRCC, tumor suppressor genes Polybromo 1 (PBRM1), BRCA1 associated protein-1 (BAP1) and SET domain containing protein 2 (SETD2) at 3p have emerged as major cancer genes, which regulate different gene expression programs [2,28]. PBRM1, BAP1, SETD2 and VHL are all on chromosome 3p and are lost in approximately 90% of sporadic RCCs [28]. In patients treated with VEGF targeted

therapy, those with mutation of PBRM1 had longer time to treatment failure compared those with wild type (Log rank, median 12.0 months versus 6.9 months, p=0.01) [29]. Patients with metastatic ccRCC with mutations in PBRM1 had increased clinical benefit to immune checkpoint therapy [30]. These patients also had increased expression of hypoxia and IL-6–JAK-STAT3 gene sets in the PBRM1-LOF tumors [30].

For more about other hereditary RCCs please refer to the review by Kallinikas et al [31].

1.3 Classification of RCC

1.3.1 TNM classification

The staging system in RCC is based on the TNM classification as an anatomical prognostic marker to give information about survival probability [32,33]. The TNM staging system describes tumor size, and define the extent of local disease and presence of metastases. The recent TNM stage system is from 2017 (8th edition) [33].

1.3.2 Histopathological classification

The consensus meetings in Heidelberg and Rochester used for the first time genetic information to classify RCC in addition to histologic appearance and architecture [34]. The agreements from these meetings were leading up to the World Health Organization (WHO) classification in 2004 [35]. The latest version from 2016 includes more than 50 variants of renal neoplasms [36]. The main groups are ccRCC (c. 70%), papillary RCC (type I and II) (c. 10-15%), chromophobe RCC (c. 5%) and collecting duct carcinoma (< 1%) [2]. Others variants are medullary carcinoma and other rare variants [36,37].

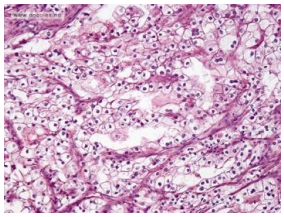
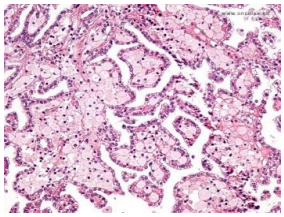
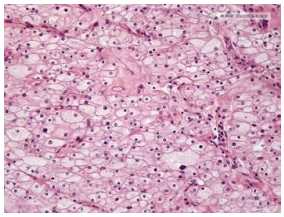
A  B  C 

Figure 2. A: Clear cell RCC, B: Papillary RCC type I, C: Chromophobe RCC.
Photos adopted with permission from oncolex.no (http://oncolex.no/Nyre/Bakgrunn/Histologi).

Clear cell RCC

Clear cell RCC (ccRCC) origins from the epithelium of the renal tubules [36]. Macroscopically, the cut surface is golden-yellow, often with hemorrhage and necrosis [38]. ccRCCs are defined as a morphologically heterogeneous malignant neoplasms composed of cells with clear and/or eosinophilic cytoplasm, and they are typically associated with a vascular network and inactivation of the VHL gene either with mutation or methylation in over 80% and upregulation of HIF1α [28,39]. Loss of the chromosome 3p and mutation of the VHL gene at chromosome 3p25 are frequently found [28]. Other tumor suppressor genes as PBRM1, BAP1 and SETD2 are also frequently deleted in ccRCC [28,38].

Papillary RCC

Papillary RCC (pRCC) is the second most commonly encountered morphotype of RCC [38]. It is divided into two subtypes, pRCC type I and type II [36]. pRCC type I is associated with activating germline mutations of *MET* and pRCC type II is associated with activation of the NF-E2-related factor 2-antioxidant responsive element (NRF2-ARE) pathway with at least three subtypes [38]. Macroscopically, pRCC is typically well circumscribed with a fibrous capsule, with a yellow or brown color [39]. Foamy macrophages and cholesterol crystals are characteristic in the fibrovasculare core of the papillo-tubular structure [39]. Hemorrhage and necrosis are often seen [39]. Microscopically, in pRCC type I, cancer cells are small, and they have scanty basophilic or pale cytoplasm and low-grade nuclei without nuclear pseudo-stratification [39]. pRCC type II is composed of cancer cells having abundant eosinophilic cytoplasm and high-grade nuclei with pseudo-stratification [39].

Chromophobe RCC

Chromophobe RCC (chRCC) is macroscopically a pale tan, homogenous and though, well-demarcated mass without a capsule [38]. Microscopically, it has prominent cell membranes, wrinkled nuclei with perinuclear halos, and pale to eosinophilic cytoplasm, and it comprises about 5 % of all renal tumors [39]. Loss of chromosomes are typical genetic changes [35,40].

1.3.3 Histopathological grading of RCC

Histologic subtyping is an important prognostic marker in RCC [41]. ccRCC subtype has a worse prognosis compared to pRCC and chRCC [41,42].

The Fuhrman grading system has been used the last four decades for nuclear grading [43]. The system scores the assessment of nuclear size, nuclear irregularity and nucleolar prominence to grade tumors into four groups [43]. A weakness with the Fuhrman grading has been the use of simultaneous assessments of the three parameters [44]. It is difficult to evaluate tumors where two or three of the nuclear parameters appear to contradict each other [44]. In addition, there may be poor inter/intra-observer reproducibility and failure to discriminate outcome adequately [45].

The Fuhrman grading system is still in use, but because of its weaknesses, the International Society of Urological Pathology (ISUP) suggested a new method of grading [44]. In the ISUP system, grade 1-3 defines tumor classes based on nucleolar prominence and grade 4 on the presence of pronounced nuclear polymorphism, tumor giant cells and/or rhabdoid and/or sarcomatoid differentiation [36]. Sarcomatoid features represent worse prognosis and it represent an ISUP score of grade 4 [44]. Microscopic appearances of coagulative necrosis predict worse prognosis for ccRCC and chRCC [44].

The ISUP system is recommended and validated for ccRCC and pRCC [44,45]. The Fuhrman and ISUP grading are not recommended for chRCC [36].

1.4 Diagnostics in RCC

A multidisciplinary team is mandatory in the diagnostic investigation, before either surgery and/or systemic treatment. The clinical presentation of the patient's symptoms, medical history and performance status gives important information. Because of increased incidental findings of RCC on CT scans, the classic triad of pain, hematuria and palpable flank mass is less common today [46,47]. RCC can present with symptoms from local tumor growth, hematuria because of bleeding, paraneoplastic manifestations or metastatic disease [38]. RCC occasionally presents with paraneoplastic manifestations like hypercalcemia, production of an adrenocorticotrophic hormone, hepatic dysfunction, polycythemia, fever, amyloidosis and weight loss [47]. Other presentations could be hypertension, night sweats, malaise and in men varicocele, usually left sided, because of obstruction of the testicular vein (2% of males) [48].

RCC can develop into an advanced stage without warning signs or abnormal blood test. This makes it difficult to detect RCC at an early stage. One-third of all patients are diagnosed with metastases and over 20% of patients undergoing nephrectomy with curative intention will develop metastases during follow-up [49]. Distant metastases occur most often in the lungs, lymph nodes, liver, bone, and the brain [49,50]. Metastatic RCC can present with several different symptoms. Lung metastases can present with coughing or dyspnea, brain metastases may present with confusion, dizziness or epileptic seizure and bone metastasis with pain or pathological fracture [36].

In addition to clinical presentation, a physical examination and laboratory findings will give information about the patient can benefit from surgery and/or systemic treatment. It is also necessary to evaluate performance status, any comorbidity and risk of complications, especially if surgery is considered.

Mandatory in the diagnostic work up is imaging. CT-scan is the preferred imaging method in RCC diagnostic and staging [38]. In patients with reduced renal function MRI is preferable to preserve kidney function, because MRI give the opportunity to a non-contrast diagnostic method [38].

A tissue biopsy is important to get the correct diagnosis and to decide treatment or no treatment [38]. Biopsies may show benign tumors in small renal masses and in that way support the use of active surveillance. It may also support use of a non-surgical method as ablative treatment or use of systemic targeted treatment [38]. At least two core biopsies should be used to ensure proper quality.

1.5 Tumor biology

The path from normal cells to cancer cells and metastatic cancer cells follow multiple steps from accumulation of genetic alterations and epigenetic modifications, like activation of oncogenes and inactivation of tumor suppressor genes. Hanahan and Weinberg presented their principles of cancer tumor biology, the hallmarks of cancer [51,52]. The first six core hallmarks established in 2000 were; sustaining proliferative signaling, evading growth suppressors, activating invasion and metastases, enabling replicative immortality, inducing angiogenesis and resisting cell death [51]. And in 2011, deregulation of cellular energetics and avoidance of immune destruction were added and also two enabling characteristics of neoplasia; tumor-promoting inflammation and genome instability and mutation [52].

1.5.1 Cell cycle regulation

To maintain tissue homeostasis and avoid neoplastic growth the proliferative activity of cells [53], cyclin proteins and the associated cyclin-dependent kinases regulates checkpoint controls that monitor the four phases of the cell cycle [54]. These are the first gap phase (G1), the synthetic phase (S), the second gap phase (G2) and mitosis

(M)) [54]. In the non-proliferating G0 state, the cells need external growth factors to enter the cell cycle and the G1 phase [54].

1.5.2 **Hypoxia, angiogenesis and VEGF**

Angiogenesis is defined as sprouting of new vessels from pre-existing vessels [55,56]. Tumors over 1-2 mm^2 are dependent on neovascularization to continue growth [57]. Folkman predicted a tumor-angiogenesis factor (TAF) responsible for tumor angiogenesis [57]. A decade later vascular permeability factor (VPF) was purified [58], and later renamed to VEGF [59]. Angiogenesis is involved in several physiological processes and is very important for the survival and proliferation of cancer cells, tumor growth and spreading [60].

The most important physiologic driver of angiogenesis is hypoxia [60]. Hypoxia is a result of imbalance between oxygen supply and consumption in tumors [60]. Cancer cells surviving in a hypoxic environment are more resistant to chemotherapy and radiotherapy [60]. These features are beneficial for cancer cells and reduce overall survival for the host [61].

The VHL tumor suppressor gene is lost or mutated in 60-90% in sporadic cases and is a major contributor to development of RCC [23]. Loss of VHL leads to a chronic stress response state in the cells trough high levels of HIF1α, a transcription factor for a number of stress response proteins, including increased production of VEGF, that is triggered as a hypoxia response because of HIF1α [62]. Other regulators of VEGF are epidermal growth factors (EGF), transforming growth factor (PDGF), insulin-like growth factor 1 (ILGF-1), and fibroblast growth factor (FGF) [62].

VEGF is produced by hypoxic tumor cells, endothelial cells and tumor associated macrophages [63]. VEGF consists of different variants. The VEGF family includes VEGF A, B, C, D and placental growth factor (PIGF) [60]. VEGF is the most important factor that induce angiogenesis [60]. VEGF has a proliferative effect on endothelial

cells, which start growing because of VEGF, increasing survival and decreasing the apoptotic rate [60]. Furthermore, it enhances vascular permeability, which in turn is related to extravasation and migration of cells from and into circulation [60].

VEGF is the most important mediator of tumor-associated angiogenesis in RCC, and VEGF receptor 2 (VEGFR2) is the main target of sunitinib. Some reports suggest a role of systemic inflammation in development and progression of RCC [64-66].

Through its major downstream target STAT3 several tumor promoting pathways are activated, including HIF1α and increased VEGF activity [67]. As a response to cellular stress, IL6 activation of the transcription factor STAT3 drives angiogenesis by inducing expression of VEGF and fibroblast growth factor (bFGF) by tumor cells, and thereby supports vascularization required for tumor growth and metastasis [68,69].

1.5.3 Inflammation

Inflammation is a result of tissue trauma when inflammatory cells infiltrate in to the tissue and production of cytokines and growth factors regulates cellular proliferation [70]. Similarly, cancer promotes development of an inflammatory microenvironment in and around tumors and as well systemic inflammation [71]. Rudolf Virchow observed lymphoreticular infiltration in cancer tissue and suggested a link between inflammation and cancer [72].

Tumor inflammation is important in both tumor promoting and anti-cancer processes. Cancer cells use the immune system in development of local tumor growth and metastasis. Cancer can avoid and suppress the immune response. Usually, the innate and adaptive immune system recognizes and eliminates tumor cells [73]. The cancer cells can stay in dormancy over many years, where tumor cells and the host immune system stay in an equilibrium until tumor cells escape from the immune system and starts to grow progressively [73,74].

Cancer-related inflammation involves the infiltration of tumor associated macrophages (TAMs), white blood cells and inflammatory cytokines, like tumor necrosis factor (TNF), interleukin 1 (IL1) and IL6, and chemokines (CCL2 and CXCL8), which facilitate tissue remodeling and angiogenesis [75]. The production of different inflammatory cytokines, chemokines, and growth factors by RCC cells and tumor stromal cells stimulates the activation, expansion, and trafficking of various immune cells into the tumor where they can promote tumor progression by enhancing angiogenesis and initiate T cell immune suppression [76,77].

IL-6 is an important tumor-promoting protein associated with stress responses, inflammation and angiogenesis [67]. Through its major downstream target STAT3 several tumor promoting pathways are activated, including HIF1α and VEGF [67]. Moreover, IL6 have direct stimulating effect on endothelial cells, and has been implicated in resistance to anti-VEGF therapy [78].

C-reactive protein (CRP) is an acute-phase protein that increases rapidly following IL6 secretion by macrophages and T cells following infection, inflammation and cancer [79].

Along with a stimulating effect on tumor associated angiogenesis, VEGF also plays an important role as a suppressor of the local immune response during wound healing as well as in tumors by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells (MDSC), regulatory T cells, and VEGF inhibits the migration of T lymphocytes to the tumor [80].

The neutrophil-to-lymphocyte ratio (NLR) is also a marker of systemic inflammation in cancer patients and was found in retrospective RCC studies to add prognostic and predictive information [81,82]. Like CRP, NLR is readily available in standard blood samples in a regular clinical setting.

1.5.4 **Activating invasion and metastases**

Cancer cells have the ability to activate invasion and disseminate to distant locations. Metastases from solid tumors are related to over 90% of cancer-associated deaths [54]. The first step of the metastatic cascade is for the cancer cells to invade surrounding tissue. An important involved program is epithelial-mesenchymal transition (EMT) [83,84]. In EMT, epithelial cancer cells use the mesenchymal properties to invade, resist apoptosis and spread to distant organs [84]. The cells loose E-cadherin, a key cell-to-cell molecule, and upregulate N-cadherin, an adhesion molecule associated with cell migration during embryogenesis and inflammation [84,85]. The reversed process is called mesenchymal-epithelial transition (MET) [86-88]. This plasticity may result in the formation of new tumor colonies of cancer cells with a similar histopathology to the primary tumor cells that never went through EMT [52,89]. Transforming growth factor β (TGF-β) signaling actives transcriptional factors, which induce gene expression patterns favoring EMT development [52,90].

In RCC, Landolt et al found increased EMT score in ccRCC [90]. The gene expression analysis related to EMT correlated to ccRCC patient survival in a public available dataset [90]. These results show how EMT in ccRCC is linked to fibrosis and associated with reduced survival [90].

1.5.5 **Sustaining proliferative signaling**

One of the most important features of cancer cells is the ability to sustain chronic proliferation [52]. In the normal tissue, growth-promoting signals are controlled. In cancer cells, they find different ways to acquire the capability to sustain proliferative signaling [52]. They can use autocrine proliferation, by producing growth factors themselves or send signals to normal tumor associated cells in the stroma to produce growth factors [52]. They can also regulate the number of receptors on cell surface, to make cancer cells more responsive [52]. And last, activation of downstream signaling pathways or change of negative-feedback mechanisms can make cancer cells

independent of exogenous growth factors [91]. In RCC, the inactivation of VHL gene and upregulation of downstream HIF1α are examples of that. IL6 is important since it stimulates tumor cell proliferation and survival by activating pathways such as JAK/STAT, Ras/Raf/MEK/MAPK and PI3K/AKT pathway via gp130 tyrosine phosphorylation [75]. In tumor cells, STAT-3 activation is mediated through autocrine production and paracrine secretion of IL6 from stroma and infiltrating inflammatory cells [75].

1.5.6 Evading growth suppressors

Cancer cells can negatively regulate cell proliferation inhibitors [52]. Tumor suppressors limit cell growth and proliferation [52]. Two of the most important are the retinoblastoma associated (Rb) and tumor protein 53 (TP53) proteins [52]. These two controls the decisions of cells to proliferate or activate senescence and apoptotic programs [52]. RB is the gate-keeper in the cell-cycle from G1 to S. TP53 can halt the cell-cycle progression, dependent on intracellular conditions dependent on genome damage, levels of nucleotide pools, growth-promoting signals, glucose or oxygenation are suboptimal [52]. If needed, TP53 can induce apoptosis [52]. More than half of human tumors contain a mutation in the TP53 gene [92,93]. A meta-analysis showed how positive TP53 expression was correlated with a poor prognosis and advanced clinicopathological features in patients with RCC [94]. IL6 is involved in hyper methylation of tumor suppressor genes via CpG methylation of the promoter region of the TP53 gene [75]. In multiple myeloma cells, IL6 facilitates the phosphorylation of Rb that promotes cell growth [75].

1.5.7 Enabling replicative immortality

The telomeres protect the ends of chromosomes from fusion and DNA degradation [52]. Telomeres are centrally involved in the capability for unlimited proliferation of cancer cells [52,95]. Normal cells have a limited number of cell growth-and-division cycles. They are related to two barriers of proliferation: senescence, a typical

irreversible entrance into a non-proliferative state and crisis, which involves cell death [52]. Telomerase is the unique DNA polymerase that extend telomeres and enables replicative immortality [52]. Telomeres can also propagate proliferation of cancer cells and not only maintain telomere length [52]. The human telomerase reverse transcriptase protein (hTERT) synthesis de novo telomere, and in a recent paper hTERT expression was significantly associated in ccRCC with an advanced stage, higher grade, presence of microvascular invasion, lymph node invasion, and metastasis [96].

1.5.8 **Resisting cell death**

Apoptosis is a normal process important for normal embryogenesis and maintaining homeostasis [52]. The programmed cell death by apoptosis represents a barrier for cancer development [52]. It is initiated in response to different stimuli like DNA damage, deregulated growth signals and hypoxia [52]. Apoptosis is controlled in two major ways. The extrinsic receiving and processing extracellular death-inducing signals, and the intrinsic, which is sensing and integrating a variety of signals of intracellular origin and more widely considered a barrier to cancer pathogenesis [52]. In cancer, IL6 is shown to induce JAK/STAT3 and NF-kB signaling to translocate STAT3 and NF-kB in the nucleus [75]. Activation of these signaling pathways results in the expression of anti-apoptotic genes (i.e. Bcl-2) [75]. Proteins of the BCL-2 family regulate the pro and anti-apoptotic signals in cancer, loss of TP53 function can increase or downregulated these proteins [75]. In RCC, TP53 may be a prognostic marker (see chapter 1.5.6).

Another important cell-physiologic response is autophagy, which is a normal process increasing substantially because of cellular stress [52]. Cells use the autophagy program cells to break down cellular organelles, like ribosomes and mitochondria, to be recycled and used for biosynthesis and energy metabolism [52]. Elevated levels of autophagy can be induced by nutrient starvation, radiotherapy and certain cytotoxic drugs, paradoxically impairing and not activate the killing actions of these stress-

inducing situations [52,97]. Further, many stressed cancer cells have been shown to shrink via autophagy to a state of reversible dormancy, and may enable regrowth of cancer cells after anti-cancer treatment [52,97].

1.5.9 Reprogramming energy metabolism

Otto Warburg found a feature of cancer cell energy metabolism, where cancer cells even in an aerobic state cancer cells can reprogram their glucose metabolism and the energy production to glycolysis [98,99]. The cancer cells do this by upregulation of glucose transports, like GLUT1, which pump glucose into cells [52]. This metabolic switch is a way for cancer cells to generate nucleosides and amino acids from glycolytic intermediates to be used in the biosynthesis of the macro molecules and organelles necessary for new cancer cells [52,100]. HIF1α signaling regulates glucose metabolism in response to hypoxia and growth factors [101]. In RCC, constitutive expression of HIF1α target genes contributes to aerobic glycolysis and IL-6 induces fatty acid oxidation in the mitochondria by activating the AMPK pathway [101].

1.6 Prognostic and predictive markers

The Biomarkers Definition Working Group uses a broad formulation;
"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [102].

1.6.1 Biomarkers

Biomarkers are used to guide treatment of cancer patients. Prognostic markers can divide the clinical outcome of different patient subgroups assuming the patients will receive either no treatment or a form of standard treatment such as surgery or radiation [103]. An excellent prognostic factor may therefore identify patients that not

need standard treatment, even if it has activity [103]. If further therapy is necessary, predictive markers, can help to identify patients who may have benefit of further treatment such as targeted therapies or immune checkpoint inhibitors [103].

Predictive biomarkers identify patients who will most likely to benefit from treatment [104,105]. A predictive biomarker can also be a prognostic biomarker.

Few biomarkers have advanced beyond the clinical validity phase, even many biomarkers have been reported [103]. The phase of clinical validity show how a biomarker has a strong association with a clinical outcome of interest [103]. According to Kern et al it is said that of all cancer biomarkers investigated only 1% get into clinical practice, and neglecting this difficulty is a general failure to validate clinically that a marker has utility [106]. The power to change a clinical decision arises from the predictive marker's value or strength to categorize among disease classifications [106]. Simon et al proposed that a prospective-retrospective study must be confirmed by at least one additional prospective-retrospective study to establish clinical utility of a marker, compared to a prospective clinical trial that directly addresses clinical utility of the marker [103,107]. The statistical power to evaluate a marker's clinical utility within a trial can be difficult if the study originally was designed to answer a question of treatment efficacy [103]. Patient populations or biomarker assays used in different studies will variate [103].

In their paper, McShane and Hayes point on three types of reporting biases, that are major threats to determine the clinical utility of tumor marker [103]. The first is the non-publication bias, where researchers decide not to report negative studies [103]. The second they call "within-publication selective reporting", where researchers select only a subset of study outcomes [103]. Authors then fail to provide a rationale for the selection of cut point [103]. The third type of bias is the "incomplete study reporting", where too few details are reported to reproduce properly [103].

In an effort to improve reporting of biomarker studies governments, journals and the research field have taken action. One method was to establish trial registries of prospective studies, like ClinicalTrials.gov in the United States, which gives an overview of unpublished studies [103].

Another important step to improve reporting was the introduction of the CONSORT (Consolidated Standards of Reporting Trials) statement [108]. The updated statement in 2010 includes a flow diagram and checklist of items to report and to describe; the flow of patients through the study, primary and secondary end points, prespecified hypotheses, key aspects of the study design and methods, the prespecified statistical analysis plan, and results [103,108,109].

Further, to have more rigorous and transparent criteria for publication of tumor-biomarker studies, the Biospecimen Reporting for Improved Study Quality (BRISQ) and the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines were developed [103,110-112]. This kind of documentation should be an essential component for publication in high-impact journals of biomarker data that contribute to support clinical utility [109].

Biomarker development failures have consequences in form of lost resources from funding, time and labor, waste of talent, and give bad credibility for the field [106]. The volume of misleading publications may raise false hopes, ethical dilemmas, triggering the wrong policy changes or unnecessary political debates [106]. To treat patients with drugs that may not have an effect are expensive. Biomarkers are important to find and use. Rini et al wrote: “The ideal biomarker would be simple, reflective of intended target inhibition, easy to measure, of low cost, and reliably present at the baseline or early after initiation of therapy” [113]. And Hayes et al argues that “When patient management is dependent on the results of a biomarker test, that test becomes as critical for patient care as a therapeutic agent” [109].

1.6.2 **Prognostic markers in metastatic RCC**

Prognostic biomarkers give information about cancer outcome, regardless of therapy [105]. To improve prognostic information, targeted treatment uses prognostic models for clinical trial design, patient consulting and risk-assessment [114].

In treatment of metastatic RCC, a widely used risk score model have been a modified version of the Memorial Sloan-Kettering Cancer Center (MSKCC) model, originally made for INFα and/or IL2 treatment of ccRCCs [114-117]. To improve the model, adjusted to anti-VEGF treatment, the International Metastatic RCC Database Consortium (IMDC) found six independent prognostic markers (see Table 1) [118]. Results of this work in the new model added elevated platelet count and excluded lactate dehydrogenase (LDH), because of lack of statistical significance in the multivariate analysis [118]. The model includes Karnofsky performance status of less than 80%, less than 1 year from diagnosis to treatment, anemia (hemoglobin concentration < lower limit of normal), hypercalcemia (corrected calcium concentration > upper limit of normal), neutrophilia (neutrophil count > upper limit of normal), and thrombocytosis (platelet count > upper limit of normal) [114,118]. The model has been externally validated [114,119].

Table 1

Parameter	**Score**
KPS < 80%	1 point
Time < 1 year from diagnosis to treatment	1 point
Hemoglobin < LLN	1 point
Calcium > ULN	1 point
Neutrophils > ULN	1 point
Platelets > ULN	1 point
IMDC score	**Total score**
Favorable	0 point
Intermediate	1-2 points
Poor	3-6 points

The TNM classification is an anatomical prognostic marker to give information about probability of survival [32,33]. Tumor size, venous invasion, renal capsular invasion, adrenal involvement and lymph node and distant metastasis are included [32].

The ISUP grading system has replaced the Fuhrman grading [44]. The grading system estimates the prognosis of the patient's disease burden. Tumor grade, subtype of RCC, sarcomatoid features, microvascular invasion, tumor necrosis and invasion of the collecting system are histological factors [44].

CRP is an acute-phase protein increasing rapidly following IL6 secretion by macrophages and T cells following infection, inflammation and cancer [79]. It is an established biomarker for systemic inflammation, available in most clinical datasets, and provides prognostic information in several cancers including RCC [120].

NLR is also a marker of systemic inflammation in cancer patients and was found to add prognostic and predictive information in RCC in retrospective studies [81,82]. Among patients with baseline NLR ≤ 3 median PFS was significantly better than patients with baseline NLR > 3 (median PFS, 14.7 months vs. 6.7 months, Log rank p $= 0.05$) [121].

Baseline health related Quality of Life (HRQoL) questionnaire is a method where patients self-determine their own function, symptoms and global health [122,123]. HRQoL evaluation of patient-reported baseline QoL symptoms can be a prognostic indicator of survival times in clinical trials and practice [123,124]. Using the FACT-Kidney Symptom Index-15 (FKSI-15) score, Cella et al found a higher score (fewer symptoms) at baseline to be linked with median PFS and OS in metastatic RCC patients [124]. Using the EORTC QLQC30, another group found that "global QoL" was prognostic for PFS [125].

IL6 and IL6Rα are negative prognostic markers of survival in both primary and metastatic RCC [71,126-128]. IL6, is an immune system related cytokine, and has a role in

inflammation, infection responses, and the regulation of metabolic, regenerative, and neural processes [67,71,129,130]. High levels of inflammation-associated cytokines are negative for the outcome of treatment [130]. Elevated IL6 has been associated with poor survival in RCC and resistance to rTKI treatment [127,131-133]. IL6 and IL6Rα co-expression might be an independent early stage immunological prognostic factor for patients with organ-confined ccRCC [134]. However, high serum IL6 was significantly associated with better PFS in patients treated with pazopanib vs. placebo [135].

Dornbush et al found an association between high expression of VEGFR2 and good treatment response [136], and Terakawa et al also found high expression of VEGFR2 to be beneficial to sunitinib treatment [137].

High expression of HIF2α and PDGFRβ in tumor tissue were associated with better response of sunitinib treatment [138]. In another study, elevated level of perivascular PDGFRβ was found to be a marker of poor prognoses in RCC [139].

1.6.3 **Predictive markers in metastatic RCC**

Predictive biomarkers identifies patients who will most likely respond to a therapeutic intervention [105]. A controlled study is mandatory to introduce a predictive marker. The best way is to have a comparison of a treatment to a control in patients with and without the biomarker. The ongoing search for biomarkers to optimize VEGF inhibitor treatment in RCCs has so far been unsuccessful in finding predictive biomarkers useful in the clinic. Until recently there is no such biomarker validated for VEGF targeted therapy in metastatic RCC [140,141]. It would be of great value to identify, with help of predictive markers, which patients are most likely to benefit from the treatment [104].

The CheckMate 214 trial is probably the only validated study demonstrating IMDC as a predictive biomarker in first-line treatment of mRCC [142,143]. In the trial, they demonstrated significantly higher OS and objective response rates with nivolumab

plus ipilimumab than with sunitinib among intermediate and poor-risk patients with previously untreated mRCC [142].

A possible predictive marker is CRP shown in a phase two study where baseline CRP was significantly associated with objective response (Mann Whitney, p = 0.01) [121]. Fujita et al. showed that normal level of baseline CRP was an independent predictive marker of response in multivariate analysis [144].

In immune checkpoint inhibitor treatment, PD-L1 expression have not been conclusive to be a predictive marker, because of effect of treatment regardless of PD-L1 tumor expression [142,145].

1.6.4 **On treatment markers**

Prognostic factors are based on patient information at baseline derived from retrospective analyses of large cohorts of patients. They are dynamic and can change during treatment [114,146].

Development of early hypertension at week 12 under sunitinib treatment was associated with an improved clinical outcome and patients with induced hypothyroidism had longer PFS [113,147,148]

Donskov et al have demonstrated how on-treatment neutropenia and hypertension were significantly associated with longer PFS and longer OS [149], and hand–foot syndrome with longer OS, independent of baseline prognostic factors, including the IMDC risk criteria [146]. Adding the five-factor biomarker profile significantly improved prognostication in the IMDC intermediate (25.7 months vs. 12.0 months) and poor (12.8 months vs. 6.4 months) risk groups and a trend was seen in the IMDC favorable risk group (38.9 months vs. 28.7 months) [146].

1.7 Treatment

Tumor stage and disease burden determine the treatment choice of RCC. Current European guidelines are used in treatment of patients with localized or metastatic RCC in Norway [37,38].

1.7.1 Treatment of localized and locally advanced RCC

Surgery

A in depth description of the surgical treatment of localized RCC is beyond the scope of this introduction. Thus, only a brief overview is presented. Open radical nephrectomy (RN) was for four decades the standard of surgical care for RCC [150]. Partial nephrectomy (PN) is now standard for organ-confined tumors at the renal poles measuring <7 cm (T1 disease) [151]. PN in any method is the treatment choice in patients with T1 tumors rather than minimal invasive RN [38]. There is no survival benefit of removing lymph nodes without evidence of lymph node metastasis [38]. There is clear evidence demonstrating that laparoscopic RN has lower morbidity than open surgery [38]. Thus, it is now recommended for patients with T2 tumor and for T1 not treatable by PN [38]. Localized (T1 and T2) tumors can be cured by surgery alone [38]. The 5-year PFS for T1 and T2 disease is 95% and 74%, respectively [152]. In patients with locally advanced (T3 and T4) there is no survival benefit to remove the adrenal or lymph nodes without evidence of adrenal or lymph node involvement [151,153].

Surgery is indicated in patients with non-metastatic RCC and venous tumor thrombus [38]. In a large study published, a higher level of thrombus was not associated with increased tumor dissemination to lymph nodes, perinephric fat or distant metastasis [38]. As most methods have similar oncological outcome, the choice of methods for locally advanced tumors will depend on the skills and experience of the surgeon [38].

Post-surgery the TNM score system, the Leibovich score system and AJCC staging give prognostic information [32,33,152,154], and can be used to stratify patients with regard

to prognosis. A risk stratified follow up regimen is recommended because of risk of relapse within the first five years after surgery [155]. Also to be mentioned, a management option in elderly and/or comorbid patients with small renal masses not candidates for surgery may be active surveillance [38]. This is done by using serial abdominal imaging of tumor [37]. As the growth of small renal masses is slow in most cases, and progression to metastatic disease is rare [38,156], many patients can avoid surgical intervention. A renal biopsy is recommended before active surveillance. Other options of poor surgical candidates are radiofrequency ablation and cryoablation [38].

Adjuvant therapy

Despite high recurrence rates for patients with locally advanced (T3 and T4) disease, adjuvant therapy has not proven to be beneficial in randomized phase III trials [153].

Several studies have failed to demonstrate significant improvement in OS using adjuvant treatment [38]. These studies included use of immunotherapy (IFN-α, IL2, autologous tumor vaccines) and use of anti-angiogenic or targeted therapy (sunitinib, sorafenib, pazopanib, girentuximab) [37,157-160]. Neither do adjuvant cytokines improve survival after nephrectomy [38].

Ravaud found, in the S-TRAC study, that a sub group of patients with locoregional RCC at high risk for tumor recurrence after nephrectomy had a longer duration of disease-free survival (DFS) than those receiving placebo [160]. The results showed a benefit of sunitinib over placebo for DFS (HR: 0.76; 95% CI: 0.59-0.98, p = 0.03) but data for OS remained immature [160]. In a follow-up study, George et al found in the sunitinib group, a potential predictive marker where increased density of CD8+ cells was beneficial for DFS [161]. The ASSURE study, was a large adjuvant study investigating sunitinib versus sorafenib versus placebo [162]. The updated analysis in 2018 did not show a significant difference in disease free survival [162].

The PROTECT study, was a study investigating pazopanib versus placebo [159]. The study did not meet the primary end point of DFS in the intention to treat group receiving 600 mg, but in the group receiving full dose therapy (800 mg) there was an improvement in DFS (HR: 0.69; 95% CI: 0.51-0.94, nominal p = 0.02) [159].

Based on the results European guidelines do not recommend sunitinib in the adjuvant treatment of high-risk RCC after nephrectomy [38].

1.7.2 Treatment of advanced/metastatic RCC

Surgery

Cytoreductive nephrectomy (CN) and metastasectomy are options in metastatic RCC [38]. Curative treatment is only possible if the removal of all tumor tissue is removed [37]. In a minority of metastatic RCC, CN and metastasectomy are indicated as palliative treatment for pain relief or uncontrolled bleeding [151]. CN with simultaneous resection of single metastasis or oligo metastases may improve survival and delay systemic therapy [38].

Earlier, IFN-α and IL2 were the systemic treatment options and two randomized studies showed a survival benefit for CN plus IFN-α versus IFN-α alone [163,164]. After the introduction of targeted therapy, TKI's were used in similar fashion, and retrospective studies reported that patients with CN had better survival than those only treated with targeted therapy [165,166]. In a meta-analysis, CN increased OS in patients with metastatic RCC that were treated with targeted therapy with CN versus those without CN [167]. However, these retrospective studies were flawed by selection bias because of patients undergoing CN had significantly better performance score compared with the non-CN patients [38].

To investigate this further, two prospective randomized clinical trials (SURTIME and CARMENA) were performed. In SURTIME, time of CN in relation to sunitinib treatment was investigated. OS was better in the group of deferred versus immediate

CN [168]. In the CARMENA study, CN plus sunitinib versus sunitinib alone in patients with metastatic RCC at presentation did not find a difference in OS [169]. Both studies were closed prematurely due to slow recruitment. Both studies have been criticized for statistical power issues and their general validity. However, based on these two studies recommendations have changed. Today CN is only recommended in highly selected patients that do not have poor IMDC risk score, small primaries, high metastatic volume and/or a sarcomatoid tumor component [166].

There are no randomized studies on metastasectomy [38]. Except for the brain and maybe bone metastases, there is generally a weak recommendation for metastasectomy [38]. In 2014, a systematic review reported that patients with metastatic disease and favorable risk score could be offered a resection or ablation to achieve complete resection [38,170]. Standard treatment is to continue regular radiological surveillance [38,153].

Systemic therapy

First line treatment

Targeted therapy is the standard of care in systemic treatment of advanced disease. Until recently, anti-angiogenic treatment was the first line treatment for all risk groups [38]. Sunitinib, a receptor tyrosine kinase inhibitor (rTKI), is currently the primary choice [116]. Similar rTKIs are sorafenib, pazopanib and axitinib [171-173]. Other important drugs are the mTOR inhibitors temsirolimus and everolimus [174,175]. Sunitinib or pazopanib is first line treatment in the IMDC favorable risk group whereas ipilimumab/nivolumab combination immunotherapy is first line treatment option in the intermediate and poor risk group [176]. Recently new studies showed improved benefit of immune checkpoint inhibitors, compared with rTKI, in first line treatment and will probably change future treatment regime [142,145,177-179].

Sunitinib (SU11248) marketed, as Sutent by Pfizer, is an oral multi targeted tyrosine kinase inhibitor. It was FDA approved 2006 and introduced as the preferred anti-angiogenesis therapy for metastatic ccRCC [27,116,180,181]. Sunitinib was superior to IFN-

α treatment (PFS, 11 months vs. 5 months, HR 0.42 (95% CI, 0.32 to 0.54; p < 0.001) [116]. It was developed as a drug targeting angiogenic receptors on endothelial cells [182-184].

Sunitinib inhibits several receptors like; VEGFR-1, -2 and -3, PDGFR α and β, fibroblast growth factor receptor 1, proto-oncogene cKIT, fms-related tyrosine kinase 3 (FLT3), ret proto-oncogene, rearranged during transfection (RET) and colony stimulating factor 1 receptor. In experiments, it is also shown to downregulate myeloid-derived suppressor cells as part of the anti-tumor effect [184].

Sunitinib reach maximum concentration 6-12 hours after administration. The drug is metabolized by cytochrome P450 3A4 (CYP3A4), which makes it important to avoid or adjust drugs or foods affecting CYP3A4. Elimination is through feces and urine. There is no need of adjustment because of renal dysfunction or mild hepatic malfunction.

The rationale behind sunitinib in metastatic ccRCC treatment is the anti-angiogenic effect. The frequent inactivation of the VHL gene in ccRCC, leads to increased levels of HIF1α and VEGF [27].

The most common adverse events are fatigue, diarrhea, stomatitis, hand-foot skin reaction and hypertension [121,185]. The sunitinib dose or administration can be adjusted for a more tolerable adverse events profile [186,187].

Pazopanib was approved after being shown to be superior to placebo (median PFS, 9.2 months vs. 4.2 months; HR, 0.46; 95% CI, 0.34-0.62; p < 0.0001) [172] and was non-inferior to sunitinib in a first line setting [171]. Sunitinib and pazopanib share similar adverse events and in one trial pazopanib was preferred over sunitinib by the patients [188].

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is a downstream effector of the PI3K/AKT pathway [153]. mTOR has been found to increase HIF at the translational level, therefore inhibition of mTOR can be useful in RCC [153,189].

Intravenous temsirolimus is a competitive inhibitor of the mTOR complex 1. It can be used as first-line treatment in poor-risk ccRCC patients and was shown prolonged survival over IFN-α among poor-risk patients [174]. Sunitinib and pazopanib have also shown effect in the poor-risk patient group and may be preferable due to an oral route of administration [49].

The monoclonal anti-VEGF treatment bevacizumab with IFN-α can be used in first-line treatment. It was approved in the first line in metastatic RCC based on two phase three trials, showing the addition of bevacizumab to IFN-α extended PFS to eight to 10 months versus five months [190,191]. The combination is less used, because of oral TKIs, like sunitinib and pazopanib in first-line treatment [153].

Second line treatment

After progress on first-line rTKI patients can have second-line rTKI or mTOR inhibitors as temsirolimus or everolimus [153,175,192]. Everolimus was approved on the basis of the phase 3 randomized trial (RECORD-1), which compared everolimus with placebo, in patients who had progress on sunitinib or sorafenib [175]. Axitinib was associated with a longer PFS than sorafenib among patients treated with one previous line of therapy (predominantly sunitinib or cytokines) [49,173].

Despite an improved outcome nearly all patients develop resistance to anti-VEGF or mTOR treatment [49]. Combination therapy to overcome resistance has been tested without better effect in three studies [193-195]. In a phase 2 study, everolimus plus lenvatinib versus everolimus showed increased PFS and OS [49,196,197]. Lenvatinib is a dual VEGF-fibroblast growth factor inhibitor and start doses were reduced to avoid high level of toxicity [49,196].

The mechanism of acquired resistance to rTKI treatment stay largely unknown, but non-VEGF pathways involved in angiogenesis, invasion and proliferation may be of importance [49]. Pathways of tyrosine kinases FGFR, MET and AXL are examples of targets involved in resistance to anti-VEGF therapy [49]. Cabozantinib is a TKI, approved for metastatic medullary thyroid cancer that inhibits VEGF, MET and AXL implicated in the pathogenesis of RCC or the development of resistance [153]. In a phase 3 study (METEOR), cabozantinib showed a significant PFS advantage versus everolimus, with median PFS 7.4 months in the cabozantinib group versus 3.8 months in the everolimus group (HR for progression or death, 0.58; 95% CI, 0.45 to 0.75; P<0.001) [198].

Immunotherapy

Immune checkpoint inhibitor

In modern immunotherapy, immune checkpoint inhibitors have shown promising results. Immune checkpoint inhibitors with monoclonal antibodies targets and blocks the inhibitory T-cell receptor PD-1, the ligand (PD-L1) or cytotoxic T-lymphocyte associated antigen 4 (CTLA-4)-signaling to restore tumor-specific T-cell immunity [38,199]. Ipilimumab is an inhibitor of antigen four receptor (CTLA-4) expressed on cytotoxic T-cell. Nivolumab and pembrolizumab inhibit the programmed death 1 (PD-1) receptor expressed on macrophages, T- and B-cells and atezolizumab (PD-L1) inhibits the ligand [142,145,177-179].

Nivolumab was shown to have better OS, better QoL and less grade 3 and 4 adverse events compared with everolimus, in metastatic ccRCC after one or two lines of targeted treatment [177]. The Checkmate 214 study (NCT 02231749) investigated the combination of nivolumab plus ipilimumab versus sunitinib study in treatment naïve advanced or metastatic ccRCC [142]. At a median follow-up of 25.2 months in intermediate and poor-risk patients, the 18-month overall survival rate was 75% (95% CI, 70-78) with nivolumab plus ipilimumab and 60% (95% CI, 55-65) with sunitinib;

the median OS was not reached with nivolumab plus ipilimumab versus 26.0 months with sunitinib (HR for death, 0.63; $p < 0.001$) [142]. Based on these results the guidelines recommend to use ipilimumab plus nivolumab in treatment-naïve patients with clear-cell metastatic RCC of the IMDC intermediate and poor risk score [38].

The latest immune checkpoint inhibitor trials show a response benefit in first-line treatment, in treatment naïve patients [179]. The Keynote 426 study, an open label phase 3 trial, found significantly increased median PFS among patients treated with pembrolizumab plus axitinib versus sunitinib (PFS, 15.1 months vs. 11.1 months; HR for progression or death, 0.69; 95% CI, 0.57-0.84; $p < 0.001$) [179]. The objective response rate was 59.3% (95% CI, 54.5-63.9) in the pembrolizumab plus axitinib group and 35.7% (95% CI, 31.1-40.4) in the sunitinib group ($p < 0.001$). The benefit was across all IMDC risk scores and regardless of PD-L1 expression [179].

The PD-L1 inhibitor avelumab was tested with axitinib versus sunitinib in another phase 3 study in treatment naïve patients with advanced RCC [178]. The study reported the results dividing PD-L1 positive tumors versus PD-L1 negative tumors [178]. In PD-L1 positive tumors, median PFS was 13.8 months with avelumab plus axitinib versus 7.2 months with sunitinib (HR for progression or death, 0.61; 95% CI, 0.56-0.84; $p < 0.001$) [178]. The objective response rate was 55.2% versus 25.5%, in the two groups respectively. The PD-L1 negative tumors were not reported as a subpopulation in the study, but only as part of overall population [178]. PFS in the overall population was 13.8 months with avelumab plus axitinib versus 8.4 months with sunitinib (HR for progression or death 0.69; 95% CI, 0.56-0.84; $p < 0.001$) [178].

In the IMmotion 151, a phase 2 trial, the results showed improved PFS for atezolizumab plus bevacizumab versus sunitinib in both PD-L1 positive patients and showed a favorable adverse event profile [145]. Median PFS was 11.2 months with atezolizumab plus bevacizumab versus 7.7 months with sunitinib (HR 0.74; 95% CI, 0.57–0.96; $p = 0.0217$) [145]. The study needs longer observation time to establish possible survival benefit [145].

Interferon-α monotherapy and combined with bevacizumab

Targeted therapy has taken over for interferon-α (IFN-α) in metastatic ccRCC [116]. A Cochrane meta-analysis confirmed that IFN-α may still be effective in some patients groups [38,200]. IFN-α had low response rate (< 15%) [115]. The best benefit seems to be for patients in the favorable-risk group according to MSKCC risk score, where the median survival time was separated by 6 months or more in between the three risk groups [117].

Along with the sunitinib versus IFN-α results [116], Escudier et.al. found better efficacy in patients treated with bevacizumab plus IFN-α versus IFN-α monotherapy [190].

Interleukin-2

Interleukin-2 (IL2) affects tumor growth by activating lymphoid cells in vivo without affecting tumor proliferation directly [201]. Treatment response rate is from 7% to 27%. High dose of IL2 have increased toxicity and often observation and treatment in intensive care unit is needed [201]. In one study there was 4% treatment related deaths [202].

Vaccines and targeted immunotherapy

Cancer vaccines can enhance anti-tumor immunity, by presenting tumor antigens to T-cells [153]. Examples of vaccines can be autologous tumor cells, peptide-based vaccines and dendritic cell-based vaccines. There are experimental studies ongoing. One study with a tumor antigen 5T4 plus a first-line standard treatment did not show survival benefit compared to placebo or first-line standard therapy [203]. In IMPRINT, a phase 3 study, IMA901 a peptide-based cancer vaccine, aiming to increase the number of tumor-specific T cells did not find a survival benefit. They tested IMA901 plus sunitinib versus sunitinib [204].

Dendritic cells can migrate in response to inflammatory signals to the lymph nodes and present T cells to stimulate an anti-tumor immune response [153]. To make an anti-cancer vaccine it is necessary to collect patient's dendritic cells and culture them in vitro, prime them with antigens and then re-infuse into the patient [153]. There was a phase 3 study (Clinicaltrials.gov, NCT01582672), investigating AGS-003, an autologous dendritic cell-based vaccine plus sunitinib versus sunitinib, but it was stopped because of lack of efficacy [205].

Chemotherapy

Chemotherapy is not recommended in modern treatment of metastatic RCC. In one study, there was small effect with 5-fluorouracil combined with immunotherapeutic agent [206] and one study showed similar OS between IFN-α versus a combination therapy of IFN-α, IL-2 and 5-fluorouracil [207]. For patients with a poor prognosis, either with sarcomatoid histology or rapidly progressive RCC, gemcitabine and doxorubicin in combination could be used [208,209].

Radiotherapy

Traditionally, RCC is regarded a relatively radioresistant disease and the use of radiotherapy has been considered without effect in curative therapy of RCC. There is some evidence that use of palliative radiotherapy to bone or brain metastases can induce significant relief from local symptoms [38].

1.8 Health-related Quality of Life

Historical development

Aristoteles (384-322 BC) thought that the role of the health-care system was to improve patient's quality of life [210]. WHO defines quality of life as: "Individuals' perceptions of their position in life in the context of the culture and value systems in

which they live, and in relation to their goals, expectations, standards, and concerns" [211,212].

In modern medicine, the Karnofsky Performance Scale (KPS), was one of the first assessments of QoL in the early 1940s [213]. KPS is part of the MSKCC risk score and the IMDC prognostic risk scores [117,118].

Morton published a list of important subjects to be included in the QoL questionnaire [214]. Today the QoL questionnaire is called health-related QoL (HRQoL), which refers to the aspects of QOL related to a health or medical setting [215,216].

HRQoL in RCC patients

In RCC, reports of QoL have been related to surgical procedures [217-219].

Considering the health of patients undergoing nephrectomy for localized tumor, Ames investigated the psychological needs using another questionnaire (The Functional Assessment of Cancer Treatment – General (FACT-G) [220,221].

The EORTC have not developed or validated a disease-specific HRQoL questionnaire in RCC patients [122]. In 2006 a symptom index where developed and validated (the Functional Assessment of Cancer Therapy (FACT) – Kidney Symptom Index (FSI). It is made of thirty-four symptoms related to the disease [124]. In research projects in our hospital, the HRQoL questionnaire has been used in RCC and head and neck cancer [222-226].

2. Aims of the thesis

2.1 General hypotheses

Hypothesis: Patients with metastatic or inoperable kidney cancer with positive biomarkers of VEGF driven angiogenesis and VEGF associated immune suppression will have improved clinical benefit from treatment with an anti-VEGF-receptor kinase inhibitor.

2.2 General aim

Primary goal: To identify predictive markers of response in first line treatment with sunitinib against kidney cancer.
Secondary goal: To estimate response, time to progression, survival and amount of patients with stable disease on treatment.

2.3 Specific aims

2.3.1 **Paper I**

To report the clinical data from hospital records including hospital blood samples, side effects and quality of life data. To evaluate the response rates from sunitinib treatment with use of the radiologic examinations.

2.3.2 **Paper II**

To investigate potential predictive markers of response by immunohistochemistry in respect to angiogenic and immunogenic responses. To investigate these candidate markers in relation to clinicopathological data of paper 1.

2.3.3 **Paper III**

To investigate potential predictive value of immune related biomarkers in serum and plasma of sunitinib response.

3. Material and methods

3.1 Study design

This PhD-project was based on a clinical phase II trial model conducted at the Oncology Department at Haukeland University Hospital in Bergen, Norway. The study was an open labelled, single arm, single institution clinical trial where patients with metastatic ccRCC in progression were treated with sunitinib monotherapy until disease progression, significant toxicity or consent withdrawal. Sunitinib was administered 50 mg/day on schedule 4 weeks on/two weeks off. Clinical evaluation was done every 6th week, and computer tomography (CT) was performed every 12th week for response evaluation. Biopsies and blood samples for research purposes were taken before the first treatment. Blood samples for research purposes were taken every 12th week. Data were collected from the hospital records and included demographics, treatment modifications, adverse events, radiologic response data and survival. Data cut-off date was July 31 2015.

3.2 Ethics

The study followed the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. The protocol was approved by the Regional Ethics Committee (REC number 080/07 and REC number 78/05)) and the Norwegian Medicines Agency. REC number 080/07 is the sunitinib study. REC number 78/05 is the kidney cancer biobank study. All participating patients provided signed informed consent before enrolment.

3.3 Patient cohort and inclusion criteria's

Between October 2007 and October 2014, a regional cohort of 77 patients with mRCC was screened for inclusion at Haukeland University Hospital, Bergen, Norway. Forty-six patients were enrolled after signing the informed consent sheet.

Inclusion criteria included previously untreated metastatic or non-resectable ccRCC, WHO performance state 0-2, no known brain metastases, evaluable tumor lesions according to RECIST (version 1.1) and no significant comorbidity or laboratory abnormalities.

Table 2

Inclusion criteria's:
Metastatic or unresectable primary tumor of clear cell renal cell carcinoma
WHO performance status 0-2
Non-earlier cytokine treatment or other cancer treatment
Radiation therapy against symptom given metastasis was possible, but not against lesion that we would like to evaluate response
Non symptom given brain metastasis, but radiation treated brain metastasis where corticosteroids is stopped is allowed
> 12 months since coronar bypass operation
> 18 years
Not pregnant or breast feeding
Clinically or radiographic measurable disease according to the RECIST
> 21 days since major surgery or damage
> 2 days since biopsy, FNAC or central venous catheter
No ongoing grade 3 bleeding
None of the following the last 12 months: -Myocardial infarction -Serious unstable angina pectoris -Symptomatic heart failure (not including EF < 50% or > 20% reduction of EF compared to beginning of treatment) -Stroke including TIA -Pulmonary embolism
No other active malignant disease or not been treated for other cancers the last five years
Uncontrolled hypertension
Uncontrolled arrhythmia, especially prolonged QT-interval and bradycardias
Laboratory values of: -Granulocytes > 1,5 x 10^9/L -Platelets > 100 x 10^9/L -Bilirubin and < 1,5x upper normal limit -ASAT < 2,5x upper normal limit -ALAT < 2,5x upper normal limit
No other compliance that could make the patient unsuitable to be included in a research protocol
Written consent from every patient

Abbreviations: WHO: World Health Organization, RECIST: Response Evaluation Criteria in Solid Tumors, FNAC: Fine-Needle Aspiration Cytology, EF: Ejection Fraction, TIA: Transient Ischemic Attack, ASAT: aspartate aminotransferase, ALAT: alanine aminotransferase.

3.4 Response assessments

3.4.1 **RECIST v1.1**

Response evaluation criteria in solid tumors (RECIST) v.1.0 was introduced in 2000 and updated to v.1.1 in 2009 [227]. We used RECIST v.1.1 [228]. The major difference between version 1.0 and 1.1 is a maximum of five lesions to follow in v.1.1 versus maximum of 10 lesions to follow in v.1.0. If the patient has more than five lesions, the physician must choose five lesions that are possible to follow up over time. In both versions, non-target lesions were to be measured in case of progression. MP did the response evaluations, with advices from OS.

Almost all patients did their computer tomography (CT) analyses at the department of radiology at Haukeland University hospital. A few patients (< 5) did their control CTs at their local radiology department because of the long travel distance. These CT scans were re-examined at Haukeland University hospital. CT contrast was used in all patients except in a few examinations due to development of renal failure.

3.4.2 **Endpoints**

The primary endpoint was objective response (OR) defined as complete response (CR) or partial response (PR) according to RECIST v.1.1, as well as clinical benefit (CB) defined as CR + PR and including stable disease (SD) for more than 6 months [228]. Disease stabilization is considered beneficial to patients experiencing progression at the time of inclusion and CB is frequently included as an additional statistical endpoint in trials investigating antiangiogenic drugs in which therapeutic activity and clinical benefit are present, even in the absence of radiological tumor shrinkage [229]. Importantly, all patients were in clinical and/or radiological progression at the time of inclusion. OR and CB were calculated based on investigator assessment. Response evaluation by CT-scan or MRI was performed every 12 weeks. In the final response evaluation, only CT-scans were used. Patients with clinically evident disease progression or death because of metastatic RCC before first radiological progression

were recorded as progressive disease (PD). Best overall response (BOR), recorded as change in size of target lesion, was unavailable in these patients. PFS was defined as the time from treatment initiation until disease progression according to RECIST v.1.1. OS was defined as the time from enrolment until death of any cause.

Response status and endpoints at data cut-off date July 31 2015, were used in **paper I**, **II** and **III**.

3.5 Toxicity

Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse events, version 3.0 (CTCAE v.3.0) [230], and were recorded at each 6-week cycle based on clinical evaluation, physical examination and outpatient ward blood tests.

Patients that stopped treatment because of toxicity before first tumor response evaluation at week 12 were not included in the analyses of response rates or PFS, but were included in the analyses of OS. The complete list of adverse events is listed in the supplemental of **paper I**.

3.6 Sampling of tissue and blood samples

The biopsy material was prepared at the Department of Pathology, Haukeland University Hospital. Two formal-fixed paraffin embedded (FFPE) blocks were collected from external hospitals and one block was missing. Paraffin-embedded tissue was available in 45/46 (97.8%) patients in total. The most recent biopsy, from the metastatic lesion (n = 29) or from the non-resectable primary tumor, performed closest to the date of clinical trial inclusion (n = 12), was selected for further analysis if several lesions were available. In addition, protein expression of the candidate markers was analyzed in primary tumors alone (n = 41). All results in **paper II** referred to the most recent biopsy unless otherwise specified. All metastases and primary ccRCCs were reclassified by a RCC pathologist (LB) using the ISUP grading

system. Biopsies were investigated and scored on the hematoxylin and eosin slides to find the best FFPE tissue block to use in immunohistochemistry

Full blood samples were collected at baseline before starting sunitinib and after every second cycle of treatment during the study time. After centrifugation, Na-heparin plasma samples were stored frozen at minus 80°Celsius. For ELISA we used the heparin plasma sample tubes, which were de-frozen in room temperature, shaken and then centrifuged for different amount of fibrin precipitation.

In **paper I**, the regular hospital blood samples were used for baseline investigations. CRP was collected in 45/46 patients and the missing patient sample was one of the responders.

The excellent staff in the research group did the work of biopsy preparation and blood sample collection.

3.7 Clinicopathological variables

The following variables were recorded: age, sex, WHO performance status, sites of metastases, number of disease sites, hypertension before treatment, IMDC risk score, time from initial diagnose, prior removal of primary tumor, adverse events, tumor size according to RECIST criteria, Quality of Life before and under treatment and all findings according to inclusion criteria.

The TNM system used in the clinic during inclusion of patients in this study, was the 6th [231] and 7th edition [232]. In this work, all patients were stage four because of active metastatic disease at time of inclusion.

3.8 Immunohistochemistry

We used tissue sections of 4-5 µm from FFPE blocks from biopsies and resections of primary and metastatic RCC. Slides were deparaffined in Xylene and rehydrated

followed by antigen retrieval in a microwave oven. Endogenous peroxidase and alkaline phosphatase were blocked before incubation with the primary antibody followed by incubation with appropriate visualization kit. For negative controls, primary antibodies were omitted or specific blocking peptides were used for Heat shock protein 27 (HSP27) and VEGF-A. Tissues from different cancer types were used as positive controls. For JAG1, endothelial cells were used as positive internal control. Slides were stained with primary antibodies of IL6Rα, IL6, JAG1, VEGF-A, VEGFR2, PDGFRβ and HSP27.

Table 3

Primary antibody	Comment	Dilution	Incubation time	Antigen retrieval	Peroxidase block	Diluent	Visualization	Chromogen/ Counterstaining	Staining method
IL6	Mouse monoclonal	1/600	30 min	Dako S1699, pH 6	Dako K4007	Dako S3022 w/background	EV+sHRP/ anti-mouse (monokl. abs) Dako K4007	DAB/ Hemotoxylin	Auto
IL6Rα	Rabbit polyclonal	1/800	Over night	Dako S1699, pH 6	Dako K4011	Dako S3022 w/background	EV+sHRP/ anti-rab (polyc.abs) Dako K4011	DAB/ Hemotoxylin	Manual
JAG1 [1]	Goat polyclonal	1/100	60 min	Dako S1699, pH 6	Dako K4011	Dako S3022 w/background	EV+sHRP/ anti-rab (polyc.abs) Dako K4011	DAB/ Hemotoxylin	Manual
VEGF-A	sc-152 (rabbit polyclonal IgG)	1/50	60 min	Dako S1699, pH 6	Dako K4011	Dako S3022 w/background	EV+sHRP/ anti-rab (polyc.abs) Dako K4011	DAB/ Hemotoxylin	Auto
VEGFR2	FLK-3 (mouse monoclonal IgG)	1/100	60 min	Dako S1699, pH 6	Dako K4007	Dako S3022 w/background	EV+sHRP/ anti-mouse (monokl. abs) Dako K4007	DAB/ Hemotoxylin	Auto
PDGFRβ	2B3 (mouse mAb)	1/100	60 min	Dako S1699, pH 6	Dako K4007	Dako S3022 w/background	EV+sHRP/ anti-mouse (monokl.abs) Dako K4007	DAB/ Hemotoxylin	Auto
HSP27 [2]	C20 (goat polyclonal IgG)	1/1600	20 min	Dako S1699, pH 6	Dako K4011	Dako S3022 w/background	EV+sHRP/ anti-rab (polyc. abs), Dako K4011	DAB/ Hemotoxylin	Auto

1 JAG1: Secondary antibody: Rabbit anti-goat (6164-01).
2 HSP27: Extra protein block: Dako X0909 and Secondary antibody: Rabbit anti-goat (6164-01).
More details provided in supplementary table 1 in **paper II** [233]

3.9 Evaluation of staining results

Evaluation of staining results was performed by a semi-quantitative method. All sections were screened at x40 and x100 magnifications to map areas of cancer tissue and normal tissue. Further, with high power magnification (x200 or x400), staining intensity and the proportion of positive tumor cells were recorded implementing a semi-quantitative grading. Staining intensity was defined as absent (0), weak (1), moderate (2) or strong (3). The proportion was rated as "no positive tumor cells" (0), "less than 10% positive tumor cells" (1), "10–50% positive tumor cells" (2) or "more than 50% positive tumor cells" (3). The staining index (SI) is the product of intensity and area (range 0–9) [234,235]. SI was used to quantify cytoplasmic staining of the antibodies. Cases were categorized into groups (absent/low vs. median/high protein expression) based on the SI distribution for each biomarker under investigation. Because of difference in distribution, there were different cut-points in the groups. The cut-points were set to: IL6Rα low (SI = 1-3) vs. median/high (SI = 4-9); IL6 absent/low (SI = 0-2) vs. median/high (SI = 3-9); JAG1 absent/low (SI = 0-2) vs. median/high (SI = 3-9); VEGF-A low (SI = 1-3) vs. median/high (SI = 4-9); VEGFR2 absent/low (SI = 0-2) vs. median/high (SI = 3-9); PDGFRβ absent/low (SI = 0-1 vs. median/high (SI = 2-9); HSP27 low (SI = 1-3) vs. median/high (SI = 4-9). In addition, protein expression in tumor associated endothelial cells was graded based on staining intensity (0-3) for VEGFR2 and PDGFRβ. The IHC protein expression was evaluated and discussed by two observers (MP, OS) blinded with temporary number tags for response data.

3.10 Analysis of blood samples

3.10.1 Enzyme linked immunosorbent assay (ELISA)

MP found different manufactures of antibodies tested in human blood samples in patients with RCC. Of them, the ELISA kits were selected based on the experience of GHN. GHN carried out the experiments to increase the quality of the work and

because of shortness of time. MP observed the method. The antibodies used were human IL6 (P05231), human IL6Rα (BMS214) and human IL6ST (EHIL6ST). Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA provided all three. For IL6 we used a ready-to-use self-coating system kit (Invitrogen) and an ELISA 96-well flat-bottom plate (Nune MaxiSorp flat-bottom, Invitrogen (catalog number 44-2404)). For IL6Rα we used a ready-to-use sandwich ELISA 96 micro well plate coated kit with human IL6Rα (Invitrogen). For IL6ST we used a ready-to-use self-coating system kit (Invitrogen). Phosphate buffered saline (PBS); containing 0.05% (v/v) Tween 20 (PBS-T) (Prod.nr. 822184, Merck, USA) was used as washing buffer. All other buffers used were from the respective ELISA kit. The staining process was performed according to the manufactures manual and was analyzed at 450 nm with a microplate reader (Molecular Devices Emax).

3.10.2 Evaluation of ELISA results

We evaluated the ELISA results according to the manufactures manual. SoftMax Pro was used to evaluate the ELISA data and then transferred to SPSS for statistical analysis. We categorized the baseline ELISA variables (pIL6, pIL6Rα, pIL6ST), into low (below median) versus high (above median). The change in pIL6, pIL6Rα and pIL6ST concentration between baseline and week 12 were divided into three categories (decrease, stable, increase). We tested the decrease group versus the stable and increase groups. The variables referred in the **paper III** are baseline values if not otherwise specified.

3.11 Health related Quality of life

3.11.1 The general HRQoL questionnaire EORTC QLQ C-30

HRQoL was assessed by a validated Norwegian version of the questionnaire of the European Organization for Research and Treatment of Cancer (EORTC QLQ-C30 v.3.0) at baseline and every 12 weeks during treatment. The QLQ-C30 contains a

global health/QoL scale, five functional scales (physical, role, cognitive, emotional, and social), three symptom scales (fatigue, pain, and nausea/vomiting) and six single items (dyspnea, insomnia, anorexia, constipation, diarrhea, and financial difficulties). The answers were selected according to a 4-point Likert format, with the exception of questions about general health and quality of life, given according to a 7-point Likert format. Scores were calculated as described in the EORTC QLQ-C30 Scoring manual (3rd edition) [236]. The C30 functional scales and the global scale were transformed so that 100% indicates best function and 0% least function of the individual QoL index, whereas the C30 symptom scales were transformed so 0% indicates the least and 100% the most symptoms. We compared the upper quartile with the lower three quartiles for the symptom sum score and the lower quartile with the upper three quartiles for the functional sum score and global health/QoL score [121].

3.12 Statistics

Sample size calculations (alpha 0.05/power 80%) indicated 20 patients per group based on candidate marker expression were needed to detect a difference between 10% and 50% of patients having a response to treatment with sunitinib. The Mann-Whitney U test was used to compare the distribution of continuous variables between two groups like responders and non-responders. Comparisons between categorical variables were performed by using the Fisher's exact test and Pearson chi-square. Spearman's rank correlation coefficient was used to test correlations between variables of interest. Logistic regression analysis was used to test the relative importance of predictive factors for sunitinib response. Cronbach's α was used to estimate the reliability of the global health score and the functional/symptomatic scores made up of more than one question. Kaplan-Meier estimates were applied for time-to-event endpoints like PFS and OS, and log rank-test was applied for testing of differences between groups. All p-values are two-sided. The significance level was 0.05 for all tests. Statistical investigation was performed using IBM SPSS Statistics version 22 (**paper I**) and version 24 because of program update (**paper II and III**) (SPSS Inc., Chicago, IL, USA).

4. Summary of the results

4.1 Paper I

Seventy-seven patients were screened and 46 patients enrolled. The median age was 63.1 (range 41.1-84.0). We observed 1 complete response (CR), 7 partial responses (PR) and 18 patients had stable disease (SD) ≥ 6 months. Twelve patients showed progressive disease (PD), of which 10 were confirmed by radiology and two were confirmed by clinical progress before week 12. Eight patients discontinued treatment before week 12 and were recorded as non-evaluable for response rates and PFS. Of these, six were due to toxicity without evidence of disease progression, one patient because of appendicitis and one protocol violation. Of interest, seven of these eight patients were females.

Median PFS was 9.1 months (range 0.5-57.3 months) and median OS was 15.4 months (range 1.8-83.9 months).

The key finding was a significant association between normal CRP (≤ 10 mg/L) at baseline and OR (CR+PR) (Fisher's exact test, p = 0.01). 7/17 (41%) of patients with normal CRP had an objective response to sunitinib, compared with 1/20 (5%) patients with elevated CRP had an objective response. Median CRP at baseline was 17.0 mg/L (range 0-235 mg/L). Seventeen of 37 patients evaluated for overall response had normal CRP. Logistic regression analysis was used to test the relative importance of the candidate predictive factors for sunitinib response (eHTN, IMDC risk score, baseline NLR, baseline CRP, baseline EORTC QoL symptom scale). Only baseline CRP was an independent predictive variable of response, with an odds ratio of 14.3 (p = 0.02) of not having an objective response if CRP was above normal (10 mg/L).

Baseline NLR was not significantly associated with OR or CB.

Treatment induced early hypertension (eHTN), defined as SBP ≥ 140 or DBP ≥ 90 mm Hg at week 6, was not significantly associated with OR or CB. In our population, the baseline blood pressure was slightly higher when compared with the clinical trial population studied [113], and 52% of our patients were hypertensive at baseline. Still, the number of patients recorded as having sunitinib induced eHTN after cycle 1 and 2, using the same criteria, was nearly the same (~80%). eHTN at week 12 was associated with improved survival, but this is most likely because of the fact that the responders in the study stayed on treatment long enough to develop hypertension. Even if pharmacodynamically interesting, as eHTN occurs after sunitinib initiation it is not going to be an applicable predictive marker in the clinic.

IMDC risk score was not significantly associated with OR or CB.

The most frequent severe adverse events (grade 3+4) were hypertension (19.6%), fatigue (15.2%), low serum platelets (15.2%), hand-foot skin reaction (10.9%) and diarrhea (10.9%). We observed one grade 5 adverse event, death because of appendicitis, probably unrelated to sunitinib treatment. Only for "Fatigue", there was a statistically significant increase in the score during treatment compared with the baseline value (Wilcoxon ranked signed test, $p = 0.04$) for the health related quality of life (HRQoL) questionnaires at baseline. A Symptom score in lower 3 quartiles was significantly associated to improved CB (Fisher's exact test, $p = 0.02$). Investigating symptom sum scores indicated the upper quartile had significantly worse OS (median 12.7 months vs. 25.2 months, Log-rank $p = 0.01$) and PFS (median 2.9 months vs. 14.7 months, Log-rank $p = < 0.01$). No such difference could be demonstrated for global health/QoL status or functional sum score.

4.2 Paper II

In **paper II**, we investigated the potential predictive value of angiogenic, inflammatory and immunogenic factors in tissue samples for response to sunitinib treatment. We used immunohistochemistry to investigate tissue expression of IL6Rα,

IL6, JAG1, VEGF-A, VEGFR2, PDGFRβ and HSP27. Low expression of IL6Rα was significantly associated with OR (Fisher's Exact test, $p = 0.03$). Sixty-six percent of the patients with response data available showed median/high expression of IL6Rα in tumor cells, and only 10% of these patients responded to treatment with sunitinib, whereas 46% of patients with low expression responded. Absent/low expression of IL6 was significantly associated with improved PFS (median PFS, 17.0 months vs. 8.7 months, Log rank, $p = 0.04$).

4.3 Paper III

The purpose of **paper III** was to test if the inflammatory factors IL6 and IL6Rα in plasma could predict treatment response to sunitinib therapy. We assessed single ELISA assays. Plasma concentration of IL6 was significantly associated with CB (Fisher's exact test, $p < 0.01$). Similarly, the continuous value of IL6 was significantly associated with CB (Mann-Whitney U-test, $p < 0.01$). Low IL6 was significantly associated with improved PFS (median PFS, 14.7 months vs. 5.3 months, Log rank, $p = 0.04$). IL6Rα was not significantly associated with OR or CB. The High change in IL6 was significantly associated with decrease of IL6Rα (Pearson chi-square, $p = 0.05$). Low change in IL6Rα was significantly associated with OR (Fisher's exact test, $p = 0.01$) and not CB. Decrease of IL6Rα was significant associated with OR (Pearson chi-square, $p < 0.01$) and not CB.

We also investigated the association to markers of interest in **paper I and II**. Low pIL6Rα at baseline was significantly associated with under median of age (Pearson chi-square, $p = 0.04$) and hypertension at baseline (Pearson chi-square, $p = 0.02$). pIL6Rα was not significantly associated with CRP and tumor tissue expression of IL6Rα or IL6.

5. Discussion

5.1 Discussion of materials and methods

5.1.1 Patients, study design and samples

The study was a single arm, single institution clinical phase II trial to investigate potential predictive biomarkers of sunitinib treatment in patients with metastatic ccRCC. Seventy-seven patients were screened and 46 enrolled. The numbers of screening failures were because of other histology than ccRCC or lack of biopsy from primary tumor or metastases. Still, there were enough patients enrolled for statistical power, to find possible biomarkers of interest. The inclusion time ranged from 2007 to 2015, in part because of the 42.8% screening failure. A great advantage of this study is the availability of tumor tissue, blood samples, clinical data, quality of life data and follow-up data.

In **paper I**, the main objective was to identify and evaluate the predictive value of candidate markers readily available in a standard clinical setting, in metastatic ccRCC patients treated with sunitinib. Candidate markers included early hypertension (eHTN), IMDC risk groups, baseline NLR, baseline CRP and baseline EORTC QoL symptom scale. Response rates according to RECIST 1.1 were used as primary endpoint. Secondary objectives were to evaluate prognostic value of the candidate markers with regard to PFS and OS. In addition, toxicity rates and HRQoL were recorded.

Because of ethical reasons, the study only had a single arm, since there was no acceptable control treatment available and placebo was impossible. All patients were treated with standard treatment of care, as sunitinib was during the entire study period.

5.1.2 Immunohistochemistry

All antibodies were first tested on sections of tissue microarrays containing cores of different tumor tissues including ccRCC.

We used protocols from the immunohistochemistry research laboratory with adjustments to optimize the procedures. The time of tissue fixation in formalin is an unknown variable when working with archival FFPE blocks. Different fixation times can result in inhomogeneous immunohistochemical staining. To optimize demasking of the epitopes, different retrieval methods like proteinase K, cooking in different buffers with pH 6 or pH 9 and heating in the microwave, were tested. Different dilutions of the primary antibody as well as varied incubation times were used to find optimal staining procedure. When tested over night the slides were incubated at 4C degrees in a fridge. Endogenous peroxidase and alkaline phosphatase were inhibited prior to incubation with the primary antibody. Detection systems based on horseradish peroxidase (HRP) in combination with 3,3'-diaminobenzidine (DAB). Final counterstaining with hematoxylin was applied on all antibodies.

5.1.3 Evaluation of staining methods and assessment of markers

Immunohistochemical staining was evaluated in a semi quantitative manner as described in chapter 3.9. MP and OS evaluated optimal primary antibody incubation time with a double light microscope. All evaluations of staining were done with the same double light microscope. For VEGF-A, VEGFR2, PDGFβR and HSP27 assessments were done by MP and OS. For IL6, IL6Rα, IL6ST and JAG1 assessment were first rated by MP and all non-conclusive cases were discussed with OS.

Because of limited amount of tumor tissue in some samples, there was not a complete set of staining in the different biomarkers **(paper II, table 2)**.

Assessment of staining index (SI) was performed using both the intensity and the area of protein expression. The method evaluates the intratumor protein expression better than the staining intensity alone [234,235]. The lack of standardized staining protocols and multiple methods of evaluation are important to consider when comparing with other reports .

5.1.4 **Analyses of blood**

ELISA is a simple and efficient assay method [237,238]. In our work we used sandwich ELISA, reported to be 2-5 times more sensitive than other ELISAs [239].

It is important to avoid or reduce analytic errors [239]. To reduce pre-analytic errors, the samples were taken following a strict protocol. We stored the supernatant at -80 Celsius. The patients were told to be fasting before taking samples. To reduce analytic errors, we used the same manufacture, the same type of plates for each antibody and the same experienced staff members. Post-analytic errors were reduced by not calculating the concentration using a curve expects program.

The choice of sample type is important in the planning phase of the study. The sampling protocol must be standardized to make sure there is little variation in handling of samples. Advantages and disadvantages of additives in various tubes must be considered because they can be an issue in later analyses.

The use of serum samples reduces this problem, but proteins can be bound by different cells during clotting. In our work, fibrin had to be removed in some samples.

ELISA kits from different companies are pre-coated with different antibodies, which may result in different detection sensitivity and protein concentration [239]. Sensitivity for binding a protein of interest may not be high enough, especially if the expected concentration is low. In single protein ELISA, reactivity of a kit with an antigen may be visually traceable and cross-reactivity therefore less relevant. Enough plasma is

needed to analyze one protein in duplicate and insufficient binding of a antigen to the antibody epitope can be problematic [239].

5.1.5 Health related Quality of life

Our findings showed how higher baseline QoL symptom scores are prognostic for PFS and OS in treatment with sunitinib. This is in line with the earlier report by Cella et al. [123]. In their report, however, a different QoL tool was used. Herrmann et al demonstrated by using EORTC QLQ-C30, that global QoL was prognostic for PFS [125]. Our study could not confirm this finding. In general, there were only small changes in QoL scores form baseline to 12 weeks. Herrmann et al. also showed a relatively small change in global QoL after 12 weeks [125]. This could be due to the administration of sunitinib (4 weeks on/ 2 weeks off), with subsequent remission of eventual treatment induced symptoms. There are indications that long-term survivors might retain a good QoL over years, as described by Carmichael et al. [240].

The QLQC30 form was not always given to the patient before information about CT scan. This may have biased the results of HRQoL data, because of information about disease status.

5.1.6 Statistics

The sample size was calculated to show predictive value of sunitinib treatment in patients with metastatic ccRCC. In addition to the lack of a control group, our study has some weaknesses. First, the number of patients included is low and thereby the study lacks the significant power to detect minor differences in response rates between groups based on the biomarkers under investigation.

Thus, our findings should be validated in an independent and larger cohort of patients. Still, our data strongly suggest that biomarkers associated with tumor immune responses might be important in patients treated with anti-VEGF therapy.

We used the Mann-Whitney U test to distinguish between responders and non-responders. In clinical practice, it is necessary to use a cut-off definition on predictive markers.

In this study, we focused on single candidate biomarkers based on our hypotheses, and not panels of multiple markers. Bonferroni testing or other test for multiple testing were unused for correction. The main reason was the small size. Perneger, a medical epidemiologist, discusses limitation of the test [241]. He argues that quote; "Bonferroni adjustments follow the original logic of statistical tests as supports of repeated decisions, but they are of little help in determining what the data say in one particular study" [241]. External validation is most important to control clinical validity.

5.2 Discussion of results

5.2.1 Angiogenesis, inflammation and anti-angiogenic treatment in RCC

In this translational study, we have investigated the predictive value of markers of anti-angiogenic treatment. Until recently, chemotherapy, palliative surgery and radiation therapy were the only treatment options for metastatic RCC, and primary therapy resistance, reduced quality of life and short survival were major challenges in this patient group. Currently, three major categories of systemic treatment exist for the largest subgroup of metastatic RCC, the ccRCC: cytokines and immune checkpoint inhibitors, anti-VEGF targeted drugs and mTOR inhibitors [242]. The two latter of these new treatment options have emerged based on knowledge of the pathogenesis of ccRCC. The VHL tumor suppressor gene is lost or mutated in 60-90% in sporadic cases [23] and is a major contributor to development of this cancer. Loss of VHL leads to a chronic stress response state in the cells trough high levels of HIF1α, a transcription factor for a number of stress response proteins, including VEGF.

In addition to being a potent angiogenic growth factor, VEGF plays a role in the local immune response in wounds and tumors by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, as well as by inhibiting the migration of T lymphocytes to the tumor [80]. RCC is regarded as highly immunogenic and angiogenic tumors, supporting VEGF as target for treatment. The VEGF receptor inhibitor sunitinib has been first line treatment for metastatic RCC [242], but a significant portion of the patients do not respond, and the search for good predictive markers of response has been disappointing. Whereas the focus in search for predictive markers has been on angiogenesis [136,243], markers of hypoxia [136,138,243], clinical markers [113,147,148], VHL mutation status [244] and single nucleotide polymorphisms (SNPs) [245,246], less focus has been on markers of immune responses.

Recently, an inverse response relationship was reported for VEGF inhibitor treatment and immune checkpoint inhibitor treatment according to IMDC risk groups [142]. New predictive biomarkers are needed to further optimize treatment for individual patients. The ongoing search for biomarkers to optimize VEGF inhibitor treatment in RCC has so far been unsuccessful in finding predictive biomarkers useful for clinical practice. In our work, we have presented results suggesting a predictive role of CRP, IL6 and IL6Rα [121,141,233]. This might indicate the immunomodulating effect of anti-VEGF therapy plays an important role in treatment response in addition to the effect on angiogenesis.

5.2.2 **Candidate markers of inflammation**

C-reactive protein (CRP) is an acute-phase protein that increases rapidly following IL6 secretion by macrophages and T-cells following infection, inflammation and cancer [79]. CRP is a negative prognostic marker in cancer [247].

Our results indicate that an inflammatory response, defined by high CRP is associated with poor response to sunitinib and poor prognosis in these patients. The response to

VEGF inhibition by sunitinib thus seems to be more pronounced in patients with a non-inflammatory state defined by normal CRP. Nevertheless, the significant association with response rates suggests CRP might be a useful marker, in addition to other clinical and biochemical features to consider prior to initiation of systemic treatment.

In our material **(paper I)**, we found normal CRP to be a possible predictive factor of response. Whereas 41% of the patients with normal CRP at baseline experienced an objective response, this was the case for only 5% of patients with CRP levels at baseline above normal. CRP was also associated with PFS and OS supporting its role as a prognostic marker as well, and this is in line with previous reports [144,248,249]. In a study by Fujita et al normal level of CRP at baseline was an independent predictive marker of response in multivariate analysis [144]. In a retrospective study of 200 patients treated with sunitinib, 61% of patients with normal CRP responded versus 32% of patients with elevated CRP [250]. In our study, CRP was correlated to several factors including other markers of systemic inflammation like high platelet counts and anemia as well as tumor load and performance status.

Interleukin-6 (IL6) is an important tumor-promoting protein associated with stress responses, inflammation and angiogenesis and has a role in the regulation of metabolic, regenerative and neural processes [67,71,129,130]. Through its major downstream target STAT3 several tumor promoting pathways are activated, including HIF1α and increased VEGF activity [67]. As a response to cellular stress, IL6 activation of the transcription factor STAT3 drives angiogenesis by inducing expression of VEGF and fibroblast growth factor by tumor cells, and thereby supports vascularization required for tumor growth and metastasis [68,69]. Our results are in support of previous reports indicating that high levels of inflammation-associated cytokines are negative for the outcome of treatment [130].

IL6 signals in cells via classic (membrane-bound) and trans-signaling (soluble) pathways [251,252]. The trans-signaling pathway is considered to be pro-inflammatory

[253]. Elevated IL6 has been associated with poor survival in RCC and resistance to rTKI treatment [127,131-133]. Tumor cells produce IL6 in response to cellular stress like hypoxia, and enhanced levels of IL6 is associated with increased tumor cell invasion [71,254]. The prognostic information of IL6 and IL6Rα is well known [71,128]. A predictive value of IL6 levels has also been reported for response to immune checkpoint inhibitors [255].

Kwon et.al. found a stimulating effect of elevated IL6 on endothelial cells, which might represent a resistance mechanism to anti-VEGF therapy [78]. Elevated levels of IL6 among patients with poor response were also found in a recent work of Mizuno, investigating angiogenic, inflammatory and immunologic markers of sunitinib treatment in 56 patients with metastatic RCC [133]. However, they did not include patients with poor IMDC risk score. Our findings may therefore show that we can include this group as well. Tran et.al. found opposite results, with a significant increase in PFS in patients treated with pazopanib versus placebo, in patients with high serum IL6 [135].

Tissue and serum levels of IL6 are elevated in RCC, and high levels of IL6 have been associated with elevated CRP and poor survival in RCC patients [126,127,131,256]. Being closely correlated to IL6 expression, increased CRP levels might therefore be a surrogate marker of IL6 driven disease, again being associated with expression of multiple angiogenic factors [257], thus less responsive to specific anti-VEGF treatment like sunitinib.

In **paper II**, we found that low tumor cell expression of IL6Rα was significantly associated with improved OR and that low tumor cell expression of IL6 was significantly associated with improved PFS and OS to sunitinib [258]. Blay et al. previously showed that a higher IL6 level correlated with increased concentration of CRP [256]. In our study, IL6 was not significantly associated with CRP.

Absent/low expression of both IL6 and IL6Rα was beneficial to treatment outcome, although not significantly associated with response rates, but the group with low expression of IL6 had almost a doubling of PFS compared to median/high expression. In line with previous reports, this suggests IL6 expression has prognostic value independent of the treatment given [126,127].

Fu et.al. found that IL6 and IL6Rα co-expression might be an independent early-stage immunologic prognostic factor for patients with organ-confined ccRCC [134]. In **paper III**, low baseline level of pIL6 was significantly associated with clinical benefit of sunitinib treatment and improved PFS. Our results are in support of previous reports indicating that high levels of inflammation-associated cytokines are detrimental for the outcome of sunitinib treatment [130].

The interleukin 6 receptor α (IL6Rα) binds to the interleukin 6 signal transducer (IL6ST), also known as glycoprotein 130 (gp130) protein receptor to transduce the signal. Dysregulation of the cytokine IL6 and its receptor is involved in the pathogenesis of several diseases, like autoimmune conditions and cancer [259]. The membrane-bound IL6Rα is found on hepatocytes and different leukocytes [260]. In trans-signaling, soluble IL6 binds to soluble IL6Rα and the complex binds to cells expressing IL6ST [261]. Soluble IL6ST is also detected in the blood and has been shown as an inhibitor of IL6 trans-signaling [262]. The prognostic value of IL6Rα expression has previously been presented, where Costes et al. found a significant association between IL6Rα expression and OS in patients with primary RCC tumors [128].

In **paper II**, we found that low expression of IL6Rα may predict response to rTKI treatment. IL6Rα was expressed in all cases, and low expression of IL6Rα was significantly associated with OR to sunitinib treatment [233]. Only 10% of patients showing increased expression of IL6Rα responded, suggesting that high IL6Rα expression might represent an essential mechanism of resistance to anti-VEGF therapy in ccRCC.

When correlating IL6Rα to the other biomarkers investigated, we found that median/high expression of IL6Rα was significantly associated with median/high expression of HSP27. Both IL6 and HSP27 signaling constitute cellular stress responses and increase the level of VEGF through activation of STAT3 [69,263]. Schuster et al. found that high HSP27 expression in melanoma metastases predicts response to anti-VEGF treatment [264]. In this work, we did not detect an association between HSP27 expression and treatment response.

In **paper III**, the baseline level of soluble IL6Rα in plasma was not significantly associated with response variables, though low pIL6Rα tended to be associated with improved OS. In **paper II**, low tumor cell expression of IL6Rα was beneficial for treatment response. The complexity of membranous and soluble IL6Rα is thoroughly discussed in several reviews [68,71,265].

Membranous IL6ST is universal expressed in human tissue [266]. IL6ST and IL6Rα form a buffer for pIL6 in the blood, and is purposed to represent a mechanism by which the organism defends itself from unspecific overstimulation by IL6 trans-signaling [265]. Even though the range of pIL6ST in our cohort was lower compared to a normal cohort, the group pIL6ST below median level had worse OS (**paper III, table 3**). This is in line with previous findings [127]. This may support the idea of a well-functioning buffer to protect against unspecific overstimulation by IL6-trans-signaling [265]. The cases with sunitinib induced reduction of pIL6Rα after two rounds of treatment had improved PFS. A reduction of pIL6Rα might be supported by the theory that trans-signaling pathways mediate cancer development [253,267]. Our results suggest that this may be used as a marker of beneficial on-treatment response, and suggest a relation between IL6Rα in tumor cells and level of circulating pIL6Rα.

Other findings

The neutrophil-to-lymphocyte ratio (NLR) is also a marker of systemic inflammation in cancer patients and was found to add prognostic [82] and predictive [81] information in

RCC in retrospective studies. Like CRP, NLR is readily available in standard blood samples in an ordinary clinical setting. Our NLR counts were comparable to previous reports in other clinical datasets [81,82]. Although significantly associated with CRP, we did not find a statistically significant association with sunitinib response or survival. The significant correlation with performance status and tumor load suggests that NLR is a nonspecific marker of disease burden.

Treatment induced early hypertension (eHTN) was not significantly associated with treatment response in our dataset. In our patient population, the baseline blood pressure was slightly higher when compared with the clinical trial population studied by Rini et al [113], and 52% of our patients were hypertensive at baseline **(paper I)**. Still, the number of patients recorded as having sunitinib induced eHTN after cycle 1 and 2, using the same criteria, was nearly the same (~80%). eHTN at week 12 was associated with improved survival, but this is most likely because of the fact that the responders in the study stayed on treatment long enough to develop hypertension. Even if pharmacodynamically interesting, as eHTN occurs *after* sunitinib initiation it is not going to be an applicable predictive marker in the clinic.

Jagged 1 (JAG1) is one of five Notch ligands. The Notch signaling pathway represent a regulator of tumor angiogenesis, stem cell self-renewal, cell fate determination, epithelial cell polarity/adhesion, cell division and apoptosis [268-271]. In metastatic ccRCC, high JAG1 was associated with poor prognosis [272]. In aggressive breast cancer cells, Sansone et.al. found that IL6 could stimulate Notch-3 dependent upregulation of JAG1 in an autocrine matter in response to hypoxic conditions [273]. In our present cohort, the expression of JAG1 was unrelated to OR, but absent/low expression of IL6 was significantly associated to absent/low expression of JAG1. These JAG1 results may support a possible interaction of JAG1, Notch and IL6 [273].

Whereas VEGF-A was expressed in all tumors, we observed no association to response, in line with other studies [136,243]. VEGF-A signals through VEGFR2 on endothelial cells to activate angiogenesis [274]. Dornbush et al. found an association

between high expression of VEGFR2 and good treatment response [136], and Terakawa et al. also found high expression of VEGFR2 to be beneficial to sunitinib treatment [137]. In the last paper, the majority of patients was in the good prognostic group whereas in ours, the majority was in the poor prognostic group [121].

PDGF receptors are key regulators of mesenchymal cells of the tumor microenvironment in several malignity's [275]. In cancers, an association between high stromal PDGFRβ expression or signaling and poor prognosis is reported [275]. However, we did not detect an association to response in our present data.

Our finding that higher baseline health related Quality of life (HRQoL) symptoms score was prognostic for PFS and OS in treatment with sunitinib is in line with the earlier report by Cella et al.[123]. In that report, however, a different QoL tool was used. Herrmann et al. demonstrated by using EORTC QLQ-C30, that "global QoL" was prognostic for PFS [125]. Our study did not confirm this finding. In general, there were only small changes in HRQoL scores form baseline to 12 weeks. Herrmann et al. also showed a relatively small change in the different HRQoL scales after 12 weeks [125]. This could be due to the administration of sunitinib (4 weeks on/2 weeks off), with subsequent remission of eventual treatment induced symptoms. There are indications long-term survivors might retain a good HRQoL over years, as described by Carmichael et al. [240].

5.2.3 **Challenges**

A major challenge in studies exploring predictive markers of treatment response in clinical data-sets is the fact that most of the candidate predictive markers are prognostic as well, thus significantly correlated with PFS and OS independent of the treatment given. Combined predictive and prognostic markers are best evaluated in two-arm trials. In single arm trials like ours, response rates according to RECIST are superior to PFS and OS as primary end-point when assessing predictive markers of treatment response [276]. Many biomarker studies in metastatic RCC have been

performed retrospectively in data-sets from large clinical trials, and these patients are frequently positively selected and do not optimally reflect the normal patient population. The strength of our study is the prospective design and the "Real-World" patient population enrolled, reflecting a normal clinical setting. When compared with large retrospective multicenter studies as well as smaller single-center studies, the majority of our patients were in the poor risk group according to IMDC criteria. Whereas the portion of poor-risk patients varies between 18 % and 33 % in comparable studies [81,114,144], 41.3% of our patients belonged to this group. In addition, all patients were in confirmed clinical and/or radiological progression at the time of inclusion. Accordingly, patients with very slow progression or stable metastases were observed without systemic treatment and screened for inclusion in the study only after confirmed disease progression. For this reason, PFS and OS were lower in our patients in comparison with clinical phase III trials. Compared with the adverse events reported in clinical trials [171], the frequency of toxicity from sunitinib in metastatic RCC recorded in our study was similar, or somewhat less frequent. Especially, the hematological toxicity including anemia, neutropenia, thrombocytopenia and lymphopenia were less frequent in our trial, although using the same criteria (CTCAE v. 3.0). The most likely explanation for this discrepancy is we assessed adverse events, including laboratory, every 6 weeks, where most of the patients were off the drug in the 4+2 weeks cycle.

In our study, the number of patients included is low and thereby the study is not sufficiently powered to detect minor differences in response rates between groups based on the biomarkers under investigation. Thus, our finding should be validated in an independent and larger cohort of patients. Validation of a biomarker is very important and many study fails in the validation process. A biomarker must have both analytical validity and clinical validity [277]. Analytic validity is how the test is accurate, reproducible and reliable [277]. Clinical validity is how the biomarker can divide a population into two separate groups with different clinical outcomes [277]. To introduce a cancer biomarker test into standard clinical management, it must also demonstrate "clinical utility" [277]. Clinical utility is defined as "the test's ability to

significantly improve measurable clinical outcomes, and its usefulness and added value to patient management decision making compared with current management without testing" [277].

Second, CRP and NLR are non-specific markers of inflammation and angiogenesis, and further studies are required to identify the key regulators controlling the systemic responses to metastatic disease. Third, the reproducibility of the quantification of protein expression used in this study also needs to be validated in a separate patient cohort.

Still, our data strongly suggest that biomarkers associated with tumor immune responses might be important in patients treated with anti-VEGF therapy.

6. Conclusions

In conclusion, in this prospective study of sunitinib in patients with metastatic ccRCC we found that patients with low expression of inflammation markers responded better on sunitinib.

In **paper I**, we found CRP was significantly associated with response rates and might serve as guidance in the selection of optimal treatment.

In **paper II**, we found that reduced expression of IL6Rα was significantly associated with improved objective response to sunitinib. Expression of IL6Rα might be a potential predictive biomarker of response to guide treatment of patients with metastatic ccRCC. We also found low expression levels of the IL6 ligand in tumor cells provided significant positive prognostic information.

In **paper III**, we found that low level of plasma IL6 is a positive predictive marker of improved response to sunitinib.

7. Future perspectives

To find predictive markers of anti-angiogenic therapy is difficult. The anti-angiogenic receptor tyrosine kinase inhibitor sunitinib is still first line treatment in metastatic RCC in Norway. Today there are no established predictive markers in clinical use. Inflammation is an important part of cancer. In our study, we found that low level of IL6 in plasma may predict treatment response of sunitinib and guide clinicians in making better treatment plans in RCC. The results suggest that up-regulation of plasma IL6 might represent an important mechanism of resistance. If validated in independent patient cohorts, the biomarker can easily be implicated into routine practice for a low cost using ELISA. Baseline measurement of this biomarker might guide clinical decision making in treatment of patients with metastatic ccRCC.

Sunitinib is overtaken by immune checkpoint inhibitors in favorable risk group [38], but many patients do still not have curation. It is therefore necessary to find the patients that will have a benefit of anti-angiogenic treatment like sunitinib or other targeted therapy. A next step may be to test treatment that bypasses the mechanism of resistance in combination with sunitinib.

8. References

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Pilskog et al. BMC Urology (2017) 17:74
DOI 10.1186/s12894-017-0267-6

BMC Urology

RESEARCH ARTICLE Open Access

Predictive value of C-reactive protein in patients treated with sunitinib for metastatic clear cell renal cell carcinoma

Martin Pilskog[1,2], Christian Beisland[3,5], Lars A. Akslen[1,4], Leif Bostad[4], Åse Haug[2], Daniel Heinrich[6], Karin M. Hjelle[3,5] and Oddbjørn Straume[1,2*]

Abstract

Background: Sunitinib has become mainstay first line treatment for patients with metastatic renal clear cell carcinoma (mRCC). Still, useful predictive markers of response are lacking and urgently needed for clinical decision making.

Methods: In the present study we investigated the predictive value of standard serum markers as well as clinical markers, including C-reactive protein (CRP), Neutrophil to Lymphocyte ratio (NLR) and early hypertension (eHTN) in an unselected prospective patient population treated with sunitinib for mRCC. Forty-six patients were enrolled in a prospective single-arm study of predictive markers for sunitinib response. Response rates according to RECIST 1.1 were used as primary end-point. Secondary objectives were to evaluate prognostic value of the candidate markers with regard to progression free survival (PFS) and overall survival (OS). In addition, toxicity rates and quality of life was recorded.

Results: Median PFS and OS was 9.1 months and 15.0 months, respectively. Of 38 patients evaluable for response, 1 patient had complete response (CR), 7 had partial response (PR), 18 had stable disease (SD) and 12 had progressive disease (PD). Normal CRP at baseline was significantly associated with objective response (CR + PR) ($p = 0.01$). Normal CRP was also significantly associated with improved PFS and OS (Log rank, $p = 0.05$ and <0.01, respectively). Early hypertension, NLR and IMDC risk score were not significantly associated with response rates or survival.

Conclusion: Baseline CRP was a significant predictive factor of sunitinib response *and* a prognostic factor of survival. Baseline CRP might be a useful biomarker in the treatment planning of mRCC. Due to the relatively small sample size, our results need to be confirmed in larger studies.

Background

The frequent inactivation of the Von Hippel Lindau (*VHL*) gene in clear cell renal cell carcinoma, leading to increased levels of hypoxia inducible factor 1 (HIF1) and vascular endothelial growth factor (VEGF), provides the rationale for treatment with antiangiogenic receptor tyrosine kinase (rTKI) inhibitors. Since the reporting of the first positive clinical trial [1], showing an overall survival benefit from a rTKI, sunitinib has become mainstay first line treatment for patients with metastatic renal cell carcinoma (mRCC). Although objective response rates are reported for around 50% of the patients, development of resistance to the treatment is a major problem [2]. Clearly, a subset of patients does not benefit from treatment with sunitinib, and side effects are frequent. Interestingly, hypertension is a common side effect of angiogenesis inhibitors and has been associated with improved treatment response [3]. In the research community, considerable effort has been made to identify and validate predictive biomarkers of response to sunitinib treatment, but so far, no biomarkers have been established as useful in clinical decision making and treatment planning.

There is increasing evidence to support an important role of systemic inflammation in development and progression

* Correspondence: Oddbjorn.straume@helse-bergen.no
[1]Centre for Cancer Biomarkers CCBIO, University of Bergen, Bergen, Norway
[2]Department of Oncology, Haukeland University Hospital, 5021 Bergen, Norway
Full list of author information is available at the end of the article

of RCC [4], as recently substantiated by positive results from a clinical trial with the PD-1 inhibitor nivolumab in mRCC [5]. VEGF does not only stimulate tumor associated angiogenesis, but also plays an important role in the local immune response in wounds (physiologic) and tumors (pathologic) by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, as well as by inhibiting the migration of T lymphocytes to the tumor [6]. Thus, it is relevant to also explore biomarkers primarily associated with inflammatory responses in the search for predictive markers for response to anti-VEGF therapy.

C-reactive protein (CRP) is an established biomarker for systemic inflammation, available in most clinical datasets, and provides prognostic information in several cancers including RCC [7]. Another biomarker of inflammation, neutrophil-to-lymphocyte ratio (NLR), adds prognostic information in RCC, and was recently suggested as a predictive marker of response to sunitinib in mRCC [8]. In the present trial we investigated the predictive value of serum markers, including CRP and NLR, in an unselected prospective patient population treated with sunitinib for mRCC. In addition, we report on toxicity and health related quality of life (HRQoL) data.

Methods

Patients and treatment

Between October 2007 and October 2014, a regional cohort of 77 patients with mRCC was screened for inclusion in this prospective study at Haukeland University Hospital, Bergen, Norway. Forty-six patients were enrolled after signing the informed consent sheet (CONSORT Flow Diagram, Additional file 1: Fig. S1). Inclusion criteria included: previously untreated metastatic or non-resectable clear cell RCC, WHO performance state 0–2, no known brain metastases, evaluable tumor lesions according to RECIST (version 1.1) and no significant comorbidity or laboratory abnormalities. See Additional file 2: Table S1 for all inclusion criteria. Sunitinib was administered 50 mg/day on schedule 4 weeks on/ two weeks off. Patients continued on treatment until disease progression, significant toxicity or consent withdrawal. Data was collected from the hospital records and included demographics, treatment modifications, adverse events, radiologic response data and survival. Data cut-off date was July 31 2015.

The main objective of this study was to identify and evaluate the predictive value of candidate markers readily available in a standard clinical setting, in mRCC patients treated with sunitinib. Candidate markers included early hypertension (eHTN), IMDC risk groups, baseline neutrophil to lymphocyte ratio (NLR), baseline CRP and baseline EORTC QoL symptom scale. Response rates according to RECIST 1.1 were used as primary endpoint. Secondary objectives were to evaluate prognostic value of the candidate markers with regard to progression free survival (PFS) and overall survival (OS). In addition, toxicity rates and HRQoL was recorded.

Assessment of response, adverse events and quality of life

The primary endpoint was objective response (OR) defined as complete response (CR) or partial response (PR) according to RECIST v.1.1 as well as clinical benefit (CB) defined as CR + PR and including stable disease (SD) for more than 6 months. Disease stabilization is considered beneficial to patients experiencing progression at the time of inclusion and CB is frequently included as an additional statistical endpoint in trials investigating antiangiogenic drugs in which therapeutic activity and clinical benefit are present, even in the absence of radiological tumor shrinkage [9]. Importantly, all patients were in clinical and/or radiological progression at the time of inclusion. OR and CB were calculated on the basis of investigator assessment. Response evaluation by CT-scan or MRI was performed every 12 weeks. Patients with clinically evident disease progression or death due to mRCC before first radiological progression were recorded as progressive disease (PD). Best overall response (BOR), recorded as change in size of target lesion, was not available in these patients. PFS was defined as the time from treatment initiation until disease progression according to RECIST v.1.1. OS was defined as the time from enrollment until death of any cause.

Standard blood samples, including CRP and neutrophil/lymphocyte counts, were taken at treatment initiation and every 6 weeks during treatment. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse events, version 3.0 (CTCAE v.3.0), and were recorded at each 6-week cycle. Early hypertension (eHTN) was particularly evaluated for its potential role as a predictive marker for treatment response. We recorded eHTN in two different ways. First, we defined eHTN as either maximum post-baseline systolic blood pressure (SBP) ≥140 mmHg or maximum post-baseline diastolic blood pressure (DBP) ≥90 mmHg recorded at week 6 and week 12 [3]. Second, we recorded eHTN as HTN ≥ grade 1 defined by CTCAE v.3.0 at week 6 and week 12. All other adverse events were recorded every six weeks throughout the entire treatment period. Patients that stopped treatment due to toxicity before 1st tumor response evaluation at week 12 were not included in the analyses of response rates or PFS, but were included in the analyses of OS.

HRQoL was assessed by a validated Norwegian version of the questionnaire of the European Organization for Research and Treatment of Cancer (EORTC QLQ-C30 v.3.0) at baseline and every 12 weeks during treatment.

The QLQ-C30 contains a global health/QoL scale, five functional scales (physical, role, cognitive, emotional, and social), three symptom scales (fatigue, pain, and nausea/vomiting) and six single items (dyspnoea, insomnia, anorexia, constipation, diarrhoea, and financial difficulties). The answers are given according to a 4-point Likert format, with the exception of questions about general health and quality of life, which are given according to a 7-point Likert format. Scores was calculated as described in the EORTC QLQ-C30 Scoring manual (3rd edition) [10]. The C 30 functional scales and the global scale were transformed so that 100% indicates best function and 0% least function of the individual QoL index, whereas the C30 symptom scales were transformed so that 0% indicates the least and 100% the most symptoms. We compared the upper quartile with the lower 3 quartiles for the symptom sum score and the lower quartile with the upper 3 quartiles for the functional sum score and global health/QoL score.

Statistical analyses

The Mann-Whitney U test was used to compare the distribution of continuous variables between two groups such as responders and non-responders. Comparisons between categorical variables were performed by using the Fisher's exact test. Spearman's rank correlation coefficient was used to test correlations between variables of interest. Logistic regression analysis was used to test the relative importance of predictive factors for sunitinib response. Cronbach's α was used to estimate the reliability of the global health score and the functional/symptomatic scores made up of more than one question. Kaplan-Meier estimates were applied for time-to-event endpoints such as PFS and OS, and log rank-test was applied for testing of differences between groups. Sample size calculations were based on a difference in response rate of 40% (i.e. 10% and 50%) between groups identified by the candidate markers. Thirty-eight patients were needed to achieve a power of 80% with an α-value of 0.05. All *p*-values are two-sided. Statistical investigation were done using IBM SPSS Statistics version 22.

Results

Patient population and treatment efficacy

The characteristics of the 46 patients enrolled in the study are presented in Table 1. In our cohort the median age was 63.1 (range 41.1–84.0). By July 31st, 2015 the median follow up time was 13.8 months (range 1.8–83.9). Twenty-six patients had prior removal of the primary tumor, twenty-four by radical and 2 by partial nephrectomy. Six patients had resection of bone metastasis, eight patients had resection of other metastasis, one patient had gamma knife radiosurgery of brain metastasis and two patients had radiation therapy against bone metastasis prior to sunitinib treatment. Median time on treatment was 5.7 months (range 0.5–63.0). Median time from first diagnosis of renal cell carcinoma to treatment was 3.2 months (range 0.3–124). Median time to treatment from diagnosis of metastasis was 1.4 months (range 0.3–66.5).

Table 1 Baseline Patients Characteristics

	Study cohort (n = 46)
Age, years	
Median	63.1
Range	41.1–84.0
Sex - No. (%)	
Male	29 (63.0)
Female	17 (37.0)
WHO performance status - No. (%)	
0	30 (65.2)
1	16 (34.8)
2	0 (0.0)
Site of metastases - No. (%)	
Brain	1 (2.2)
Lung	35 (76.1)
Pleura	3 (6.5)
Liver	4 (8.7)
Bone	16 (34.8)
Lymph nodes	28 (60.9)
Number of disease sites - No. (%)	
1	10 (21.7)
2	11 (23.9)
≥ 3	25 (54.3)
Hypertension before treatment - No. (%)	
Yes	24 (52.2)
No	22 (47.8)
IMDC risk score – No. (%)	
Good	7 (15.2)
Intermediate	16 (34.8)
Poor	21 (45.7)
Missing	*2 (4.3)*
Time from initial diagnosis - No. (%)	
≤ 12 months	33 (71.7)
> 12 months	13 (28.3)
Prior removal of primary tumor - No. (%)	
Radical nephrectomy	24 (52.2)
Partial nephrectomy	2 (4.3)
No	20 (43.5)

Abbreviations: *WHO* World Health Organisation, *IMDC* International Metastatic Renal Cell Carcinoma Database Consortium

By July 31 2015, median progression free survival (PFS) was 9.1 months (range 0.5–57.3) and median overall survival (OS) was 15.4 months (range 1.8–83.9). At data cut-off, 9 patients were still alive, and six patients were still on sunitinib treatment without signs of progression. Twenty-three patients started second line systemic treatment. We observed 1 complete response (CR), 7 partial responses (PR) and 18 patients had stable disease (SD) ≥ 6 months. Twelve patients showed progressive disease (PD), of which 10 were confirmed by radiology and 2 were confirmed by clinical progress before week 12. Eight patients stopped treatment before week 12 and were recorded as non-evaluable for response rates and PFS. Of these, six were due to toxicity without evidence of disease progression, one patient due to appendicitis and one protocol violation. Of interest, seven of these eight patients were females.

Predictive value of pre-treatment clinical and biochemical markers and survival analyses

The correlations between clinical, as well as biochemical markers assessed ahead of treatment initiation and sunitinib response are given in Table 2. The association between clinical, as well as biochemical markers assessed ahead of treatment initiation, PSF and OS is given in Table 3.

C-reactive protein (CRP)

Median CRP at baseline was 17.0 mg/L, range 0–235 mg/L. Seventeen of 37 patients evaluated for overall response had normal CRP (≤10 mg/L). Normal CRP at baseline was significantly associated with OR (CR + PR) (Fisher's exact test, $p = 0.01$) (Fig. 1). Seven/17 (41%) of patients with normal CRP had an objective response to sunitinib, compared with 1/20 (5%) patients with elevated CRP had an objective response. Logistic regression analysis was used to test the relative importance of the candidate predictive factors for sunitinib response(eHTN, IMDC risk groups, baseline NLR, baseline CRP, baseline EORTC QoL symptom scale). Only CRP level at baseline was an independent predictive variable of response, with an odds ratio of 14.3 ($p = 0.02$) of not having an objective response if CRP was above normal (10 mg/L). CRP at baseline was significantly correlated with several other variables including age, function sum score, symptom sum score, performance status and tumor load (Additional file 3: Table S2). Median PFS was significantly longer among patients with normal CRP at baseline (median 14.7 vs 5.3 months, log rank $p = 0.05$). Similarly, an improved OS was found in patients with normal CRP at baseline (median 26.0 vs 12.1 months, log-rank $p < 0.01$) (Fig. 2 a, b).

Neutrophil to lymphocyte ratio (NLR)

Twenty-two of 34 (64%) patients evaluated for response and available NLR, had NLR ≤3 at baseline (median = 2.7, range 1.0–7.9) and NLR at baseline was not significantly correlated with OR or CB. Eighty-three % of patients evaluated for response had NLR ≤3 at week 6 (median = 1.6, range 0.4–5.9) and this was not significantly correlated to OR or CB. A shift from NLR >3 at baseline to ≤3 at week 6 ($n = 10$) was not significantly associated with OR or CB. Median PFS among patients with baseline NLR ≤3 was significantly better than patients with baseline NLR >3 (median 14.7 vs 6.7 months, log rank $p = 0.05$). A borderline association was present between baseline NLR ≤3 and OS (median 25.2 vs 13.2 months, log-rank $p = 0.06$). High NLR at baseline was significantly correlated with increased tumor load ($r = 0.43$, $p = 0.005$, Spearman).

Treatment induced early hypertension (eHTN)

Applying the first definition of eHTN (SBP ≥140 or DBP ≥90 mmHg at week 6 and week 12) seventeen of 32 patients (53%) evaluated for response had eHTN after week 6. Median SBP over DBP was 145 (range: 120–170) mmHg over 89 (range: 60–170) mmHg, and was not significantly associated to OR or CB. Using the same definition at week 12, fifteen of 19 patients (79%) had eHTN. Median SBP over DBP was 142 (range: 120–170) mmHg over 88 (range: 65–107) mmHg, and was significantly associated with improved CB, but not OR (Fischer's exact test $p = 0.04$ and $p = 0.53$, respectively) (Table 2). The second definition (based on CTCAE v 3.0) of eHTN was not significantly associated OR or CB (data not presented). eHTN at week 12 was associated with improved PFS and OS (Table 3). All seven patients with increased blood pressure during the two first cycles used anti-hypertensive drug(s) at baseline.

Risk scores

The distribution of IMDC risk score [11] is given in Table 1. IMDC risk score was not significantly associated with OR or CB. Good IMDC risk score versus intermediate and poor was not significantly correlated to PFS (median 20.4 vs 9.1 vs 8.4 months, log-rank $p = 0.10$), but was significantly associated with OS (median 67.9 vs 12.7 vs 13.7 months, log-rank $p = <0.01$). We found similar results for MSKCC risk score and WHO performance status (PS) (data not presented).

Metastatic sites

The distribution of metastatic sites is given in Table 1. There was no significant association between metastatic site and response rates. Although present in only 3 patients, pleura metastasis was significantly associated with reduced PFS (median 2.6 vs 9.1 months, log rank

Table 2 Univariate analyses of clinical and biochemical markers in relation to response to sunitinib

Variable	Best overall tumor response (RECIST ver. 1.1)					
	OR[1] n(%)	SD[2]+PD[3] n(%)	*p* value[4]	CB[5] n(%)	PD[3] n(%)	*p* value
Total	8(21)	30(79)		26(68)	12(32)	
Age			0.69			0.73
< 63.1	4(17)	19(83)		15(65)	8(35)	
≥ 63.1	4(27)	11(73)		11(73)	4(27)	
Sex			0.17			0.45
Female	4(40)	6(60)		8(80)	2(20)	
Male	4(14)	24(86)		18(64)	10(36)	
Number of disease sites			0.71			0.31
≤ 2	3(18)	14(82)		10(59)	7(41)	
> 2	5(24)	16(76)		16(76)	5(24)	
Prior nephrectomy			0.26			0.73
Yes	6(29)	15(71)		15(71)	6(29)	
No	2(12)	15(88)		11(65)	6(35)	
Pretreatment hypertension[6]			0.70			0.30
Yes	5(26)	14(74)		15(79)	4(21)	
No	3(16)	16(84)		11(58)	8(42)	
Treatment induced eHTN[7] ≤ week 6			1.00			0.70
Yes	4(23)	13(77)		13(76)	4(24)	
No	4(27)	11(73)		10(67)	5(33)	
Treatment induced eHTN[8] ≤ week 12			0.53			0.04
Yes	5(33)	10(67)		13(87)	2(13)	
No	0(0)	4(100)		1(25)	3(75)	
IMDC risk			0.77			0.46
Good	2(29)	5(71)		6(86)	1(14)	
Intermediate	3(25)	9(75)		9(75)	3(25)	
Poor	3(18)	14(82)		10(59)	7(41)	
NLR baseline ≤3			1.00			0.46
Yes	5(23)	17(77)		16(73)	6(27)	
No	3(25)	9(75)		7(58)	5(42)	
NLR week 6 ≤ 3			0.32			0.15
≤ 3	7(23)	23(77)		22(73)	8(27)	
> 3	0(0)	6(100)		2(33)	4(67)	
NLR shifted from >3 to ≤3 at week 6			1.00			0.06
Yes	2(25)	6(75)		6(75)	2(25)	
No	0(0)	3(100)		0(100)	3(100)	
NLR shifted from ≤3 to >3 at week 6			1.00			0.50
Yes	0(0)	2(100)		1(50)	1(50)	
No	5(26)	14(74)		14(74)	5(26)	
CRP ≤10 (mg/L)			0.01			0.09
Yes	7(41)	10(59)		14(82)	3(18)	
No	1(5)	19(95)		11(55)	9(45)	

Table 2 Univariate analyses of clinical and biochemical markers in relation to response to sunitinib *(Continued)*

EORTC QoL symptom scale at BL[9]			0.31		0.02
Upper quartile	0(0)	7(100)	2(29)	5(71)	
Lower 3 quartiles	8(27)	22(73)	24(80)	6(20)	

Abbreviations: *IMDC* International Metastatic Renal Cell Carcinoma Database Consortium, *LDH* Lactate dehydrogenase, *ULN* Upper limit of normal, *LLN* lower limit of normal, *NLR* neutrophil/lymphocyte ratio, *CRP* C-reactive protein, *BL* baseline
[1]Objective response (Complete + Partial response)
[2]Stable disease
[3]Progressive disease
[4]Fisher's exact test
[5]Clinical benefit (OR + SD)
[6]Defined as on anti-hypertensive treatment before initiation of sunitinib
[7]Defined as systolic blood pressure(SBP) ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg ≤ week 6
[8]Defined as ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg ≤ week 12
[9]Quality of Life

p = 0.05) and OS (median 10.8 vs 17.5 months, log-rank p = 0.04). Presence of lung metastasis was significantly associated with reduced OS (median 13.2 vs 48.2 months, log-rank p = 0.04). Other metastatic sites (brain, bone, liver or lymph nodes) were not significantly associated with PFS or OS.

Toxicity

Adverse events occurring during treatment with sunitinib according to CTCAE v.3.0 are summarized in Additional file 4: Table S3. The most common adverse effects of lower grade (1 + 2) were nausea (52.2%), anemia (47.8%), fatigue (45.7%) and diarrhea (39.1%). The most common severe adverse effects (grade 3 + 4) were hypertension (19.6%), fatigue (15.2%), low serum platelets (15.2%), hand-foot skin reaction (10.9%) and diarrhea (10.9%). We observed one grade 5 adverse effect (death due to appendicitis) probably not related to sunitinib treatment.

Health related quality of life

The results of the HRQoL questionnaires at baseline (n = 45) and at the first treatment evaluation (n = 28, after 12 weeks) are presented in Additional file 5: Table S4. Only for "Fatigue", there was a statistically significant increase in the score during treatment compared with the baseline value (p=0.041, Wilcoxon ranked signed test). The Cronbach- α of the indices derived by more than one question showed acceptable/good values (0.74-0.89), except for "cognitive function" (0.30) and "social functioning" (0.56). The Cronbach- α of the sum scores of functional indexes (0.80) and symptom indexes (0.79) were acceptable/good. In contrast to Global health status/QoL and Functional scale, a Symptom sum score below median was significantly associated to improved CB (Fisher's exact test, p = 0.02, Table 2). Investigating symptom sum scores indicated that the upper quartile had significantly worse OS (median 12.7 vs 25.2 months, log-rank p = 0.01) and PFS (median 2.9 vs 14.7 months, log-rank p = <0.01). No such difference could be demonstrated for global health/QoL status or functional sum score (data not shown).

Discussion

Until recently, palliative surgery, radiation therapy and chemotherapy were the only treatment options for metastatic RCC (mRCC), and primary therapy resistance, reduced quality of life and short survival were major challenges in this patient group. Currently, three major categories of systemic treatment exist for the largest subgroup of mRCC, the clear cell carcinomas: cytokines and immune checkpoint inhibitors, anti VEGF targeted drugs and mTOR inhibitors [12]. The two latter of these new treatment options have emerged based on recent knowledge of the pathogenesis of clear cell renal cancer. The von Hippel-Lindau (VHL) tumor suppressor gene is lost or mutated in 60–90% in sporadic cases [13] and is a major contributor to development of this cancer. Loss of VHL leads to a chronic stress response state in the cells trough high levels of HIF1-α, a transcription factor for a number of stress response proteins, including vascular endothelial growth factor (VEGF). In addition to being a potent angiogenic growth factor, VEGF plays a role in the local immune response in wounds and tumors by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, as well as by inhibiting the migration of T lymphocytes to the tumor [6]. Renal cell cancer is regarded as highly immunogenic and angiogenic tumors, supporting VEGF as a promising target for treatment. The VEGF receptor inhibitor Sunitinib is currently first line treatment for mRCC [12], but a significant portion of the patients do not respond, and the search for good predictive markers of response has been disappointing so far. Whereas most focus in the search for predictive markers has been on angiogenesis, less focus has been on markers of immune responses. In the current study, we evaluated readily available clinical and biochemical markers, associated with systemic inflammation, for their association with response to sunitinib.

Table 3 Survival analyses according to clinical and biochemical variables

Variable	PFS[1]			OS[2]		
	Median	95% CI[3]	*p*-value[4]	Median	95% CI	*p*-value
Age			0.29			0.47
< 63.1	8.7	6.2–11.2		17.5	5.5–29.4	
≥ 63.1	20.4	3.2–37.7		15.0	12.4–17.6	
Sex			0.03			0.87
Female	NR	-		12.7	7.3–18.2	
Male	8.7	6.6–10.7		18.0	10.5–25.4	
Number of disease sites			0.80			0.52
≤ 2	12.9	2.1–23.7		17.5	11.3–23.7	
> 2	9.1	8.3–9.8		13.9	10.2–17.6	
Prior nephrectomy			0.07			<0.01
Yes	14.7	5.9–23.5		26.0	20.1–31.8	
No	8.7	3.1–14.3		10.8	4.5–17.0	
Pretreatment hypertension			0.42			0.79
Yes	17.0	6.2–27.7		18.0	9.4–26.5	
No	8.4	3.7–13.1		11.6	5.3–17.9	
Treatment induced early hypertension[5] at week 6			0.68			0.85
Yes	14.7	9.6–19.8		18.0	3.5–32.5	
No	8.7	3.9–13.5		12.1	3.8–20.4	
Treatment induced early hypertension[5] at week 12			<0.01			<0.01
Yes	14.7	10.1–19.3		26.0	24.1–27.9	
No	2.6	1.9–3.3		7.7	4.4–11.0	
IMDC risk score			0.10			<0.01
Good	20.4	13.1–27.7		67.9	38.4–97.5	
Intermediate	9.1	6.0–12.2		12.7	10.6–14.9	
Poor	8.4	0–17.7		13.7	5.4–22.1	
NLR baseline ≤3			0.05			0.06
Yes	14.7	8.8–20.6		25.2	10.6–39.8	
No	6.7	2.0–11.4		13.2	10.3–16.1	
NLR week 6 ≤ 3			0.09			<0.01
Yes	10.8	6.0–15.7		25.2	13.7–36.7	
No	1.8	0.2–3.5		3.8	3.3–4.3	
NLR shifted from >3 to ≤3 week 6			<0.01			<0.01
Yes	8.4	6.0–10.7		13.2	7.0–19.4	
No	1.3	0.9–1.7		3.6	0.8–6.4	
NLR shifted from ≤3 to >3 at week 6			0.75			0.16
Yes	NR	-		4.0	2.3–5.7	
No	14.7	9.2–20.3		26.0	24.2–27.7	
CRP			0.05			<0.01
≤ 10 (mg/L)	14.7	2.5–26.9		26.0	0.6–51.4	
> 10 (mg/L)	5.3	0.9–9.8		12.1	8.8–15.5	
EORTC QoL symptom scale at BL			<0.01			0.01
Upper quartile	2.8	2.2–3.5		12.7	4.8–20.7	
Lower 3 quartiles	14.7	4.6–24.8		25.2	12.7–37.7	

Abbreviations: *IMDC* International Metastatic Renal Cell Carcinoma Database Consortium, *LDH* Lactate dehydrogenase, *ULN* Upper limit of normal, *LLN* lower limit of normal, *NLR* neutrophil/lymphocyte ratio, *CRP* C-reactive protein, *NR* Not reached, *BL* Baseline

[1]Progression free survival

[2]Overall survival

[3]Confidence interval

[4]Log rank test

[5]Defined as systolic blood pressure(SBP) ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg

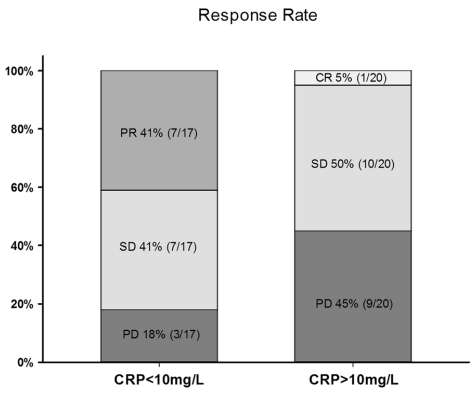

Fig. 1 Response rates as a function of baseline CRP levels. Patients with a baseline CRP ≤ 10 mg/L (normal) showed an objective response rate (CR + PR) of 41% whereas patients with elevated CRP showed an objective response rate of 5%. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease

C-reactive protein (CRP) is an acute-phase protein that increases rapidly following interleukin-6 secretion by macrophages and T cells following infection, inflammation and cancer [14]. CRP is a negative prognostic marker in most cancers. In the present study we found normal CRP to be a possible predictive factor of response. Whereas 41% of the patients with normal CRP at baseline experienced an objective response, this was the case for only 5% of patients with CRP levels at baseline above normal. CRP was also associated with PFS and OS supporting its role as a prognostic marker as well, and this is in line with previous reports [15–17]. Our finding supports the results of a recent study by Fujita et al. where normal level of CRP at baseline was an independent predictive marker of response by multivariate analysis [15]. In a retrospective study of 200 patients treated with sunitinib 61% of patients with normal CRP responded vs 32% of patients with elevated CRP [18]. In our trial, CRP was correlated to several factors including other markers of systemic inflammation such as high platelet counts, anemia as well as tumor load and performance status. Thus, CRP might represent a marker of disease burden identifying a patient subpopulation with poor prognosis, less likely to respond. Nevertheless, the significant association with response rates suggests that CRP might be a useful marker, in addition to other clinical and biochemical features to consider prior to initiation of systemic treatment. IL-6 is an important tumor-promoting protein associated with stress responses, inflammation and angiogenesis [19]. Through its major downstream target STAT3 several tumor promoting pathways are activated, including HIF1-α and VEGF [19]. Moreover, IL6 have direct stimulating effect on endothelial cells, and has been implicated in resistance to anti-VEGF therapy [20]. Being closely correlated to IL6 expression, increased CRP levels might therefore be a surrogate marker of IL6 driven disease, again being associated with expression of multiple angiogenic factors [21], thus less responsive to specific anti-VEGF treatment like sunitinib. Our results indicate that an inflammatory response, defined by high CRP is associated with poor response to sunitinib and poor prognosis in these patients. The effect of sunitinib on inhibiting the angiogenesis supporting and immunosuppressive effect of VEGF, thus seem to be more pronounced in patients with a non-inflammatory state defined by normal CRP.

The neutrophil-to-lymphocyte ratio (NLR) is also a marker of systemic inflammation in cancer patients and was found to add prognostic [22] and predictive [8] information in RCC in retrospective studies. Like CRP, NLR is readily available in standard blood samples in a regular clinical setting. Our NLR counts were comparable to what has been reported in other clinical datasets.

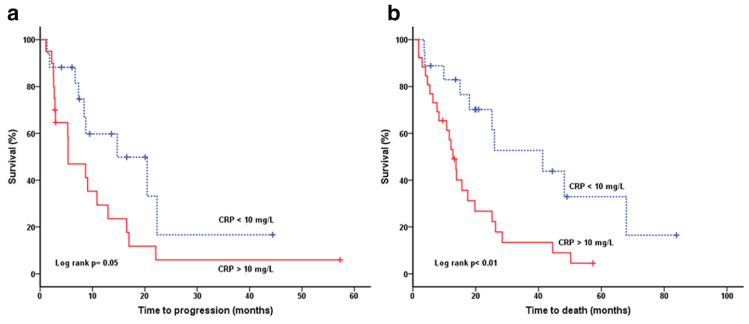

Fig. 2 Kaplan-meier estimates of **a**) progression free survival (PFS) and **b**) overall survival (OS) grouped by CRP level. The normal CRP (≤ 10 mg/L) cohort showed significantly improved PFS and OS compared with patients with elevated CRP

Although significantly associated with CRP, we did not find a statistically significant association with sunitinib response or survival. The significant correlation with performance status and tumor load suggests that NLR is a nonspecific marker of disease burden. Still, due to relatively small sample size and low statistical power, our data must be interpreted carefully.

Treatment induced early hypertension (eHTN) was not significantly associated with treatment response in our dataset. In our patient population, the baseline blood pressure was slightly higher when compared with the clinical trial population studied by Rini et al. [3], and 52% of our patients were hypertensive at baseline. Still, the number of patients recorded as having sunitinib induced eHTN after cycle 1 and 2, using the same criteria, was nearly the same (~80%). eHTN at week 12 was associated with improved survival, but this is most likely due to the fact that the responders in the study stayed on treatment long enough to develop hypertension. Even if pharmacodynamically interesting, as eHTN occurs *after* sunitinib initiation it is not going to be an applicable predictive marker in the clinic.

Our finding that higher baseline HRQoL symptoms score is prognostic for PFS and OS in treatment with Sunitinib is in line with the earlier report by Cella et al. [23]. In that report, however, a different QoL tool was used. Herrmann et al. demonstrated by using EORTC QLQ-C30, that "global QoL" was prognostic for PFS [24]. Our study did not confirm this finding. In general, there were only small changes in HRQoL scores form baseline to 12 weeks. Herrmann et al. also showed a relatively small change in the different HRQoL scales after 12 weeks [24]. This could be due to the administration of Sunitinib (4 weeks on/ 2 weeks off), with subsequent remission of eventual treatment induced symptoms. There are indications that long-term survivors might retain a good HRQoL over years, as described by Carmichael et al. [25].

A major challenge in studies exploring predictive markers of treatment response in clinical data-sets is the fact that most of the candidate predictive markers are prognostic as well, thus significantly correlated with PFS and OS independent of the treatment given. Combined predictive and prognostic markers are best evaluated in two-arm trials. In single arm trials such as ours, response rates according to RECIST are superior to PFS and OS as primary end-point when assessing predictive markers of treatment response. Many biomarker studies in mRCC have been performed retrospectively in data-sets from large clinical trials, and these patients are frequently positively selected and do not optimally reflect the normal patient population. The strength of our study is the prospective design and the "Real-World" patient population enrolled, reflecting a normal clinical setting. When compared with large retrospective multicenter studies as well as smaller single-center studies, the majority of our patients were in the poor risk group according to IMDC criteria. Whereas the portion of poor-risk patients varies between 18 and 33% in comparable studies [8, 11, 15], 46% of our patients belonged to this group. In addition, all patients were in confirmed clinical and/or radiological progression at the time of inclusion. Accordingly, patients with very slow progression or stable metastases were observed without systemic treatment and screened for inclusion in the study only after confirmed disease progression. In comparison with clinical phase III trials, PFS and OS were lower in our patients. Compared with the adverse events reported in clinical trials [26], the frequency of toxicity from sunitinib in metastatic renal cell cancer recorded in our study was similar, or somewhat less frequent. Especially, the hematological toxicity including anemia, neutropenia, thrombocytopenia and lymphopenia was less frequent in our trial, although using the same criteria (CTCAE v. 3.0). The most likely explanation for this discrepancy is that we assessed adverse events, including laboratory, every 6 weeks, where most of the patients were off the drug in the 4 + 2 weeks cycle.

In addition to the lack of a control group, our study has some weaknesses. First, the number of patients included is low and thereby the study lacks the significant power to detect minor differences in response rates between groups based on the biomarkers under investigation. Thus, our finding should be validated in an independent and larger cohort of patients. Second, CRP and NLR are non-specific markers of inflammation and angiogenesis, and further studies are required to identify the key regulators controlling the systemic responses to metastatic disease. In this report, we focused on biomarkers available in standard clinical blood samples routinely used in the clinic. Further studies of candidate biomarkers in serum and plasma, such as IL6 and IL8 are ongoing.

Conclusion

In conclusion, in this prospective study of sunitinib in patients with mRCC we found that normal level of s-CRP at baseline is significantly associated with improved response rates and might serve as guidance in the selection of optimal treatment. Still, due to the relatively small sample size and low statistical power, our results need to be confirmed in larger studies.

Additional files

Additional file 1: Fig. S1. CONSORT 2010 Flow Diagram. (DOCX 36 kb)

Additional file 2: Table S1. Inclusion criteria. (DOCX 17 kb)

Additional file 3: Table S2. Correlations between CRP and other variables. (DOCX 15 kb)

Additional file 4: Table S3. Adverse effects. (DOCX 20 kb)

Additional file 5: Table S4. Summary of quality of life (QoL) scores. (DOCX 14 kb)

Abbreviations
IMDC: International Metastatic Renal Cell Carcinoma Database Consortium; MSKCC: Memorial Sloan Kettering Cancer Center

Funding
The study was supported by grants from The Norwegian Research Council, The Norwegian Cancer Society as well an unrestricted grant from Pfizer Norway. The authors alone are responsible for the content and writing of the paper. This work was partly supported by the Research Council of Norway through its Centres of Excellence funding scheme, project number 223250.

Availability of data and materials
All participants in the study signed an informed consent sheet providing the investigators permission to create a clinical dataset. According to the approval from the local ethical committee as well as from the hospital data protection board, the clinical dataset cannot be made publicly available.

Authors' contributions
MP, CB, LAA, OS: Conception and design, acquisition of data, analysis and interpretation of data, project funding, and were major contributors in writing the manuscript. LB performed the histological examination of the H&E stained tumor biopsies, and was a major contributor in writing the manuscript. ÅH, DH and KMH collected the clinical data including the QoL data, and were major contributors in writing the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate
The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. The protocol was approved by the Regional Ethics Committee (REK number 080/07) and the Norwegian Medicines Agency. All participating patients provided signed informed consent before enrolment.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

Author details
[1]Centre for Cancer Biomarkers CCBIO, University of Bergen, Bergen, Norway. [2]Department of Oncology, Haukeland University Hospital, 5021 Bergen, Norway. [3]Department of Urology, Haukeland University Hospital, 5021 Bergen, Norway. [4]Department of Pathology, Haukeland University Hospital, 5021 Bergen, Norway. [5]Department of Clinical Medicine, University of Bergen, Bergen, Norway. [6]Department of Oncology, Akershus University Hospital, 1478 Lørenskog, Norway.

Received: 3 January 2017 Accepted: 28 August 2017
Published online: 31 August 2017

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CONSORT 2010 Flow Diagram

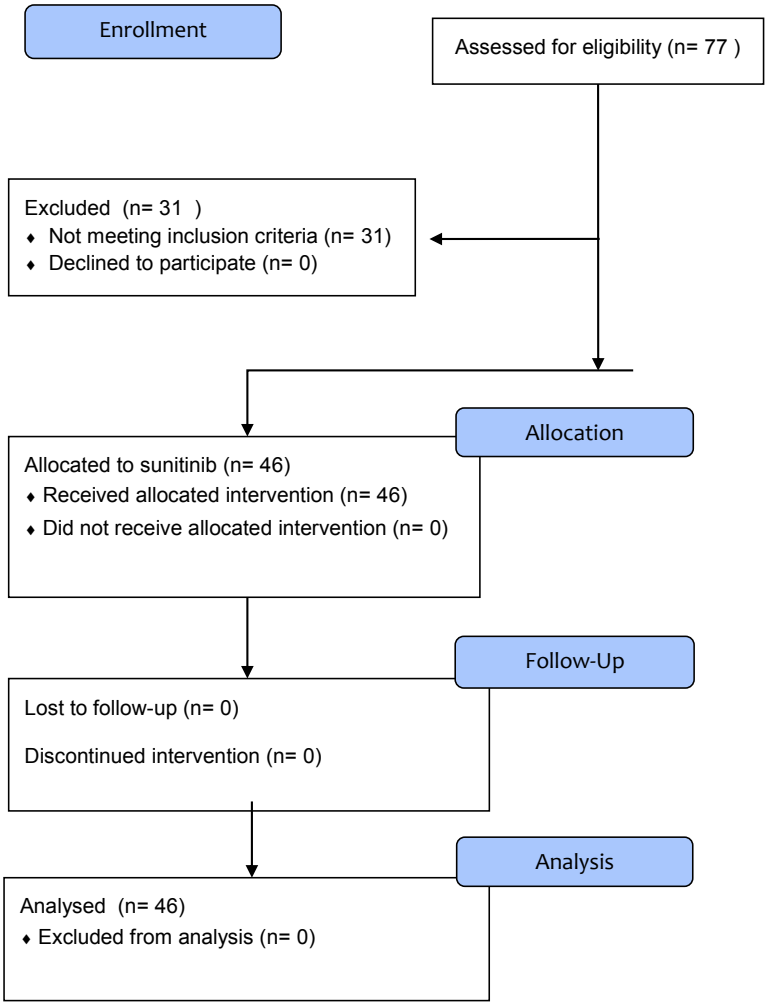

Figure S1. 77 patients with metastatic renal cell carcinoma were screened for inclusion in the prospective clinical study. 46 patients were enrolled after signing the informed consent sheet. 31 patients did not meet the inclusion criteria; not clear cell mRCC: n= 17, WHO performance state >2: n= 6, brain metastasis: n= 1, deep vein thrombosis: n= 1, no available biopsy: n= 2, impaired kidney function: n= 1, died during screening: n= 1, included in other study: n= 1.

Table S1 – Inclusion criteria

Metastatic or unresectable primary tumor of clear cell renal cell carcinoma

WHO performance status 0-2

Non-earlier cytokine treatment or other cancer treatment

Radiation therapy against symptom given metastasis was possible, but not against lesion that we would like to evaluate response

Non symptomatic brain metastasis, but radiation treated brain metastasis where corticosteroids is stopped is allowed

>12 months since coronar bypass operation

>18 years

Not pregnant or breast feeding

Clinically or radiographic measurable disease according to the RECIST

>21 days since major surgery or damage

>2 days since biopsy, FNAC or central venous catheter

No ongoing grade 3 bleeding

None of the following the last 12 months:

-Myocardial infarction

-Serious unstable angina pectoris

-Symptomatic heart failure (not including EF <50% or >20% reduction of EF compared to beginning of treatment)

-Stroke including TIA

-Pulmonary embolism

No other active malignant disease or not been treated for other cancers the last five years

Uncontrolled hypertension

Uncontrolled arrhythmia, especially prolonged QT-interval and bradycardias

Laboratory values of:

-Granulocytes >1,5 x 10^9/L

-Platelets >100 x 10^9/L

-Bilirubin and <1,5x upper normal limit

-ASAT <2,5x upper normal limit

-ALAT <2,5x upper normal limit

-Creatinine <1.5x upper normal limit

-International Normalized Ratio (INR) < 1,5x upper normal limit

No other compliance that could make the patient unsuitable to be included in a research protocol

Written consent from every patient

Abbrevations: WHO: World Health Organisation, RECIST: Response Evaluation Criteria in Solid Tumours, FNAC: Fine-Needle Aspiration Cytology, EF: Ejection Fraction, TIA: Transient Ischemic Attack, ASAT: aspartate aminotransferase, ALAT: alanine aminotransferase.

Table S2. Correlations between CRP and other variables

Variable	CRP at baseline	
	Corr. coeff	*p*-value[1]
Age	-0.38	0.01
IMDC risk score	0.61	<0.001
NLR baseline	0.19	0.24
Sum function score	-0.38	0.01
Sum symptom score	0.35	0.02
S-Platelets	0.45	0.002
S-Creatinine	-0.53	<0.001
S-Hemoglobin	-0.59	<0.001
S-Calcium	0.47	0.002
S-Albumin	-0.74	<0.001
Tumor load	0.35	0.02
	Median	*p*-value[2]
Sex		0.60
Male	17 mg/L	
Female	16 mg/L	
WHO performance status		0.008
0	9 mg/L	
1	78 mg/L	

[1]Spearman correlation. [2]Mann Whitney test. Abbreviations: IMDC: International Metastatic Renal Cell Carcinoma Database Consortium. NLR: Neutrophil/ Lymphocyte Ratio.

Table S3. Adverse effects

	Total	Grade 1+2	Grade 3+4
Laboratory			
Hemoglobin	22(50.0)	22 (47.8)	1 (2.2)
Lymphopenia	4 (8.7)	2 (4.3)	2 (4.3)
Neutropenia	11 (23.9)	10 (21.7)	1 (2.2)
Platelets	14 (30.4)	7 (15.2)	7 (15.2)
Cardiac			
Hypertension	13 (28.3)	4 (8.7)	9 (19.6)
Hypertension (prior to 12 weeks)	7 (15.2)	2 (4.3)	5 (10.9)
Left ventricular systolic dysfunction	2 (4.3)	1 (2.2)	1 (2.2)
Constitutional symptoms			
Fatigue	28 (60.9)	21 (45.7)	7 (15.2)
Fever	3 (6.5)	2 (4.3)	1 (2.2)
Weight loss	3 (6.5)	3 (6.5)	0 (0)
Dermatology/skin			
Desquamation	9 (19.6)	9 (19.6)	0 (0)
Hand-foot skin reaction	17 (37.0)	12 (26.1)	5 (10.9)
Hypopigmentation	7 (15.2)	7 (15.2)	0 (0)
Rash (other)	5 (10.9)	5 (10.9)	0 (0)
Endocrine			
Thyroid function (high)	2 (4.3)	2 (4.3)	0 (0)
Thyroid function (low)	9 (19.6)	9 (19.6)	0 (0)
Gastrointestinal			

Anorexia	8 (17.4)	8 (17.4)	0 (0)
Constipation	4 (8.7)	4 (8.7)	0 (0)
Dehydration	3 (6.5)	2 (4.3)	1 (2.2)
Diarrhea	23 (0.5)	18 (39.1)	5 (10.9)
Dry mouth	2 (4.3)	2 (4.3)	0 (0)
Gingiva	2 (4.3)	2 (4.3)	0 (0)
Heartburn	8 (17.4)	6 (13.0)	2 (4.3)
Mucositis (clinical exam)	12 (26.1)	11 (23.9)	1 (2.2)
Mucositis (symptomatic/functional)	10 (21.7)	10 (21.7)	0 (0)
Nausea	24 (52.2)	24 (52.2)	0 (0)
Taste alteration (dysgeusia)	12 (26.1)	12 (26.1)	0 (0)
Vomiting	6 (13.0)	5 (10.9)	1 (2.2)
Infection			
Infections	7 (15.2)*	5 (10.9)	1 (2.2)
Lymphatics			
Periorbital edema	3 (6.5)	3 (6.5)	0 (0)
Neurology			
Dizziness	2 (4.3)	2 (4.3)	0 (0)
Mood alteration, depression	2 (4.3)	2 (4.3)	0 (0)
Neuropathy, motor	3 (6.5)	0 (0)	3 (6.5)
Ocular/visual			
Watery eye	3 (6.5)	3 (6.5)	0 (0)
Pain			
Gastrointestinal pain	12 (26.1)	11 (23.9)	1(2.2)
General pain	3 (6.5)	3 (6.5)	0 (0)

Headache	3 (6.5)	3 (6.5)	0 (0)
Musculoskeletal pain	11 (23.9)	8 (17.4)	3 (6.5)
Pulmonary/upper respiration			
Dyspnea	2 (4.3)	1 (2.2)	1 (2.2)
Renal			
Cystitis	2 (4.3)	2 (4.3)	0 (0)

*One with Grade 5 (death due to appendicitis)

Table S4. The table shows the score (mean±SEM) of the global health status/ QoL, functional scales and symptom scales according to the EORTC QLQ-C30 scoring manual at baseline and after 12 weeks. The p-value is based on the non-parametric Wilcoxon signed rank sum test for the 28 patients evaluated at both time points.

	Baseline (n=45)	12 weeks (n=28)	p-value
Global health status / QoL	63±3	63±5	0.447
Functional sum score	75±3	77±4	0.219
Symptom sum score	20±3	22±4	0.166
Functional Scales			
Physical function	73±3	74±5	0.977
Role function	62±5	70±6	0.138
Emotional function	77±3	81±4	0.415
Cognitive function	89±2	88±3	0.064
Social function	72±4	72±5	0.565
Symptom Scales			
Fatigue	34±4	39±5	0.041*
Nausea / vomiting	9±3	9±3	0.468
Pain	32±5	32±5	0.705
Dyspnoea	20±4	19±5	0.565
Insomnia	22±4	24±6	0.554

Appetite loss	16±4	24±5	0.110
Constipation	19±4	23±7	0.233
Diarrhorea	15±3	21±5	0.364
Financial problems	10±3	12±5	1.000

II

The Journal of Pathology: Clinical Research
J Path: Clin Res 2018
Published online in Wiley Online Library
(wileyonlinelibrary.com). DOI: 10.1002/cjp2.96

ORIGINAL ARTICLE

Tumour cell expression of interleukin 6 receptor α is associated with response rates in patients treated with sunitinib for metastatic clear cell renal cell carcinoma

Martin Pilskog[1,2], Leif Bostad[3], Reidunn J Edelmann[1], Lars A Akslen[1,3], Christian Beisland[4,5] and Oddbjørn Straume[1,2]*

[1] *Centre for Cancer Biomarkers CCBIO, University of Bergen, Bergen, Norway*
[2] *Department of Oncology, Haukeland University Hospital, Bergen, Norway*
[3] *Department of Pathology, Haukeland University Hospital, Bergen, Norway*
[4] *Department of Urology, Haukeland University Hospital, Bergen, Norway*
[5] *Department of Clinical Medicine, University of Bergen, Bergen, Norway*

**Correspondence to: Oddbjørn Straume, Department of Oncology, Haukeland University Hospital, 5021 Bergen, Norway. E-mail: Oddbjorn.straume@helse-bergen.no*

Abstract

Clear cell renal cell carcinoma (ccRCC) is the most common type of renal cell carcinoma, and anti-angiogenic treatment is currently first line therapy for metastatic ccRCC (mccRCC). Response rates and duration of response show considerable variation, and adverse events have a major influence on patient quality of life. The need for predictive biomarkers to select responders to receptor tyrosine kinase inhibitors upfront is urgent. We investigated the predictive value of immunohistochemical biomarkers associated with angiogenesis and systemic inflammation in mccRCC. Forty-six patients with metastatic or non-resectable ccRCC treated with sunitinib were included. Metastatic and/or primary tumour tissue was stained by immunohistochemistry for selected markers related to angiogenesis [vascular endothelial growth factor A (VEGF-A), VEGF receptor 2 (VEGFR2), platelet-derived growth factor receptor β (PDGFRβ), and heat shock protein 27 (HSP27)] and immune responses [Interleukin 6 receptor α (IL6Rα), interleukin-6 (IL6), and jagged1 (JAG1)]. The predictive potential of the candidate markers was assessed by correlations with response rates (RECIST). In addition, progression free survival (PFS) and overall survival (OS) were analysed. Low tumour cell expression of IL6Rα was significantly associated with improved response to sunitinib (Fisher's exact test, $p = 0.03$), but not with PFS or OS. Median/high expression of IL6Rα showed significant association with median/high expression of VEGF-A and HSP27. Furthermore, low expression of IL6 was significantly associated with improved PFS, but not OS or response rates. High expression of IL6 was significantly associated with high expression of JAG1, VEGF-A, VEGFR2, and PDGFRβ. Loss of tumour cell expression of IL6Rα in mccRCC patients treated with sunitinib predicts improved treatment response, and might represent a candidate predictive marker.

Keywords: clear cell renal cell carcinoma; sunitinib; interleukin 6; interleukin 6 receptor α; response rates; biomarker; anti-angiogenesis; inflammation; immunohistochemistry; treatment efficacy

Received 13 November 2017; Revised 9 January 2018; Accepted 16 January 2018

Conflict of interest statement: M Pilskog has received consultation fees from Novartis and Pfizer. The other authors declare no conflicts of interest.

Introduction

Clear cell renal cell carcinoma (ccRCC) is known to be an immunogenic cancer [1]. Recently, nivolumab, an immune checkpoint inhibitor, was shown to improve overall survival (OS) in second line treatment of metastatic disease [2]. Immunotherapy, such as interferon and interleukin-2 therapy, was the only treatment choice up until 2007 when anti-angiogenic receptor tyrosine kinase inhibitors (rTKI) showed superior efficacy [3]. rTKIs are currently first line treatment options for metastatic disease. Still, response rates and duration of response show considerable variation among patients, and adverse events have a major influence on quality of life [4]. Despite scientific efforts to identify clinically useful

predictive markers of response to anti-angiogenic treatment, no such indicators have currently been successful. Among many, the focus has been on angiogenesis markers [5,6], markers of hypoxia [5–7], clinical markers [8–10], *VHL* mutation status [11], and single nucleotide polymorphisms [12,13], but immune response-related markers are less studied.

Vascular endothelial growth factor (VEGF) is the most important mediator of tumour-associated angiogenesis in renal cell carcinoma (RCC), and VEGF receptor 2 (VEGFR2) is the main target of sunitinib. Some reports suggest a role of systemic inflammation in development and progression of RCC [1,14,15]. Along with a stimulating effect on tumour-associated angiogenesis, VEGF also plays an important role in the local immune response during wound healing as well as in tumours by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, and VEGF inhibits the migration of T lymphocytes to the tumour [16].

In a recent study, we investigated the role of systemic inflammation in metastatic ccRCC (mccRCC) treated with sunitinib [4]. We found a significant correlation between low serum C-reactive protein (CRP) and objective response (OR). CRP is a relevant biomarker for systemic inflammation [17]. Tissue and serum levels of interleukin-6 (IL6) are elevated in RCC, and high levels of IL6 are associated with elevated CRP in RCC patients [18,19]. IL6 has a role in inflammation, infection responses, and the regulation of metabolic, regenerative, and neural processes [20–23]. In RCC, IL6 is secreted when cells are exposed to hypoxia, and enhanced levels of IL6 result in RCC cell invasion [23,24]. IL6 has also been shown to be closely related to HIF-1α as well as increased VEGF activity [25]. IL6 signals in cells via classic (membrane-bound) and trans-signalling (soluble) pathways [26,27]. Interleukin 6 receptor α (IL6Rα) binds to the gp130 protein receptor to transduce the signal. Membrane-bound IL6Rα is found on hepatocytes and different leukocytes [28]. In trans-signalling, soluble IL6 binds to soluble IL6R and the complex binds to cells expressing gp130 [29]. Takenawa *et al* have previously shown the presence of IL6Rα on RCC cells [18] and Costes *et al* reported a prognostic value of IL6 and IL6R in primary RCC [30].

Another important signalling system and regulator of tumour angiogenesis, stem cell self-renewal, epithelial cell polarity, cell division, and apoptosis is the Notch signalling pathway [31–34]. Thus, IL6 might trigger a potential autocrine or paracrine Notch-3/jagged1 (JAG1) loop to boost stem/progenitor self-renewal in the mammary gland [35].

Here, we enrolled patients with mccRCC treated with the VEGFR inhibitor sunitinib in a prospective clinical study, and analysed an expanded panel of candidate predictive biomarkers related to VEGF associated angiogenesis, inflammation, and tumour immune responses.

Materials and methods

Patients and treatment

Forty-six patients with mccRCC were enrolled in an open-label, single-arm phase II study at Haukeland University Hospital, Norway. Between 2007 and 2015, mccRCC patients with radiologically confirmed progressive disease were treated with sunitinib 50 mg/day on schedule 4 weeks on/two weeks off until disease progression, significant toxicity, or consent withdrawal. Study design, inclusion criteria, and clinical response data were reported earlier [4]. In summary, we observed 1 complete response (CR), 7 partial responses (PR), and 18 patients with stable disease (SD) ≥ 6 months. Twelve patients showed progressive disease (PD). Eight patients stopped treatment before week 12 and were recorded as non-evaluable for response rates and progression free survival (PFS). Thus, 38 patients were available for response evaluation. Treatment response was recorded according to RECIST 1.1 and the frequency of OR (CR + PR) was used as primary endpoint. The evaluation of the prognostic value of the biomarkers concerning PFS and OS were secondary endpoints. Clinical information is provided in Table 1.

Ethics

The study followed the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. The protocol was approved by the Regional Ethics Committee (REK number 080/07 and REK number 78/05) and the Norwegian Medicines Agency. All participating patients provided signed informed consent before enrolment.

Tissue samples

Tumour tissue was available in 45/46 (97.8%) patients in total. The most recent biopsy, the metastatic lesion ($n = 29$), or the non-resectable primary tumour diagnosed closest to the date of clinical trial inclusion ($n = 12$), was selected for further analysis if

Table 1. Baseline patients characteristics

	Study cohort ($n = 46$) *n* (%)
Sex	
Male	29 (63.0)
Female	17 (37.0)
Age, years	
Median	63.1
Range	41.1–84.0
IMDC* risk score	
Good	7 (15.2)
Intermediate	16 (34.8)
Poor	21 (45.7)
Missing	*2 (4.3)*
WHO** performance status	
0	30 (65.2)
1	16 (34.8)
2	0 (0.0)
Number of disease sites	
1	10 (21.7)
2	11 (23.9)
≥3	25 (54.3)

*International Metastatic Renal Cell Carcinoma Database Consortium.
**World Health Organisation.

several lesions were available. In addition, protein expression of the candidate markers was analysed in primary tumours alone ($n = 41$). All results in this paper refer to the most recent biopsy unless otherwise specified. All metastases and primary ccRCCs were reclassified by an experienced pathologist based on haematoxylin and eosin-stained sections (LB).

Immunohistochemistry (IHC)

Tissue sections (4–5 μm) were stained with primary antibodies for interleukin-6 receptor α (IL6Rα), IL6, JAG1, vascular endothelial growth factor A (VEGF-A), VEGFR2, platelet-derived growth factor receptor β (PDGFRβ), and heat shock protein 27 (HSP27). Slides were deparaffinized in xylene and rehydrated followed by antigen retrieval in a microwave oven. Endogenous peroxidase and alkaline phosphatase were blocked before incubation with the primary antibody followed by incubation with the appropriate visualization kit. Details are provided in supplementary material, Table S1. For negative controls, primary antibodies were omitted or specific blocking peptides for HSP27 and VEGF-A were used. Tissues from different cancer types were used as positive controls. For JAG1, endothelial cells were used as positive internal control.

Evaluation of tissue staining results

All sections were screened at ×40 and ×100 total magnifications to map areas of cancer tissue and normal tissue. Further, with high power magnification (×200 or ×400), staining intensity and the proportion of positive tumour cells were recorded using a semi-quantitative grading. Staining intensity was defined as absent (0), weak (1), moderate (2), or strong (3). The proportion was rated as 'no positive tumour cells'(0), 'less than 10% positive tumour cells'(1), '10–50% positive tumour cells'(2), or 'more than 50% positive tumour cells'(3). The staining index (SI) is the product of intensity and proportion (range 0–9) [36]. SI was used to quantify cytoplasmic staining of IL6Rα, IL6, JAG1, VEGF-A, VEGFR2, PDGFRβ, and HSP27. Cases were categorized into groups (absent/low versus median/high protein expression) based on the SI distribution for each biomarker under investigation. Thus, cut-points were set to: IL6Rα low (SI = 1–3) versus median/high (SI = 4–9); IL6 absent/low (SI = 0–2) versus median/high (SI = 3–9); JAG1 absent/low (SI = 0–2) versus median/high (SI = 3–9); VEGF-A low (SI = 1–3) versus median/high (SI = 4–9); VEGFR2 absent/low (SI = 0–2) versus median/high (SI = 3–9); PDGFRβ absent/low (SI = 0–1 versus median/high (SI = 2–9); and HSP27 low (SI = 1–3) versus median/high (SI = 4–9). In addition, protein expression in tumour-associated endothelial cells was graded based on staining intensity (0–3) for VEGFR2 and PDGFRβ. The IHC protein expression was evaluated and discussed by two observers blinded with temporary number tags for response data.

Statistical analyses

Comparisons between categorical variables were performed by Fisher's exact test. In the analyses of the IHC markers, we dichotomized the index score into absent/low versus median/high protein expression and tested the different groups against the frequency of OR in the patients. Logistic regression analysis was used to test the relative importance of predictive factors for sunitinib response. Sample size calculations (alpha 0.05/power 80%) indicated that 20 patients per group based on candidate marker expression were needed to detect a difference between 10 and 50% of patients having an OR to treatment with sunitinib. Thus, 46 patients were enrolled. Kaplan–Meier estimates were constructed for time-to-event endpoints such as PFS and OS, and log rank-test was applied for testing of differences between groups. Log-rank was applied for testing of differences between groups for PFS and OS. All *P* values are two-sided. Statistical investigations were performed using IBM SPSS Statistics version 24.

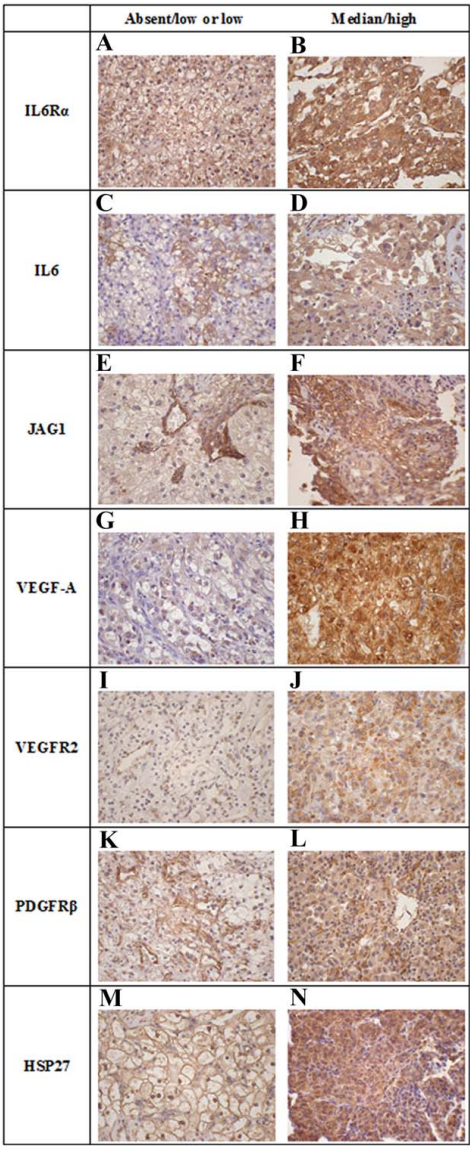

Figure 1. Expression of tumour biomarkers by IHC. Representative microscopic images for IL6Rα (A,B), IL6 (C,D), JAG1 (E,F), VEGF-A (G,H), VEGFR2 (I,J), PDGFRβ (K,L), and HSP27 (M,N) in tumour tissue. All pictures taken with x400 magnification; A, G, and M represent low tumour expression of the marker applied; C, E, G, I, K, and M represent absent/low tumour expression of the marker applied; and B, D, F, H, J, L, and N represent median/high tumour expression of the marker applied.

Results

Evaluation of IHC

IL6Rα

Thirty-eight of 41 (92.7%) cases had significant tumour tissue for quantification of IL6Rα. IL6Rα was expressed in the cytoplasm and membrane in 38/38 (100%) (median SI = 6) (Figure 1A, B).

Low expression of IL6Rα was significantly associated with OR (Fisher's exact test, $p = 0.03$) (Table 2). Sixty-six percent of the patients with response data available showed median/high expression of IL6Rα in tumour cells, and only 10% of these patients responded to treatment with sunitinib, whereas 46% of patients with low expression responded (Table 2) (Figure 2). Logistic regression analysis was used to test the relative importance of the candidate predictive factors [International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk groups, baseline CRP, baseline European Organization for Research and Treatment of Cancer Quality of Life (EORTC QoL) symptom scale and IL6Rα] for OR to sunitinib. Of these, IL6Rα was the only significant predictive factor of OR in the

Table 2. IHC biomarkers in relation to response

Variable	Best overall tumour response (RECIST ver. 1.1) OR* *n* (%)	SD** + PD† *n* (%)	*P* value††
IL6Rα			**0.03**
SI‡ = 1–3	5(46)	6(54)	
SI = 4–9	2(10)	19(90)	
IL6			0.39
SI = 0–2	5(31)	11(69)	
SI = 3–9	2(13)	13(87)	
JAG1			1.00
SI = 0–2	4(22)	14(78)	
SI = 3–9	3(23)	10(77)	
VEGF-A			1.00
SI = 1–3	2(25)	6(75)	
SI = 4–9	5(20)	20(80)	
VEGFR2			0.66
SI = 0–2	3(27)	8(73)	
SI = 3–9	4(18)	18(82)	
PDGFRβ			1.00
SI = 0–1	3(23)	10(77)	
SI = 2–9	4(25)	12(75)	
HSP27			0.38
SI = 1–3	4(33)	8(67)	
SI = 4–9	3(15)	17(85)	

*Objective response (complete + partial response).
**Stable disease.
†Progressive disease.
††Fisher's exact test.
‡Staining index. Statistically significant comparisons ($p < 0.05$) are shown in bold.

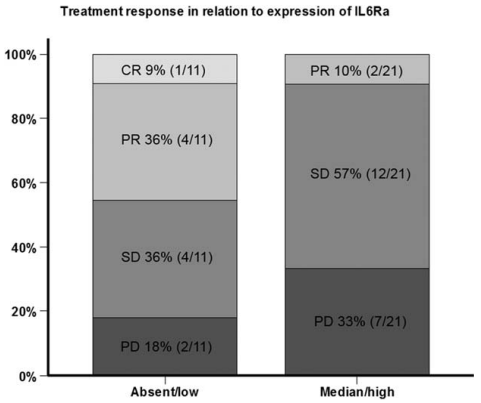

Figure 2. Treatment response in relation to expression of IL6Rα. Histogram showing the difference between absent/low and median/high expression of IL6Rα in tumour cells according to response. Ten percent of patients with median/high expression experienced an objective response to treatment with sunitinib compared with 46% of patients with absent/low expression.

final model, with an odds ratio of 7.9 ($p = 0.03$). There was no statistically significant association between IL6Rα and PFS or OS (Table 3). Median/high expression of IL6Rα was significantly associated with median/high expression of HSP27 (Fisher's exact test, $p = 0.01$) (Table 4). IL6Rα was not significantly associated with serum CRP (s-CRP) or lactate dehydrogenase (s-LDH).

Table 3. Survival analyses according to biomarker expression

	PFS*			OS**		
Variable	Median	95% CI†	*P* value††	Median	95% CI	*P* value
IL6Rα			0.21			0.22
SI‡ = 1–3	17.0	12.5–21.4		41.3	0.0–93.0	
SI = 4–9	8.7	5.8–11.6		13.7	11.0–16.5	
IL6			**0.04**			0.20
SI = 0–2	17.0	8.7–25.2		18.0	0.0–51.9	
SI = 3–9	8.7	6.8–10.6		11.6	8.8–14.4	
JAG1			0.80			0.63
SI = 0–2	12.9	6.3–19.6		13.7	11.2–16.2	
SI = 3–9	16.5	0.0–35.5		19.7	7.6–31.9	
VEGF-A			0.19			0.27
SI = 1–3	5.3	–		48.2	9.9–86.5	
SI = 4–9	12.9	6.4–19.5		13.2	8.7–17.7	
VEGFR2			0.09			0.45
SI = 0–2	17.0	6.5–27.5		15.6	8.8–22.4	
SI = 3–9	9.1	5.9–12.2		12.1	5.9–18.3	
PDGFRβ			0.72			0.29
SI = 0–1	10.8	3.7–17.9		13.7	11.0–16.5	
SI = 2–9	14.7	2.8–26.6		25.2	3.0–47.4	
HSP27			0.86			0.62
SI = 1–3	9.1	1.6–16.5		25.2	0.0–53.3	
SI = 4–9	12.9	4.0–21.9		13.9	11.1–16.7	

*Progression free survival.
**Overall survival.
†Confidence interval.
††Log rank test.
‡Staining index. Statistically significant comparisons ($p < 0.05$) are shown in bold.

Table 4. Analyses of patient characteristics and IHC biomarkers in relation to IL6Rα

	Interleukin-6 receptor α		
Variable	SI* = 0–3 *n*(%)	SI = 4–9 *n*(%)	*P* value**
Sex			0.20
Female	6(43)	8(57)	
Male	7(29)	17(71)	
Age			1.00
< median	7(35)	13(65)	
≥ median	6(33)	12(67)	
IMDC† risk			0.23
Good	4(67)	2(33)	
Intermediate	2(20)	8(80)	
Poor	5(33)	10(67)	
WHO†† performance status – No. (%)			1.00
0	9(36)	16(64)	
1	4(31)	9(69)	
2	–	–	
Number of disease sites – No. (%)			0.43
1	4(44)	5(56)	
2	3(50)	3(50)	
≥3	6(26)	17(74)	
IL6			0.09
SI = 0–2	9(75)	3(25)	
SI = 3–9	11(44)	14(56)	
JAG1			0.08
SI = 0–2	10(77)	3(23)	
SI = 3–9	10(44)	13(56)	
VEGF-A			0.18
SI = 1–3	4(33)	8(67)	
SI = 4–9	3(12)	22(88)	
VEGFR2			1.00
SI = 0–2	5(42)	7(58)	
SI = 3–9	9(36)	16(64)	
PDGFRβ			0.30
SI = 0–1	3(27)	8(73)	
SI = 2–9	11(48)	12(52)	
HSP27			**0.01**
SI = 1–3	8(67)	4(33)	
SI = 4–9	5(20)	20(80)	

*Staining index.
**Fisher's exact test.
†International Metastatic Renal Cell Carcinoma Database Consortium.
††World Health Organisation. Statistically significant comparisons ($p < 0.05$) are shown in bold.

IL6

Thirty-eight of 41 (92.7%) cases had sufficient tumour tissue for quantification of IL6. Cytoplasmic IL6 was expressed in 36/38 (94.7%) (median SI = 2) (Figure 1C, D). IL6 was not significantly associated with OR (Table 2). Absent/low expression of IL6 was significantly associated with improved PFS

Table 5. Analyses of patient characteristics and IHC biomarkers in relation to IL6

Variable	Interleukin 6 SI* = 0–2 n(%)	SI = 3–9 n(%)	P value**
Sex			0.20
Female	10(67)	5(33)	
Male	10(44)	13(56)	
Age			0.12
< median	8(40)	12(60)	
≥ median	12(67)	6(33)	
IMDC† risk			0.37
Good	5(83)	1(17)	
Intermediate	4(46)	6(54)	
Poor	9(60)	6(40)	
WHO†† performance status – No. (%)			1.00
0	13(54)	11(46)	
1	7(50)	7(50)	
2	–	–	
Number of disease sites - No. (%)			0.19
1	7(78)	2(22)	
2	4(57)	3(43)	
≥3	9(41)	13(59)	
IL6Rα			0.09
SI = 1–3	9(75)	3(25)	
SI = 4–9	11(44)	14(56)	
JAG1			**0.04**
SI = 0–2	14(74)	5(26)	
SI = 3–9	6(35)	11(65)	
VEGF-A			**0.02**
SI = 1–3	8(89)	1(11)	
SI = 4–9	11(39)	17(61)	
VEGFR2			**0.05**
SI = 0–2	11(73)	4(27)	
SI = 3–9	8(36)	14(64)	
PDGFRβ			0.17
SI = 0–1	10(71)	4(29)	
SI = 2–9	9(45)	11(55)	
HSP27			0.18
SI = 1–3	9(69)	4(31)	
SI = 4–9	11(44)	14(56)	

*Staining index.
**Fisher's exact test.
†International Metastatic Renal Cell Carcinoma Database Consortium.
††World Health Organisation. Statistically significant comparisons ($p < 0.05$) are shown in bold.

(median 17.0 versus 8.7 months, log rank $p = 0.04$) (Table 3). There was a significant association between absent/low expression of IL6 and absent/low expression of JAG1 (Fisher's exact test, $p = 0.04$), low expression of VEGF-A (Fisher's exact test, $p = 0.02$), and absent/low expression of VEGFR-2 (Fisher's exact test, $p = 0.05$) (Table 5). No statistically significant correlation was present between IL6 and IL6Rα (Table 4), s-CRP, or S-LDH.

JAG1

Thirty-eight of 41 (92.7%) cases had significant tumour tissue for quantification of JAG1. JAG1 was expressed in the cytoplasm and membrane in 28/38 (73.7%) (median SI = 2) (Figure 1E, F). There was no significant association between JAG1 expression and OR, PFS, or OS (Table 2 and 3). Absent/low expression of JAG1 was significantly associated with low expression of VEGF-A (Fisher's exact test, $p = 0.01$), whereas median/high expression of JAG1 tended to be associated with median/high expression of IL6Rα (Fisher's exact test, $p = 0.08$) (Table 5).

VEGF-A

Thirty-nine of 41 (95.1%) cases had significant tumour tissue for quantification of VEGF-A. VEGF-A was expressed in the cytoplasm of tumour cells in all patients (median SI = 6) (Figure 1G, H). The expression of VEGF-A was not significantly associated with OR, PFS, or OS (Tables 2 and 3).

VEGFR2

Thirty-nine of 41 (95.1%) cases had significant tumour tissue for quantification of VEGFR2. VEGFR2 was expressed in the cytoplasm and membrane in 31/39 (79.5%) (median SI = 3) (Figure 1I, J). No significant association between VEGFR2 expression and OR was present (Table 2). Cytoplasmic VEGFR2 expression was not significantly associated with OS or PFS (Table 3).

PDGFRβ

Thirty-four of 41 (82.9%) cases had significant tumour tissue for quantification of PDGFRβ. PDGFRβ was expressed in the cytoplasm and membrane in 21/34 (61.8%) (median SI = 2) (Figure 1K, L). PDGFRβ expression was not significantly associated with OR, PFS or OS (Tables 2 and 3).

Hsp27

Thirty-nine of 41 (95.1%) cases had significant tumour tissue for quantification of HSP27. HSP27 was expressed in the cytoplasm of tumour cells in all patients (median SI = 6) (Figure 1M, N). HSP27 expression was not significantly associated with OR, OS, or PFS (Tables 2 and 3).

Discussion

Ever since rTKI was introduced as first line treatment for mRCC, an intensive search for predictive markers has been performed to optimize treatment. In a recent paper, we found evidence for a predictive role for CRP in sunitinib treatment of RCC suggesting a potential role for markers of anti-tumour immune responses [4]. Lymphocytic infiltration in RCC has

been shown to be associated with poor survival [37] and IL6 might be used as a surrogate marker of host immunity in patients with RCC [38]. Tissue and serum levels of IL6 are elevated in RCC, and high levels of IL6 have been associated with elevated CRP in RCC patients [18,19]. Dysregulation of the cytokine IL6 and its receptor is involved in the pathogenesis of several diseases, such as autoimmune conditions and cancer [39]. In classic signalling, IL6 binds to the complex of IL6Rα and gp130 to induce intra-cellular signals [29,40]. Almost all cells in the body express gp130 [28], but only some have the IL6Rα subunit to bind IL6 [39].

Here, we investigated the expression of IL6Rα in RCC tumour cells and found that expression of IL6Rα may predict response to rTKI treatment. In our study, IL6Rα was expressed in all cases, and low expression of IL6Rα was significantly associated with OR to sunitinib treatment. Only 10% of patients showing increased expression of IL6Rα responded, suggesting that high IL6Rα expression might represent an important mechanism of resistance to anti VEGF therapy in ccRCC. The strong association between IL6Rα expression and treatment response, as well as the lack of a significant association with PFS and OS, suggest that IL6Rα expression adds more predictive than prognostic information in patients with mccRCC treated with sunitinib. Costes *et al* found a significant association between IL6R expression and OS in patients with primary RCC tumours. In their study, all patients underwent nephrectomy and six out of 38 patients had metastatic disease [30]. Eighteen percent of the 38 patients and 83% of the 6 patients with synchronous metastatic disease showed positive expression of IL6Rα. In our cohort of patients with primary inoperable or metastatic disease, 100% of the patients showed positive IL6Rα expression.

Regarding the IL6 ligand, absent or low expression was associated with disease outcome in the survival analyses of PFS. The group with low expression of IL6 had almost a doubling of PFS compared to median/high expression. In line with previous reports [18,19], this suggests that IL6 expression has prognostic value independent of the treatment given.

Elevated serum IL6 has been associated with poor survival in RCC [19,30,41]. Tumour cells produce IL6 in response to cellular stress such as hypoxia, and enhanced levels of IL6 are associated with increased tumour cell invasion [23,24]. Kwon *et al* found elevated IL6 to have a stimulating effect on endothelial cells, and this may be a reason for resistance to anti-VEGF therapy [42]. As a response to cellular stress, IL6 activation of the transcription factor STAT3 drives angiogenesis by inducing expression of VEGF and fibroblast growth factor by tumour cells, and thereby supports vascularization required for tumour growth and metastasis [43,44]. Fu *et al* found that IL6 and IL6Rα co-expression might be an independent early-stage immunological prognostic factor for patients with organ-confined ccRCC [45]. Tran *et al* showed a significant increase in PFS in patients treated with another rTKI (pazopanib) versus placebo, when analysing patients with high serum IL6 [46]. Our results are in support of previous reports indicating that high levels of inflammation-associated cytokines are detrimental for the outcome of sunitinib treatment [21].

When correlating IL6Rα to the other biomarkers under investigation, we found that median/high expression of IL6Rα was significantly associated with median/high expression of HSP27. Both IL6 and HSP27 signalling constitute cellular stress responses and increase the level of VEGF through activation of STAT3 [44,47]. Schuster *et al* found that high HSP27 expression in melanoma metastases predicts response to anti-VEGF treatment [48]. In the present study, we did not find an association between HSP27 expression and treatment response. Blay *et al* previously showed that a higher IL6 level correlated with increased concentration of CRP [49]. In our study, IL6 was not significantly associated with CRP.

JAG1 is one of five Notch ligands. The Notch signalling pathway is a regulator of tumour angiogenesis, stem cell self-renewal, cell fate determination, epithelial cell polarity/adhesion, cell division, and apoptosis [31–34]. In mccRCC, high JAG1 was associated with poor prognosis [50]. In aggressive breast cancer cells, Sansone *et al* found that IL6 could stimulate Notch-3-dependent upregulation of JAG1 in an autocrine matter in response to hypoxic conditions [35]. In our present cohort, the expression of JAG1 was not related to OR, but absent/low expression of IL6 was significantly associated to absent/low expression of JAG1. These JAG1 results may support a possible interaction of JAG1, Notch, and IL6 [35].

Moreover, absent/low expression of IL6 was shown to be significantly associated with low expression of VEGF-A and absent/low expression of VEGFR2, further supporting an important role of IL6 signalling the regulation of angiogenesis in mRCC.

Along with a stimulating effect on tumour-associated angiogenesis, VEGF-A also plays an important role in the local immune response during wound healing as well as in tumours by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, and inhibiting the migration of T lymphocytes to the tumour [16]. Whereas VEGF-A was expressed in all

tumours, we found no association between the level of expression and response, in line with other studies [5,6]. VEGF-A signals through VEGFR2 on endothelial cells to activate angiogenesis [51]. Dornbush *et al* found an association between high expression of VEGFR2 and good treatment response [6], and Terakawa *et al* also found high expression of VEGFR2 to be beneficial to sunitinib treatment [52]. In the last paper, the majority of patients were in the good prognostic group whereas in ours, the majority were in the poor prognostic group [4].

PDGF receptors are key regulators of mesenchymal cells of the tumour microenvironment in several malignancies [53]. In cancers, an association between high stromal PDGFRβ expression or signalling and poor prognosis is reported [53]. Still, we did not find an association with response in our present data.

In addition to the lack of a control group, our study has some weaknesses. First, the number of patients included is low and thereby the study lacks the statistical power to detect minor differences in response rates between groups based on the biomarkers under investigation. Thus, our findings should be validated in an independent and larger cohort of patients.

Second, the reproducibility of the quantification of protein expression used in this study also needs to be validated in a separate patient cohort. Still, our data suggest that both angiogenesis and tumour immune responses play important roles in anti-VEGF therapy.

Whereas expression levels of the IL6 ligand in tumour cells provided significant prognostic information, reduced expression of its receptor IL6Rα was significantly associated with response to sunitinib, thereby suggesting that upregulation of IL6Rα might represent an important mechanism of resistance. Expression of IL6Rα might be a potential predictive biomarker to guide treatment of patients with mccRCC.

Acknowledgements

The study was supported by grants from The Norwegian Research Council and The Norwegian Cancer Society. The authors alone are responsible for the content and writing of the paper. This work was partly supported by the Research Council of Norway through its Centres of Excellence funding scheme, project number 223250.

Author contributions statement

MP conceived, carried out experiments, evaluated the experiments, and analysed data. LB investigated the material upon experiments. RJE contributed with ideas. LAA contributed in writing the paper. CB contributed in writing the paper. OS conceived, evaluated the experiments, and analysed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL ONLINE

Table S1. Details of antibodies and protocols used for immunohistochemistry

Table S1. Details of antibodies and protocols used for immunohistochemistry

Primary antibody	Comment	Product code	Source	Dilution	Incubation time	Antigen retrieval	Peroxidase block	Extra protein block	Diluent	Secondary antibody	Visualisation	Chromogen/ Counterstaining	Method
HSP27	C20 (goat polyclonal IgG)	sc-1048	Santa Cruz Biotechnology; Santa Cruz, USA	1/1600	20 min	Dako S1699, pH 6	Dako K4011	Dako X0909	Dako S3022 w/background	Rabbit anti-goat (6164-01)	EV + sHRP/ anti-rab (polyc. abs) Dako K4011	DAB/ hemotoxylin	Autostainer
PDGFRβ	2B3 (mouse mAb)	3175S	Cell Signaling Technology, Inc.; Danvers, USA	1/100	60 min	Dako S1699, pH 6	Dako K4007	–	Dako S3022 w/background	–	EV + sHRP/ anti-mouse (monokl. abs) Dako K4007	DAB/ hemotoxylin	Autostainer
VEGF-A	sc-152 (rabbit polyclonal IgG)	sc-152	Santa Cruz Biotechnology; Santa Cruz, USA	1/50	60 min	Dako S1699, pH 6	Dako K4011	–	Dako S3022 w/background	–	EV + sHRP/ anti-rab (polyc. abs) Dako K4011	DAB/ hemotoxylin	Autostainer
VEGFR2	FLK-3 (mouse monoclonal IgG)	sc-6251	Santa Cruz Biotechnology; Santa Cruz, USA	1/100	60 min	Dako S1699, pH 6	Dako K4007	–	Dako S3022 w/background	–	EV + sHRP/ anti-mouse (monokl. abs) Dako K4007	DAB/ hemotoxylin	Autostainer
IL6	Mouse monoclonal	Ab9324	Abcam	1/600	30 min	Dako S1699, pH 6	Dako K4007	–	Dako S3022 w/background	–	EV + sHRP/ anti-mouse (monokl. abs) Dako K4007	DAB/ hemotoxylin	Autostainer
IL6Rα	Rabbit polyclonal	Ab128008	Abcam	1/800	Over night	Dako S1699, pH 6	Dako K4011	–	Dako S3022 w/background	–	EV + sHRP/ anti-rab (polyc. abs) Dako K4011	DAB/ hemotoxylin	Manually
JAGGED 1	Goat polyclonal	Sc-6011	Santa Cruz	1/100	60 min	Dako S1699, pH 6	Dako K4011	–	Dako S3022 w/background	Rabbit anti-goat (6164-01)	EV + sHRP/ anti-rab (polyc. abs) Dako K4011	DAB/ hemotoxylin	Manually

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Cancer Treatment and Research Communications 19 (2019) 100127

Contents lists available at ScienceDirect

Cancer Treatment and Research Communications

journal homepage: www.elsevier.com/locate/ctarc

Elevated plasma interleukin 6 predicts poor response in patients treated with sunitinib for metastatic clear cell renal cell carcinoma

Martin Pilskog[a,b], Gry Hilde Nilsen[d], Christian Beisland[c,d], Oddbjørn Straume[a,b,*]

[a] Centre for Cancer Biomarkers, University of Bergen, Norway
[b] Department of Oncology, Haukeland University Hospital, Bergen, Norway
[c] Department of Urology, Haukeland University Hospital, Bergen, Norway
[d] Department of Clinical Medicine, University of Bergen, Norway

ARTICLE INFO

Keywords:
Biomarker
Inflammation
ELISA
Kidney cancer
Treatment
Targeted therapy

ABSTRACT

Introduction: Clear cell renal cell carcinoma (ccRCC) is the most common type among renal cell carcinomas, and anti-angiogenic treatment is currently first line therapy in metastatic ccRCC (mccRCC). Response rates and duration of response show considerable variation, and adverse events have major influence on patient's quality of life. The need for predictive biomarkers to select those patients most likely to respond to receptor tyrosine kinase inhibitors (rTKI) upfront is urgent. We investigated the predictive value of plasma interleukin-6 (pIL6), interleukin-6 receptor α (pIL6Rα) and interleukin 6 signal transducer (pIL6ST) in mccRCC patients treated with sunitinib.

Material and methods: Forty-six patients with metastatic or non-resectable ccRCC treated with sunitinib were included. Full blood samples were collected at baseline before start of sunitinib and after every second cycle of treatment during the study time. pIL6, pIL6R and pIL6ST at baseline and week 12 samples were analysed by ELISA. The predictive potential of the candidate markers was assessed by correlation with response rates (RECIST). In addition, progression free survival (PFS) and overall survival (OS) were analysed.

Results: Low pIL6 at baseline was significantly associated with improved response to sunitinib (Fisher's exact test, $p < 0.01$). Furthermore, low pIL6 at baseline was significantly associated with improved PFS (log rank, $p = 0.04$). In addition, patients with a decrease in concentration of pIL6R between baseline and week 12 showed significantly improved PFS (log rank, $p = 0.04$) and patients with high pIL6ST at baseline showed significantly improved OS (log rank, $p = 0.03$).

Conclusion: Low pIL6 at baseline in mccRCC patients treated with sunitinib predicts improved treatment response, and might represent a candidate predictive marker.

Introduction

Renal cell carcinoma (RCC) is the 7th most common cancer type among men and 10th most common among women worldwide [1]. 70–85% of RCC are clear cell RCC (ccRCC). After anti-angiogenic receptor tyrosine kinase inhibitors (rTKIs) showed superior efficacy over interferon and interleukin-2 therapy, currently rTKIs are first line treatment option for ccRCC [2]. Due to the diversity of treatment response and toxicity among patients, the research community investigates potential predictive markers of response to antiangiogenic treatment.

Vascular endothelial growth factor (VEGF) is the most important mediator of tumour-associated angiogenesis in renal cell carcinoma [2]. In addition, some reports suggest a role of systemic inflammation in development and progression of RCC [3–5]. Along with a stimulating effect on tumour associated angiogenesis, VEGF also plays an important role in the local immune response during wound healing as well as in tumours by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, and VEGF inhibits the migration of T lymphocytes to the tumour [6].

Inflammation is one of the hallmarks of cancer, involved in development and maintenance of cancer [7]. In a recent study, we found a significant correlation between low serum C-reactive protein (CRP) and objective response (OR) in mccRCC patients treated with sunitinib [8]. CRP is regarded a relevant biomarker for systemic inflammation [9]. Interleukin-6 (IL6) has a role in inflammation, infection responses and the regulation of metabolic, regenerative and neural processes [10–13]. Tissue and serum levels of IL6 are elevated in RCC and secreted when

* Corresponding author.
E-mail address: Oddbjorn.straume@helse-bergen.no (O. Straume).

https://doi.org/10.1016/j.ctarc.2019.100127

cells are exposed to hypoxia. Enhanced level of IL6 results in RCC cell invasion [10,14]. IL6 is also shown to be closely related to hypoxia-inducible factor 1- α (HIF-1α) as well as increased VEGF activity [15]. The prognostic information of IL6 and IL6-receptor (IL6R) is well known [10,16]. A predictive value of IL6 levels has also been reported for response to immune checkpoint inhibitors [17]. IL6 signals in cells via classic (membrane-bound) and trans-signalling (soluble) pathways [18,19]. The interleukin 6 receptor α (IL6Rα) binds to the interleukin 6 signal transducer (IL6ST), also known as glycoprotein 130 (gp130) protein receptor to transduce the signal. In a recent study, we identified ccRCC tumour cell expression of IL6Rα as a predictive marker of response to sunitinib treatment [20]. The membrane-bound IL6Rα is found on hepatocytes and different leukocytes [21]. In trans-signalling, soluble IL6 binds to soluble IL6R and the complex binds to cells expressing IL6ST [22]. Soluble IL6ST is also detected in the blood and has been shown as an inhibitor of IL6 trans-signalling [23].

In the present work, we investigated the predictive and prognostic value of plasma levels of IL6, IL6R and IL6ST in mccRCC treated with sunitinib.

Material and methods

Patients and treatment

Between 2007 and 2015, forty-six patients with radiologically confirmed progressive mccRCC were enrolled in an open-label, single-arm phase II study at Haukeland University Hospital, Norway. Treatment was given as sunitinib 50 mg/day on schedule four weeks on/ two weeks off until disease progression, significant toxicity or consent withdrawal. The study has previously been reported elsewhere [8,24]. In summary, we observed 1 complete response (CR), 7 partial responses (PR) and 18 patients with stable disease (SD) ≥ 6 months. Twelve patients showed progressive disease (PD). Eight patients stopped treatment before week 12 and were recorded as non-evaluable for response rates and PFS. In response analyses objective response (OR) is CR and PR together versus SD and PD and clinical benefit (CB) is CR, PR and SD together versus PD. Clinical information is provided in Table 1.

Ethics

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. The protocol was approved by the Regional Ethics Committee (REK number 080/07 and REK number 78/05) and the Norwegian Medicines Agency. All participating patients provided signed informed consent before enrolment.

Table 1
Baseline patients characteristics.

	Study cohort (n = 46)
Sex - no. (%)	
Male	29 (63.0)
Female	17 (37.0)
Age, years	
Median	63.1
Range	41.1–84.0
IMDC[a] risk score – No. (%)	
Good	7 (15.2)
Intermediate	16 (34.8)
Poor	21 (45.7)
Missing	2 (4.3)
WHO[b] performance status - No. (%)	
0	30 (65.2)
1	16 (34.8)
Number of disease sites - No. (%)	
1	10 (21.7)
2	11 (23.9)
≥3	25 (54.3)

[a] International Metastatic Renal Cell Carcinoma Database Consortium.
[b] World Health Organization.

Blood samples

Full blood samples were collected at baseline before start of sunitinib and after every second cycle of treatment during the study time. After centrifugation, Na-heparin plasma samples were stored frozen at −80 °C. For ELISA we used the heparin plasma sample tubes, which was de-frozen in room temperature, shaken and then centrifuged for different amount of fibrin precipitation.

Enzyme-linked immunosorbent assay (ELISA)

The antibodies used were human IL6 (P05231), human IL6R (BMS214) and human IL6ST (EHIL6ST). Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA provided all three. For IL6 we used a ready-to-use self-coating system kit (Invitrogen) and an ELISA 96-well flat-bottom plate (Nune MaxiSorp flat-bottom (catalog number 44–2404), Invitrogen). For IL6R we used a ready-to-use sandwich ELISA 96 micro well plate coated kit with human IL6R (Invitrogen). For IL6ST we used a ready-to-use self-coating system kit (Invitrogen). Phosphate buffered saline (PBS); containing 0.05% (v/v) Tween 20 (PBS-T) (Prod.nr. 822,184, Merck, USA) was used as washing buffer. All other buffers used were from the respective ELISA kit. The staining process was performed according to the manufactures manual and was analysed at 450 nm with a microplate reader (Molecular Devices Emax).

Evaluation of ELISA results

We evaluated the ELISA results in according to the manufactures manual. SoftMax Pro was used to evaluate the ELISA data and then transferred to SPSS for statistical analysis. We categorized the baseline ELISA variables (pIL6, pIL6R, pIL6ST), into low (below median) versus high (above median). The change in pIL6, pIL6R and pIL6ST concentration between baseline and week 12 were divided into three categories (decrease, stable, increase). We tested the decrease group versus the stable and increase groups. The variables referred in the paper are baseline values if not otherwise specified.

Tumour tissue samples and data

Immunohistochemically tumour tissue expression of interleukin-6 receptor α (IL6Rα), interleukin-6 (IL6), jagged1 (JAG1), vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor 2 (VEGFR2), platelet-derived growth factor receptor β (PDGFRβ) and heat shock protein 27 (HSP27) are previously published [24].

Statistical analyses

Comparisons between categorical variables were performed by using the Fisher's exact test and Pearson chi-square. The Mann–Whitney U test was used to compare the distribution of continuous variables between two groups such as responders and non-responders. Logistic regression analysis was used to test the relative importance of predictive factors for sunitinib response. Sample size calculations (alpha 0.05/ power 80%) indicated that 20 patients per group based on candidate marker expression were needed to detect a difference between 10% and 50% of patients having a response to treatment with sunitinib. Kaplan–Meier estimates were constructed for time-to-event endpoints such as PFS and OS, and log rank-test was applied for testing of differences between groups. All p-values are two-sided. Statistical investigations were done using IBM SPSS Statistics version 24.

Table 2
Plasma biomarkers in relation to response.

Variable	Best overall tumor response (RECIST ver. 1.1)		
	CB[a]	PD[b]	*p* value[c]
	n(%)	*n*(%)	
pIL6[d] baseline			**<0.01**
Low	17(94)	1(6)	
High	8(42)	11(58)	
pIL6R[e] baseline			0.73
Low	15(71)	6(29)	
High	11(65)	6(35)	
pIL6ST[f] baseline			0.48
Low	10(63)	6(38)	
High	16(76)	5(24)	
Change in pIL6 between baseline and week 12			0.54
Decrease	5(71)	28(29)	
Stable	9(90)	1(10)	
Increase	5(71)	2(29)	
Change in pIL6R between baseline and week 12			0.53
Decrease	8(89)	1(11)	
Stable	7(78)	2(22)	
Increase	6(67)	3(33)	
Change in pIL6ST between baseline and week 12			0.26
Decrease	6(86)	1(14)	
Stable	9(75)	3(25)	
Increase	2(40)	3(60)	
	CB	PD	*p* value[g]
pIL6 baseline			**<0.01**
Mean value	6.13	14.82	
pIL6R baseline			0.59
Mean value	189.30	183.47	
pIL6ST baseline			0.21
Mean value	179.43	158.63	

[a] Clinical benefit (Complete + Partial response + Stable disease).
[b] Progressive disease.
[c] Fisher's exact test.
[d] Plasma Interleukin 6.
[e] Plasma Interleukin 6 receptor.
[f] Plasma Interleukin 6 signal transducer.
[g] Mann-Withney U test.

Results

pIL6

Forty-five of 46 (98%) cases had heparin plasma available for quantification of pIL6 at baseline. Median value was 6.90 pg/ml (range 0.9–36.5 pg/ml). Twenty-six of 46 (56%) cases had heparin plasma available for quantification of pIL6 at week 12. Median value was 8.90 pg/ml (range 1.0–18.8 pg/ml). Low baseline pIL6 was significantly associated with clinical benefit (CB) (Fisher's exact test, $p = <0.01$) (Table 2). Similarly, the continuous values of pIL6 was significantly associated with CB (Mann–Whitney *U* test, $p = <0.01$) (Table 2). Logistic regression analysis was used to test the relative importance of the following candidate predictive factors for clinical benefit to sunitinib; International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk groups, baseline CRP, baseline European Organization for Research and Treatment of Cancer Quality of Life (EORTC QoL) symptom scale and pIL6. Of these, pIL6 was the only significant predictive factor for CB in the final model, with an odds ratio of 23.4 ($p = <0.01$). Low pIL6 was significantly associated with improved PFS (median 14.7 months vs 5.3 months, log rank, $p = 0.04$) (Table 3). Low pIL6 was not significantly associated with OS (Tables 2 and 3).

Low pIL6 was significantly associated with normal CRP (Pearson chi-square, $p = 0.05$), but not with tumour tissue expression of IL6 or IL6Rα (Supplementary Table 1).

pIL6R

Forty-six of 46 (100.0%) cases had significant heparin plasma for quantification of pIL6R at baseline. Median value was 190.40 ng/ml (range 115.0–288.9 ng/ml). Twenty-eight of 46 (60.9%) cases had significant heparin plasma for quantification of pIL6R at week 12. Median value was 164.13 ng/ml (range 94.7–248.4 ng/ml). pIL6R was not significantly associated with CB (Table 2). Low pIL6R tended to be associated with improved OS (median 26.3 months vs 13.7 months, log rank, $p = 0.06$) and not with PFS (Table 3).

Low pIL6R at baseline was significantly associated with age under median (Pearson chi-square, $p = 0.04$)). pIL6R was not significantly associated with s-CRP or tumour tissue expression of IL6 or IL6Rα (Supplementary Table 2).

pIL6ST

Forty-five of 46 (98.0%) cases had significant heparin plasma for quantification of pIL6ST at baseline. Median value was 170.94 ng/ml (range 102.2–260.84 ng/ml). Twenty-nine of 46 (63.0%) cases had significant heparin plasma for quantification of pIL6ST at week 12. Median value was 161.84 ng/ml (range 120.58–291.84 ng/ml). pIL6ST was not significantly associated with CB (Table 2). High pIL6ST was significantly associated with improved OS (median 25.2 months vs 12.7 months, log rank, $p = 0.04$) and tended to be associated with improved PFS (median 12.9 months vs 8.4 months, log rank, $p = 0.06$) (Table 3). pIL6ST was not significantly associated with s-CRP or tumour tissue expression of IL6 or IL6Rα (Supplementary Table 3).

Change in candidate biomarkers between baseline and week 12

Twenty-five of 46 (54.3%) cases had significant heparin plasma for quantification of pIL6 from baseline and week 12. Median value of the change in pIL6 between baseline and week 12 was 1.99 pg/ml (range: −13.4 to +11.9 pg/ml). The change in pIL6 was not significantly associated with PFS or OS (Table 3).

Twenty-eight of 46 (60.9%) cases had significant heparin plasma for quantification of change in pIL6R between baseline and week 12. Median value of the change in pIL6R between baseline and week 12 was −24.63 ng/ml (range −63.8 to 17.5 ng/ml). The cases with decrease had significantly better PFS than cases with stable or increased change of pIL6R between baseline and week 12 (median missing vs 8.7 months, log rank, $p = 0.04$). We found no association with OS (Table 3).

Twenty-nine of 46 (63.0%) cases had significant heparin plasma for quantification of change in pIL6ST between baseline and week 12. Median value of the change in pIL6ST between baseline and week 12 was −2.40 ng/ml (range −108.68 to 137.87 ng/ml). We found no association with PFS or OS (Table 3).

Discussion

Recently, an inverse response relationship was reported for VEGF inhibitor (VEGFi) treatment and immune checkpoint inhibitor treatment according to IMDC risk groups [25], and new predictive biomarkers are needed to further optimize treatment for individual patients. The ongoing search for biomarkers to optimize VEGFi treatment in renal cell carcinomas has so far been unsuccessful in finding predictive biomarkers useful for clinical practice. In a previous paper, we presented results suggesting a predictive role of CRP [8]. This might indicate that the immunomodulating effect of anti VEGF therapy plays an important role in treatment response in addition to the effect on angiogenesis. In the follow-up investigation, we found that low tumour cell expression of IL6Rα was significantly associated with improved objective response to sunitinib and low tumour cell expression of IL6 was significantly associated with PFS and OS [24]. In our present work, we investigated the plasma level of baseline IL6, IL6R and IL6ST in the

Table 3
Survival analyses according to pIL6, pIL6R and pIL6ST.

Variable	PFS[a] Median	95% CI[c]	*p*-value[d]	OS[b] Median	95% CI	*p*-value
pIL6[e] baseline			**0.04**			0.11
Low	14.7	1.9–27.6		25.2	15.6–34.8	
High	5.3	1.8–8.8		8.3	3.0–13.6	
pIL6R[f] baseline			0.12			0.06
Low	14.7	1.6–27.8		26.3	4.7–47.9	
High	8.7	7.6–9.8		13.7	11.0–16.5	
pIL6ST[g] baseline			0.06			**0.04**
Low	8.4	3.0–13.8		12.7	8.3–17.2	
High	12.9	5.9–20.0		25.2	15.7–34.7	
Change in pIL6 between baseline and week 12			**0.03**			0.63
Decrease	8.4	0.5–16.2		25.2	9.5–40.9	
Stable/Increase	17.0	9.0–25.0		19.7	03–39.2	
Change in pIL6R between baseline and week 12			**0.04**			0.40
Decrease	–[h]	–[h]		26.0	24.3–27.6	
Stable/Increase	8.7	7.4–9.9		17.5	7.2–27.7	
Change in pIL6ST between baseline and week 12			0.30			0.95
Decrease	16.5	1.9–32.2		19.7	9.0–30.5	
Stable/Increase	8.4	6.0–10.7		13.9	9.3–18.5	

[a] Progression free survival.
[b] Overall survival.
[c] Confidence interval.
[d] Log rank test.
[e] Plasma interleukin 6.
[f] Plasma interleukin 6 receptor.
[g] Plasma interleukin 6 signal transducer.
[h] Median survival cannot be calculated, due to less than 50% censored.

same cohort with established metastatic ccRCC. Low baseline level of pIL6 was significantly associated with clinical benefit of sunitinib treatment and improved PFS.

Tissue and serum levels of IL6 are elevated in RCC, and high levels of IL6 have been associated with elevated CRP in RCC patients [26,27]. IL6 signals cells via membrane-bound (classic) and soluble (trans-signalling) pathways [18,19]. The trans-signalling pathway is considered to be pro-inflammatory [28]. Elevated IL6 has been associated with poor survival in renal cell carcinoma and resistance to TKI treatment [29–32]. Tumour cells produce IL6 in response to cellular stress such as hypoxia, and enhanced levels of IL6 is associated with increased tumour cell invasion [10,14]. Kwon et al. found a stimulating effect of elevated IL6 on endothelial cells, which might represent a resistance mechanism to anti-VEGF therapy [33]. As a response to cellular stress, IL6 activation of the transcription factor STAT3 drives angiogenesis by inducing expression of VEGF and fibroblast growth factor (bFGF) by tumour cells, and thereby supports vascularization required for tumour growth and metastasis [34,35]. Our results are in support of previous reports indicating that high levels of inflammation-associated cytokines are negative for the outcome of treatment [13]. Elevated levels of IL6 among patients with poor response was also found in a recent work of Mizuno, investigating angiogenic, inflammatory and immunologic markers of sunitinib treatment in 56 patients with metastatic RCC [31]. However, they did not include patients with poor IMDC prognostic score. Our findings may therefore show that we can include this group as well. Tran et al. found opposite results, where a significantly increase in PFS in patients treated with another rTKI (pazopanib) versus placebo, in patients with high serum IL6 [36].

The baseline value of soluble IL6R in plasma was not significantly associated with response variables in this study, though low pIL6R tended to be associated with improved OS. In our previous paper, low tumour cell expression of IL6Rα was beneficial for treatment response. The prognostic value of IL6R expression have previously been presented, where Costes et al. found a significant association between IL6R expression and OS in patients with primary RCC tumours [16]. The complexity of membranous and soluble IL6R is well discussed in several reviews [10,34,37].

Membranous IL6ST is ubiquitous expressed in human tissue [38]. IL6ST and IL6R form a buffer for pIL6 in the blood, and is purposed to be a mechanism by which the organism protects itself from unspecific overstimulation by IL6ST [37]. Soluble IL6ST is an inhibitor of IL6. Even though the range of pIL6ST in our cohort was lower than a normal cohort, the group with under median level of pIL6ST level had worse OS (Table 3), in line with previous findings [29]. This may support the idea of a well-functioning buffer to protect against unspecific overstimulation by IL6-trans-signaling [37].

The cases with sunitinib induced reduction of pIL6R after two rounds of treatment had improved PFS. A reduction of pIL6R might be supported by the theory that trans-signaling pathways mediates cancer development [28,39]. Our results suggest that this might be used as a marker of beneficial on-treatment response, and suggest a relation between IL6Rα in tumour cells and level of circulating pIL6R.

In addition to the lack of a control group, our study has some weaknesses. First, the number of patients included is low and thereby the study lacks the statistical power to detect minor differences in response rates between groups based on the biomarkers under investigation. Thus, our findings should be validated in an independent and larger cohort of patients. Still, our data strongly suggest that biomarkers associated with tumour immune responses might be important in patients treated with anti-VEGF therapy.

Conclusion

Low level of plasma IL6 provides significant predictive information about response to sunitinib, and our data thereby suggest that up-regulation of IL6 might represent an important mechanism of resistance. Baseline measurement of this biomarker might guide clinical decision making in treatment of patients with mccRCC.

Clinical practice points

The anti-angiogenic receptor tyrosine kinase inhibitor sunitinib is first line treatment in metastatic renal cell carcinoma. Today there are no established predictive markers in clinical use. Inflammation is an

important part of cancer. In our study, we find that low level of interleukin 6 in plasma may predict treatment response of sunitinib and guide clinicians in making better treatment plans in renal cell carcinoma. The results suggest that up-regulation of plasma IL6 might represent an important mechanism of resistance. If validated in independent patient cohorts, the biomarker can easily be implicated into routine practice for a low cost using ELISA.

Conflict of interest

Pilskog, M has received consultation fees from Novartis and Pfizer. The other authors declare no conflicts of interest.

MicroAbstract

Anti-angiogenic treatment is first line treatment in metastatic renal cell carcinoma. There are presently no clinically useful predictive markers. In this study, we evaluate markers of tumor immune responses and angiogenesis. We find that low level of plasma interleukin-6 may predict response to sunitinib treatment. These results might represent an important mechanism of resistance.

Acknowledgments

The study was supported by grants from The Norwegian Research Council and The Norwegian Cancer Society. The authors alone are responsible for the content and writing of the paper. This work was partly supported by the Research Council of Norway through its Centres of Excellence funding scheme, project number 223250.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ctarc.2019.100127.

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Supplementary tables

Supplementary table 1 Biomarkers in relation to plasma interleukin 6

Variable	Plasma interleukin 6		
	≤ median n(%)	> median n(%)	*p*-value[1]
Plasma markers			
Baseline pIL6R[2]			0.77
Low	11(48)	12(52)	
High	12(55)	10(45)	
Baseline pIL6ST[3]			0.55
Low	10(46)	12(54)	
High	13(59)	9(41)	
Change in pIL6R between baseline and week 12			0.41
Decrease	6(67)	3(33)	
Stable/Increase	7(41)	10(59)	
Change in pIL6ST between baseline and week 12			0.41
Decrease	5(71)	2(29)	
Stable/Increase	11(50)	11(50)	
Clinicopathologic markers at baseline:			
Sex			0.37
Female	7(41)	10(59)	
Male	16(57)	12(43)	
Age			0.77
≤ median	11(48)	12(52)	
> median	12(55)	10(45)	
IMDC[4] criteria			0.17[5]
Favourable	4(80)	1(20)	
Intermediate	9(69)	4(31)	
Poor	8(42)	11(58)	
WHO[6] performance status - No. (%)			0.54
0	16(55)	13(45)	
1	7(44)	9(56)	
Hypertension baseline			0.14
Yes	15(63)	9(37)	
No	8(38)	13(62)	
s-CRP[7]			0.07
≤ 10	12(71)	5(29)	
> 10	11(41)	16(59)	
NLR[8] ratio > 3 vs ≤ 3			0.53
≤ 3	11(46)	13(54)	
> 3	10(59)	7(41)	
Number of disease sites - No. (%)			0.57[5]
1	4(40)	6(60)	
2	5(46)	6(55)	
≥3	14(58)	10(42)	
Quality of life at baseline			
Function scale			0.73
Lower 3 quartiles	5(46)	6(54)	
Upper quartile	18(55)	15(45)	
Symptom scale			0.30
Lower 3 quartiles	19(58)	14(42)	
Upper quartile	4(36)	7(64)	
Immunohistochemistry -tumor expression of			
Interleukin 6			1.00

SI[8][9] = 0-2	10(50)	10(50)	
SI = 3-9	9(53)	8(47)	
Interleukin 6 receptor α			1.00
SI = 1-3	6(46)	7(54)	
SI = 4-9	12(50)	12(50)	
Jagged 1			0.74
SI = 0-2	10(48)	11(52)	
SI = 3-9	9(56)	7(44)	
VEGFA[10]			1.00
SI = 1-3	3(43)	4(57)	
SI = 4-9	15(48)	16(52)	
VEGFR2[11]			1.00
SI = 0-2	7(50)	7(50)	
SI = 3-9	11(46)	13(54)	
PDGFRβ[12]			0.49
SI = 0-1	6(43)	8(57)	
SI = 2-9	11(58)	8(42)	
HSP27[13]			1.00
SI = 1-3	7(54)	6(46)	
SI = 4-9	12(48)	13(52)	

[1]Fisher's exact test. [2]Plasma Interleukin 6 receptor [3]Plasma Interleukin 6 signal transducer [4]The International Metastatic Renal Cell Carcinoma Database Consortium. [5]Pearson Chi-Square [6]World Health Organization [7]C-reactive protein. [8]Neutrophil/lymphocyte ratio. [9]Staining index. [10]Vascular endothelial growth factor A. [11]Vascular endothelial growth factor receptor 2. [12]Platelet derived growth factor receptor β. [13]Heat-shock-protein 27.

Supplementary table 2 Biomarkers in relation to plasma interleukin 6 receptor

	Plasma interleukin 6 receptor		
	≤ median n(%)	> median n(%)	*p*-value[1]
ELISA markers:			
Baseline pIL6[2]			0.77
Low	11(48)	12(52)	
High	12(55)	10(45)	
Baseline pIL6ST[3]			0.08
Low	8(35)	15(65)	
High	14(64)	8(36)	
Change in pIL6 between baseline and week 12			0.66
Decrease	3(43)	4(57)	
Stable/Increase	10(59)	7(41)	
Change in pIL6ST between baseline and week 12			0.39
Decrease	5(71)	2(29)	
Stable/Increase	10(46)	12(54)	
Clinicopathologic markers at baseline:			
Sex			1.00
Female	9(53)	8(47)	
Male	14(48)	15(52)	
Age			0.08
≤ median	15(65)	8(35)	
> median	8(35)	15(65)	
IMDC[4] criteria			0.71[5]
Favorable	4(67)	2(33)	
Intermediate	7(54)	6(46)	
Poor	9(47)	10(53)	
WHO[6] performance status - No. (%)			0.76
0	16(53)	14(47)	
1	7(44)	9(56)	
Hypertension			**0.04**
Yes	16(67)	8(33)	
No	7(32)	15(68)	
s-CRP[7]			0.76
≤ 10	8(44)	10(56)	
> 10	14(52)	13(48)	
NLR[8] ratio > 3 vs ≤ 3			1.00
> 3	9(53)	8(47)	
≤ 3	12(48)	13(52)	
Number of disease sites - No. (%)			0.33[5]
1	6(60)	4(40)	
2	7(64)	4(36)	
≥3	10(40)	15(60)	
Quality of life at baseline:			
Function scale			0.09
Lower 3 quartiles	3(27)	8(73)	
Upper quartile	20(59)	14(41)	
Symptom scale			0.31
Lower 3 quartiles	19(56)	15(44)	
Upper quartile	4(36)	7(64)	
Immunohistochemistry -tumor expression of:			
Interleukin 6			0.75

SI[9] = 0-2	12(60)	8(40)	
SI = 3-9	9(50)	9(50)	
Interleukin 6 receptor α			1.00
SI = 1-3	7(54)	6(46)	
SI = 4-9	13(52)	12(48)	
Jagged 1			0.75
SI = 0-2	12(57)	9(43)	
SI = 3-9	8(47)	9(53)	
VEGFA[10]			0.42
SI = 1-3	5(71)	2(29)	
SI = 4-9	16(50)	16(50)	
VEGFR2[11]			1.00
SI = 0-2	8(53)	7(57)	
SI = 3-9	13(54)	11(46)	
PDGFRβ[12]			0.73
SI = 0-1	7(50)	7(50)	
SI = 2-9	12(60)	8(40)	
$HSP27_{13}$			0.74
SI = 1-3	8(62)	5(38)	
SI = 4-9	13(50)	13(50)	

[1]Fisher's exact test. [2]Plasma Interleukin 6. [3]Plasma Interleukin 6 signal transducer [4]The International Metastatic Renal Cell Carcinoma Database Consortium. [5]Pearson Chi-square [6]World Health Organization [7]C-reactive protein. [8]Neutrophil/lymphocyte ratio. [9]Staining index. [10]Vascular endothelial growth factor A. [11]Vascular endothelial growth factor receptor 2. [12]Platelet derived growth factor receptor β. [13]Heat-shock-protein 27.

Supplementary table 3 Biomarkers in relation to plasma Interleukin 6 signal transducer

	Plasma Interleukin 6 signal transducer		
	≤ median n(%)	> median n(%)	*p*-value[1]
ELISA markers:			
Baseline pIL6[2]			0.55
Low	10(44)	13(56)	
High	12(57)	9(43)	
Baseline pIL6R[3]			0.08
Low	8(36)	14(64)	
High	15(65)	8(35)	
Change in pIL6 between baseline and week 12			1.00
Decrease	3(50)	3(50)	
Stable/Increase	8(40)	12(60)	
Change in pIL6R between baseline and week 12			0.37
Decrease	1(41)	6(86)	
Stable/Increase	8(38)	13(62)	
Clinicopathologic markers at baseline:			
Sex			0.07
Female	12(71)	5(29)	
Male	11(39)	17(61)	
Age			0.08
≤ median	8(36)	14(64)	
> median	15(65)	8(35)	
IMDC[4] criteria			0.28[5]
Favorable	2(33)	4(67)	
Intermediate	6(46)	7(54)	
Poor	12(67)	6(33)	
WHO[6] performance status - No. (%)			0.53
0	14(47)	16(53)	
1	9(60)	6(40)	
Hypertension			0.14
Yes	9(39	14(61)	
No	14(64)	8(36)	
s-CRP[7]			1.00
≤ 10	9(50)	9(50)	
> 10	14(54)	12(46)	
NLR[8] ratio > 3 vs ≤ 3			1.00
> 3	13(54)	11(46)	
≤ 3	10(59)	7(41)	
Number of disease sites - No. (%)			**0.04**[5]
1	5(56)	4(44)	
2	2(18)	9(82)	
≥3	16(64)	9(36)	
Quality of life at baseline:			
Function scale			0.16
Lower 3 quartiles	8(73)	3(27)	
Upper quartile	14(42)	19(58)	
Symptom scale			0.49
Lower 3 quartiles	7(64)	4(36)	
Upper quartile	15(46)	18(55)	
Immunohistochemistry -tumor expression of:			
Interleukin 6			1.00

SI[9] = 0-2	10(53)	9(47)	
SI = 3-9	10(56)	8(44)	
Interleukin 6 receptor α			1.00
SI = 1-3	6(40)	6(50)	
SI = 4-9	14(56)	11(44)	
Jagged 1			0.33
SI = 0-2	9(45)	11(55)	
SI = 3-9	11(65)	6(35)	
VEGFA[10]			1.00
SI = 1-3	4(57)	3(43)	
SI = 4-9	16(52)	15(48)	
VEGFR2[11]			0.20
SI = 0-2	10(67)	5(33)	
SI = 3-9	10(44)	13(57)	
PDGFRβ[12]			0.49
SI = 0-1	8(57)	6(43)	
SI = 2-9	8(42)	11(58)	
HSP27[13]			0.16
SI = 1-3	4(33)	8(67)	
SI = 4-9	16(62)	10(39)	

[1]Fisher's exact test. [2]Plasma interleukin 6. [3]Plasma Interleukin 6 receptor [4]The International Metastatic Renal Cell Carcinoma Database Consortium. [5]Pearson Chi-square [6]World Health Organization [7]C-reactive protein. [8]Neutrophil/lymphocyte ratio. [9]Staining index. [10]Vascular endothelial growth factor A. [11]Vascular endothelial growth factor receptor 2. [12]Platelet derived growth factor receptor β. [13]Heat-shock-protein 27.

Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

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ISBN: 9788230845295 (print)
9788230857816 (PDF)