Cytokines in Renal Cell Carcinoma

With emphasis on Vascular Endothelial Growth Factor (VEGF) and Interleukin 6 (IL-6)

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Thesis for the degree of Philosophiae Doctor (PhD)
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The work for this thesis is carried out at the Department of Urology, Haukeland University Hospital and through the PhD-program at the Department of Clinical Medicine (K1), University of Bergen.

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Gígja Guðbrandsdóttir

Abstract

Aims: In the first three papers we aimed to investigate if different circulating cytokines in blood drawn preoperatively can predict outcome after surgery in patients with renal cell carcinoma (RCC). The last paper aimed to evaluate the volatility of different cytokines and their receptors before, during and after surgery.

Material and Methods: In the three first studies (**paper I-III**), we used data from our kidney cancer database at Haukeland University Hospital. From the database, we identified 159 patients treated with partial, radical or a cyto-reductive nephrectomy at our institution between January 2007 and March 2010, who had signed informed consent forms and a preoperative drawn frozen blood sample were available.
In the last study (**paper IV**), 40 patients with renal tumors who were scheduled for open surgery with partial or radical nephrectomy were prospectively included between April 2018 and June 2019. Blood samples were taken pre-operatively, intra-operatively (simultaneously from the renal vein (RV) and a peripheral vein) and at control 4-6 weeks post-surgery.
The blood samples in all papers were analyzed and cytokines detected and measured using Luminex immune-bead technology and high-sensitivity kit from Invitrogen/Biosource. In **paper I-III**, the patients were followed up until death or the end of each study period.

Results: In **paper I**, a high level of circulating VEGF were an independent predictor ($p=0.017$) for cancer specific survival (CSS) in a multivariate analysis. Furthermore, VEGF together with the well-established prognostic factors tumor T-stage and nuclear grade, predicted disease recurrence in patients presumed to be radically treated ($p=0.03$, $p=0.011$ and $p=0.008$, respectively).
In **paper II**, a high level of IL-6 and IL-27 predicted disease recurrence in presumed radically treated patients ($p=0.001$ and $p=0.026$, respectively). In particular, the

predictions among patients with large tumors (>7 cm) were excellent for both IL-6 and IL-27 (p=0.014 and p=0.001, respectively).

In **paper III**, higher circulating levels of IL-33Rα are associated with worse prognosis (p=0.034). However, the demonstrated impact of IL-33Rα was dependent on the overall cytokine profile, including seven IL6 family members (IL-6, IL-6Rα, gp130, IL-27, IL-31, CNTF, and OSM), two IL-1 subfamily members (IL-1Rα and IL-33Rα), and TNFα.

In **paper IV**, among clear-cell RCC patients, the intraoperative RV concentration of IL-6 was significantly higher than in both the pre- and postoperative samples (p=0.005 and p=0.032, respectively). Furthermore, the intraoperatively ratio between the RV and the peripheral sample differed significantly from the expected value of 1, indicating that at least a fraction of the increased IL-6 levels intraoperatively originates from the tumor cells or the tumor environment. Other cytokines and receptors remained stable across all measurements.

Conclusions: In **paper I**, preoperative high levels of circulating VEGF predicted both an increased risk of disease recurrence and a worse CSS. In **paper II**, among presumed radically treated RCC patients, higher levels of circulating IL-6 and IL-27, predicted both disease recurrence and impaired CSS.

In **paper III**, based on differences in the overall acute phase cytokine profile, we were able to classify RCC patients into two main subsets that differed significantly with regard to prognosis. In addition, a high IL-33Rα predicted worse survival.

In **paper IV**, while most cytokines and receptors remained remarkably stable, serum levels of IL-6 increased during renal tumor surgery. This increase may at least in part be attributed to the RCC tumor cells or the immediate tumor environment.

In conclusion, the studied cytokines seem to play an important biological role in RCC and may be useful for outcome prediction in RCC patients.

List of Publications

I. Guðbrandsdottir G, Hjelle KM, Frugård J, Bostad L, Aarstad HJ, Beisland C. *Preoperative high levels of serum vascular endothelial growth factor are a prognostic marker for poor outcome after surgical treatment of renal cell carcinoma.* Scand J Urol 2015; 49(5): 388-94.

II. Guðbrandsdottir G, Aarstad HH, Bostad L, Hjelle KM, Aarstad HJ, Bruserud Ø, Tvedt THA, Beisland C. *Serum levels of the IL-6 family of cytokines predict prognosis in renal cell carcinoma (RCC).* Cancer Immunol Immunother 2021; 70 (1): 19-30.

III. Aarstad HH, Guðbrandsdottir G, Hjelle KM, Bostad L, Bruserud Ø, Tvedt THA, Beisland C. *The Biological Context of C-Reactive Protein as a Prognostic Marker in Renal Cell Carcinoma: Studies on the Acute Phase Cytokine Profile.* Cancers (Basel) 2020 Jul 19; 12(7): 1961.

IV. Guðbrandsdottir G, Aarstad HH, Hjelle KM, Førde K, Reisæter LAR, Bostad L, Aarstad HJ, Beisland C. *The levels of IL-6 and soluble IL-33R are increased in the renal vein during surgery for clear cell renal cell carcinoma.* Cytokine 2021; 144:155586.

Contents

Abbreviations

ASA	American society of anesthesiologist's classification
BS	Blood Sample
CCRCC	Clear Cell Renal Cell Carcinoma
CCI	Charlson's comorbidity index
CE	Contrast enhancement
CH	Chromophobe
CI	Confidence Interval
CLC/NNT-1	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
CRP	C-reactive protein
CSS[1]	Cancer specific survival
CSF	Colony stimulating factor
CT	Computer Tomography
CT-1	Cardiotrophin-1
DSS[2]	Disease specific survival
ECOG PS	Eastern Cooperative Oncology Group performance status
GP	Glycoprotein
HR	Hazard ratio
IHC	Immunohistochemistry
IL	Interleukin
ISUP	International Society of Urological Pathology

[1, 2] CSS is used in the introduction and paper I and DSS used in paper II-IV. The meaning of these abbreviations are synonymous.

IQR	Interquartile range
JAK	Janus kinases
LIF	Leukemia inhibitory factor
LR	Likelihood ratio test
MR	Magnetic resonance
NG	Nuclear grade
OS	Overall survival
OSM	Oncostatin M
PD	Programmed death
PDGFR	Platelet-derived growth factor receotor
PFS/RFS	Progression/recurrence free survival
P	Papillary
R	Receptor
RCC	Renal Cell Carcinoma
RV	Renal vein
S	Soluble
STAT	Signal transducers and activators of transcription
TGF	Transforming growth factor
TKI	Tyrosine kinase inhibitor
TNF	Tumor necrosis factor
TNM	Tumor nodes metastasis
VEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindau

1. Introduction

1.1. The basic function of the immune system

The immune system's role is to participate in identifying and neutralizing live foreign objects, primarily parasites, virus and bacteria. To be able to do that, it has to be able to know the self from the non-self. The first lines of defenses is exclusion. Second line of defenses is the natural immune system and the third line of defense is specific immunity [1].

The second line of defense may be defined as the innate immune system. The innate immune system has a limited number of pattern recognition receptors that respond rapidly to invasion, which is often able to eliminate intruders. The innate immune system is consistent between two healthy individuals. The limited number of receptors leads to a limited diversity in response. When the innate system meets the same intruder again it reacts the same way and at the same time; thus, it has limited ability to learn. Recent studies point towards that the innate immunity can be trained and adaptive which challenges the theory of limited learning abilities [2].

The third line of defense is the adaptive immune system, which through its bone marrow-derived and thymus-derived lymphocytes (B-cells and T-cells) generates receptors during development. These receptors are generated by rearrangement and a rejoining of a relatively small number of genes that lead to a big variety of receptors. The adaptive immune system has an immunological memory which makes the response quicker and more effective when the body encounters a foreign object it has previously been exposed to [1].

The immune system's ability to recognize the self from the non-self is crucial. Diseases like rheumatoid arthritis, type 1 diabetes mellitus, multiple sclerosis and systemic lupus erythematosus develop when the immune system attacks the patients' bodies [1].

One of the immune system's roles is to balance the growth of cells. When the immune system is not capable of balancing cell growth and cell death, it leads to cancerous cells. When the first cell divides the DNA error is introduced into the daughter cells and will be copied further [3].

Oncogenes were first recognized in viruses capable of transforming cells or inducing tumors in animals. In tumor cells, these genes are often mutated or expressed at high levels. Oncogenes are classified into three categories: cancer causing genes that stimulate cell division, tumor suppressor genes and apoptosis regulators [4].

There are many known oncogenes, and many of them are disease-specific. Epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor (VEGF) are all receptor tyrosine kinases that play a role in many cancer forms, e.g., colorectal cancer, breast cancer, melanomas, ovarian cancer and head and neck cancer and renal cell carcinoma (RCC) [5]. Oncogenes can also be growth factors, such as c-Sis, which induces cell proliferation in glioblastomas, fibrosarcomas and melanomas [6]. These oncogenes stimulate cell growth and proliferation.

P53 is one of the main tumor suppressor proteins; it is mutated in 70% of all cancers. P53 is not necessary for normal cells to grow and divide. P53 role is to identify, stop the cell cycle and fix DNA injury; if it does not succeed, the cell will go into apoptosis. P53 is activated through many phosphorylation events and posttranslational modifications when there is a cellular stress signal, e.g., DNA damage, hypoxia, oxidative stress, oncogenic stress and ribosomal stress. P53 is under the strict control of negative regulators (MDM2 and MDMX) [7].

1.2. Acute phase reaction and inflammation

Acute phase reaction is defined by increased levels of several serum proteins (>25%) as a response to tissue injury or inflammation. The reaction is induced by cytokines,

which are produced at the site of inflammation [8]. The acute phase reaction involves various serum proteins, including increased levels of coagulation factors, transport proteins, anti-proteases, complements factor, C-reactive protein (CRP), serum amyloid A and ferritin, as well as pro-inflammatory cytokines [9]. In the acute phase reaction, there is also a decrease in several proteins, e.g., albumin and transferrin [8]. The overall acute phase protein profile depends on the nature of the initial inflammatory occurrence, how it induces a systemic response and the variation in the response. All these factors is reflected in the overall profile of acute phase proteins [8].

The role of inflammation in cancer is extensive, with the presence of inflammatory cells often proceeding cancer development [10]. Inflammation can help cancer cells to escape apoptosis, to grow uncontrolled and to allow the cancer cells to disseminate, as well as changing or deregulating tumor surveillance [10]. There are many known cancer that are associated to infections, e.g., hepatitis B and C and liver cancer, Helicobacter pylori and ventricle cancer, human papillomavirus and head and neck cancer, in addition to others linked to chronic inflammation, such as colitis-associated cancer coli [10].

Inflammation plays a role in many urological cancers. In RCC, the surrogate marker for inflammation CRP has been shown to be both predictive and prognostic [11]. Two studies have shown that CRP is produced by RCC cells [12, 13]. High preoperative CRP levels in patients with urothelial cancer in the upper tract, which undergo nephroureterectomy, are found to be an independent prognostic factor for cancer-specific survival (CSS). CRP is also predictive of CSS in bladder cancer, both in patients with local disease and are treated with cystectomy, as well as those who undergo chemoradiotherapy. In patients with locally advanced or metastatic bladder cancer who receive chemotherapy, CRP predicts overall survival (OS) [11]. In prostate cancer, the primary focus has been on prostate specific antigen, although studies show that

CRP can be used as a marker in metastatic setting, and in castration-resistant disease [11].

1.3. Cytokines

Cytokines are small proteins (5-20 kDA) that enable the cells of the immune system to communicate. Cytokines are also essential to cell and tissue growth, migration, development and differentiation [14]. They are involved in autocrine, paracrine and endocrine signaling as immunomodulation agents. Cytokines have several characteristics; they exert their function by the ligation of membrane-bound receptors, as their production can be upregulated through stimulations, effect most locally and exert their biological effect by regulation gene expression [15]. They have been recognized for over 35 years [16]. They were first thought to only be excreted by immune cells and therefore first called lymphocyte activating factor, and then T-cell growth factor before they were called lymphokines. Today, cytokines associated to the immune system include chemokines, interferons, interleukins, lymphokines and tumor necrosis factor (TNF) [10]. Cytokines may be produced by every cell, with the exception of red blood cells. Many cell can respond to them [17], whereas specific cytokines can be produced by many different cells [18]. This makes the classification of cytokines demanding. One classification is based on protein structure with Table 1 showing the key member of each cytokine family and their common characteristics [15, 19].

Cytokines have many functions in health and diseases; those that use cytoplasmic tyrosine kinase have been grouped into types based on their structure and their receptors: type 1 (which has four α-helixes) and type 2. Table 2 gives an overview on the main hematopoietic cytokines, their receptors and effects, all of which are one of the main focuses in this thesis.

Interleukin (IL) nomenclature was evolved to deal with the multiple biological properties of cytokines [17]. Each interleukin can have a wide range of functions, and are often are grouped into families because of their common structure, function or common β-part of the membrane receptor, e.g., IL-1 or IL-6 families.

Family	Key members	Common characteristics
TNF receptor superfamily	TNF-α TNF-β CD40-Ligand Fas Ligand	- Shares structural homology to TNF. - Has three β-sheets. - Needs a cluster of receptors for signaling.
IL-1 cytokine superfamily	IL-1β IL-1RA IL-36α IL-37	- A conserved cytoplasmic Toll/IL-1R domain and three extracellular IG- like domains in the receptors. - The cytokines adopt a signature β-trefoil fold of 12 anti-parallel β-strands. - Further divided into IL-1, IL18 and IL-36 subfamilies.
The cysteine-knot growth factor superfamily	TGF-β β-HCG PDGF-β	- Contains six cysteine residues that form a cysteine-knot conformation. - This class includes otherwise structurally unrelated subfamilies.
IL-17 cytokine superfamily	IL-17A-E	- Contains five cysteines residues at their C-terminal ends, and form a cysteine-knot-fold structure.
Chemokines	CCL-1 CXCL 1 CX3CL1	- Small molecules characterized by domains containing four cysteine residues that secure a 3-dimensional structure. - Their cell surface receptors are linked to G-proteins. - -Divided into subgroups based on the spatial position of the cysteine residues.
Type 1 and type 2 hematopoietic cytokines	Type 1: IL-2, IL-3 and IL-6 subfamilies Type 2: Interferons and IL-10 subfamily	- Divided to type 1 and 2 based on their architecture of the extracellular segments. - Signal transduction occurs via JAK/STAT. - Type 1 cytokines have a typical α-helix bundle structure. - Receptors are often a ligand-specific binding protein, and a signal-transducing protein, which is shared with other family members. - Sub-classification is based on the signal-transducing receptor chain.

Table 1: Classification of cytokine families based on structure (adapted from [20])

Cytokines	Transmembrane signal transducer	Non-receptor tyrosine kinase	Transcription factor
	IL-2 cytokine family		
IL-2			
IL-7			STAT5
IL-9	Common gamma chain	JAK1, JAK2	
IL-15	(CD 131/IL-2RG)		
IL-4			STAT6
IL-21			STAT1, STAT3
	IL-6 cytokine family		
IL-6			
IL-11			
IL-27	Glycoprotein 130	JAK1	STAT1, STAT3, STAT5
LIF	(CD13/gp130)		
CNTF			
OCM			
	IL-12 cytokine family		
IL-12	IL-12Rβ1 or IL-12Rβ2		
IL-23	WSX1 or gp130	JAK1, JAK2	STAT1, STAT3, STAT4
IL-35			
	IL-3/IL-5 cytokine family		
IL-3			
IL-5	IL-5 receptor-β	JAK2	STAT5
GM-CSF			

Table 2: An overview of the sub-families of type 1 hematopoietic cytokines. In the table, the members of sub-families are listed together with proteins used for signal transduction, used for tyrosine kinase and transcription factors [20].

1.3.1. Interleukin 6 – family cytokines

The IL-6 cytokine family has nine members, IL-6, IL11, IL-27, IL-31, oncostatin M (OSM), ciliary neutrophilic factor (CNTF), Leukemia inhibitory factor (LIF) cardiotrophin 1 (CT-1) and cardiotrophin-like cytokine (CLC). All of the members have a four helix structure. It is the largest group of cytokines that use the same receptor,

glycoprotein 130 (gp130) or the g130-like protein (IL-31R) for intracellular signaling. They only have a 10-20% sequence identity [21]. The IL-6 and the IL-12 cytokine families resemble each other structurally and functionally; both have the helix bundle structure, and the IL-12 family receptor subunits share a modular homology with gp130.

1.3.1.1. Interleukin-6 (IL-6)

IL-6 consists of 184 amino acids that are glycosylated, as the molecular weight varies depending on the amount of glycosylation (22-28 kDA) [22]. IL-6 can be found in all organs, including the brain [23]. IL-6 was cloned and reported by Hirano et al. in 1986, and mapped to 7p15-p21 chromosome [24].

IL-6 is a cytokine produced by macrophages, Th2 cells, B cells, astrocytes, endothelial cells, adipocytes and some tumor cells [25]. In acute inflammation, macrophages and monocytes are the primary producer of IL-6, but T cells play that role in chronic inflammation [26]. IL-6 levels in the blood of healthy individuals are in between 1-6 pg/ml [27]. IL-6 is secreted by skeletal muscles in response to exercise, and can be up to 100-fold in serum and 500-fold in the muscle [28-30]. Inflammatory stimuli are the main driver of IL-6 production. In the acute phase response, there is an increased production of the acute phase protein, such as C-reactive protein (CRP), serum amyloid P, ferritin, mannose binding protein and fibrinogen in the liver. This production is stimulated by IL-6 and IL-6, and CRP correlates in many studies [31]. IL-6 and CRP have also been linked to mental depression, with a higher value giving a greater depression, although the causal direction and pathway are not known (does depression stimulate inflammation or does inflammation predispose depression) [32]. IL-6 has been correlated with the extent of tissue damage during surgery and evert outcome [33].

IL-6 has two different ways to initiate cell signaling, classic and trans-signaling. IL-6 stimulates by *classic* signaling, in which it binds to a membrane-bound IL-6 receptor expressed in only a few cells (hepatocytes, neutrophils, monocytes, macrophages and some lymphocytes) [34]. The alternative IL-6 *trans*-signaling is more generalized, and binds the membrane signal-transducing receptor glycoprotein 130kDa (gp130) through the sIL-6R. Hence, in short, IL-6 promotes general inflammation [35]. Soluble gp130 can bind to sIL-6 and prevent IL-6 from binding to sIL-6R, thereby inhibiting trans-signaling [25]. Sgp130 presents in high serum concentrations, and under normal circumstances the concentration is twice that of IL-6 [34].

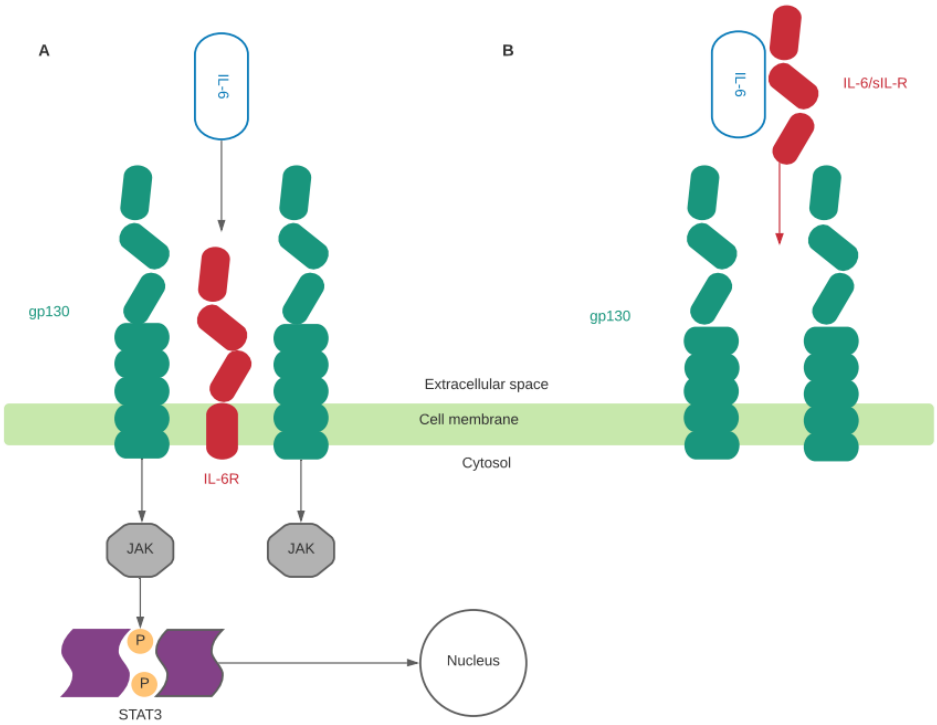

Figure 1: IL-6 signaling. A) Classical signaling, which provides a docking site to STAT3, which is then phosphorylated and translocates to the nucleus; B) Trans-signaling.

IL-6 promotes a differentiation of immunocompetent cells, induces an acute phase reaction and hematopoiesis [24, 35, 36]. IL-6 has also been shown to promote tumor proliferation, metastases and symptoms of cachexia [25].

IL-6 has an effect on cancer progression by initiating three primary oncogenic pathways, JAK/STAT3, Ras/MAPK and P13K-PkB/Akt signaling. STAT3 regulates cell proliferation, differentiation, apoptosis and angiogenesis by upregulating VEGF, metastasis and drug resistance [24]. Ras/MAPK activation leads to cell proliferation, differentiation, survival and apoptosis, in addition to angiogenesis by stimulation VEGF. Through P13A/Akt IL-6, tumor cell proliferation, apoptosis, invasion and metastasis are activated [24]. IL-6 has therefore many different pathways to affect cancer development, and the drugs applied usually only works on one of these pathways; therefore, one must attack cancer in more than one way. In the tumor microenvironment, IL-6 negatively regulates apoptotic processes, thus making cells more resistant to cell death [10].

High levels of IL-6 are suggested to be related to both worse outcomes and a higher tumor burden in prostate cancer [37], lymphoma [38], melanoma [39] and RCC [40] among other cancer diseases. Regarding RCC, IL-6 is secreted from RCC exposed to hypoxia and hypothesized to result in RCC invasion [41]. In RCC, both serum IL-6 and CRP have been associated with extended tumor stage, as well as grade, tumor burden and metastatic progression [42]. In the same series, there was an association between a high s-IL-6 and a short time to the development of metastases. Furthermore, Dosquet et al. have shown in a univariate analysis that the absence of IL-6 in blood signaled a better prognosis [43]. In conclusion, substantial evidence supports IL-6 as a growth factor in RCC patients [40].

1.3.1.2. Other IL-6 family cytokines

The IL-6 family cytokine has several members, of which IL-11, CNTF, LIF, OSM, CLC, CT-1, NNT-1 and IL-27 all share the membrane protein gp130. The receptor signaling complexes for IL-6 and IL-11 contain a gp130 homodimer, whereas other family members signal via a heterodimeric receptor complex containing gp130 [44]. IL-27 acts through a receptor consisting of IL-27Rα and gp130, which mediates signaling mostly through STAT1 and STAT3, though similarly to IL-6. IL27-Rα is present on B, T and natural killer cells, neutrophils, monocytes and mast cells, as well as in lower levels in macrophages, hepatocytes, keratinocytes and endothelial cells [45]. IL-27 has demonstrated antitumor activity in prostate cancer, multiple myeloma, non-small cell lung cancer and ovarian cancer cell lines [45]. In contrast, high serum levels of IL-27 in breast and gastroesophageal cancer are correlated with an advanced stage [45].

IL-11 has been studied in regard to ccRCC, and turned out to be an indicator of poor prognosis [46]. One study published by Pu et al. [47] showed that two polymorphisms in the IL-27 gene were associated with an increased risk for RCC [47]. To the best of our knowledge, other members of the IL-6 family have not been a matter of published RCC survival studies.

1.3.2. IL-1 family cytokines

The Interleukin-1 family consists of pro- and anti-inflammatory proteins that play a key role in the innate immunity [48]. There are 11 members in the IL-1 family. Seven of those members (IL-1α, IL-1β, IL-18, IL-33, IL36α, IL-36β and IL-36γ) are a pro-inflammatory agonist, and four (IL-1Ra, IL-36Ra, IL-37 and IL-38) exert anti-inflammatory activity [19].

IL-1 and IL-1Ra are synthesized and release as a response to the same stimuli. Mice that are IL-1Ra-deficient have spontaneously exhibited chronic inflammatory

polyarthropathy [19]. IL-1 receptor activation (IL-1RA) is essential in the induction of fever [48].

IL-18 is expressed by many inflammatory cell types, and is an inducer of interferon-γ production. Its activity can be neutralized by an IL-18 binding protein, which binds to IL-18 with a high affinity. IL-18 expression correlates with activity in inflammatory diseases, such as Crohn's and rheumatoid arthritis [19].

IL-33 exerts its function through its receptor ST2. In 2005, the ST2/ IL-1 receptor ligand 1 was discovered to be IL-33 [49]. The IL-33R is released by macrophages, fibroblasts and monocytes. The levels of soluble IL-33R are increased in many inflammatory conditions, such as rheumatoid arthritis, systemic lupus erythematous, asthma, trauma and sepsis [19]. IL-33R is a biomarker in cardiovascular disease, and has a critical role in lung, liver and head and neck squamous cancer [50].

IL-36 is a pro-inflammatory cytokine, which is a common mediator in the innate and adaptive immune responses [19]. IL-37 (originally IL-7) is found in monocytes, tonsil plasma cells and breast cancer cells, and also exerts anti-inflammatory functions [19]. IL-38 binds to the same IL-1 receptor as IL-1Ra, and is thought to play a role in the pathogenesis of inflammatory diseases [19].

IL-1, tumor necrosis factor-alpha (TNFα) and IL-6 are all acute phase cytokines that have been linked to cachexia in cancer [51]. Unfortunately, there is a sparse amount of literature on IL-1 family cytokines and RCC [52].

1.3.3. Other cytokines

The transforming growth factor β (TGFβ) serum concentration is often raised in bladder and renal cell carcinoma, and is associated with poor prognosis. It can be produced by tumor cells [52].

Interleukin-2 is mainly produced by $CD4^+$ and $CD8^+$ dendritic cells and natural killer cells. IL-2 has been known for over 35 years, with IL-2R consisting of three subunits. IL-2 is essential for the development of Treg cells. IL-2 is a B cell growth factor, stimulates antibody synthesis and stimulates the proliferation and differentiation of natural killer cells, thereby increasing their cytolytic functions [19].

IL-8 is induced by TNF-α and IL-1 and is produced by RCC cancer cells, but has not been linked to worse outcomes [52].

Interleukin-10 is upregulated by TGFβ, and is usually immunosuppressive. IL-10 inhibits major inflammatory cytokines like IL-1, IL-6, IL-12 and TNF-α, while IL-6 stimulates IL-10 in some cancer cells such as RCC. Circulating IL-10 is therefore raised in RCC, and has been associated with a worse prognosis [52].

Interferon-α can be produced by all nucleated cells, and all cells can respond to interferon. Interferon-α is critical to the stimulation of dendritic cells and the activation of naïve T cells, B-cell development and antibody production. High levels of interferons have been found in patients with autoimmune disease [19].

Several cytokines have a decreased serum value in RCC, e.g., interferon α and IL-12 [52]. Cytokines have a relatively long half time of elimination (6-15 hours for IL-6) [53].

1.4. C-reactive protein (CRP)

CRP is an acute phase protein (206 amino acids and a molecular weight of 23 kDa), which is a well-established inflammatory marker, and is increased in inflammation, injury and infections. CRP is the only acute phase biomarker used as a routine in clinical practice [9]. It was discovered in 1930 in patients with pneumococcal pneumonia, and got its name because of its reaction to the cell wall of pneumocollcal C polysaccharide [11]. CRP can be found in several isoforms, and is usually a non-

glycosylated protein with one disulfide bond and a ligand-binding surface that binds two calcium ions. The pentameric isoform of hepatic origin is the one that is mostly seen in plasma [8].

CRP is produced by hepatocytes stimulated by IL-6, but is not sufficient alone. Interleukin-1 and TNF also stimulate CRP synthesis [54]. There are other cells that can release CRP, such as smooth muscle cells, macrophages, endothelial cells, lymphocytes and adipocytes to name just a few [8]. Some renal cancer cells are able to produce CRP [11]. CRP has a half-life of 19 hours [54]. CRP has been studied in a wide variety of diseases. CRP can be increased in inflammatory diseases like rheumatoid arthritis, psoriasis and endocarditis, as well as in diseases such as Alzheimer's, cardiovascular disease and depression [55]. CRP has a tendency to be higher in those who are older, and has been correlated to aging and frailty [8]. Moreover, inflammation is associated with obesity, smoking and type 2 diabetes [8].

CRP has been associated with a higher mortality in 90% of published articles on solid tumors and CRP, mostly in gastrointestinal malignancies and kidney cancer. It has been shown that a high CRP predicts a worse prognosis in lung, pancreas, hepatocellular and bladder cancers [54].

CRP in kidney cancer has been related to a worse survival, both with OS and CSS [55]. A systematic review from 2015 showed that CRP was a prognosticator in 90% of the studies on RCC, in addition to being a strong predictor of survival in a multivariate analysis. There has been few studies on tumor recurrence and prognosis, but those that have been done have shown that CRP was predictive [54].The Glasgow Prognostic score is a score based on CRP: albumin ratio, in which a high CRP and low albumin yields the highest score. The score has been shown to predict an adverse prognosis for patients with both operable and inoperable solid tumors [8].

1.5. von Hippel Lindau

The relationship between clear cell RCC and von Hippel Lindau (VHL) disease is well known. The loss of VHL function is present in 60–80% of sporadic clear cell RCC [56]. It is a tumor suppressor gene that encodes the VHL protein, which in a normal state targets hypoxia-inducible transcription factors (HIF). HIF regulates the vascular endothelial growth factor (VEGF). Under hypoxic conditions, HIF is upregulated, whereas the VHL protein degrades it. When the VHL protein is defective, HIF accumulates and increases the expression of VEGF [56, 57]. HIF can also directly inhibit tumor apoptosis in RCC [58].

1.6. Vascular endothelial growth factor (VEGF)

VEGF was originally discovered as a vascular permeability factor secreted by tumor cells that augmented vascular leakage [59]. VEGFs represent a family of peptides important for embryo development, angiogenetic homeostasis among adults and wound healing [59]. VEGF-A (23 kDA) is considered the single most important molecule that regulates vascular development and angiogenesis in the adult. It primarily binds to VEGF receptor 2 (VEGFR 2), but also to VEGFR 1 and non-tyrosine kinase neuropilin-1. VEGFR 2 is a tyrosine kinase receptor with an extracellular ligand-binding domain, a transmembrane domain that facilitates dimerization and an intracellular kinase domain activated on ligand binding. This leads to a comprehensive intracellular signaling. VEGF-A mediates its effect on endothelium via VEGFR 2 [60]. VEGF is considered to be a primary tumor promoting cytokine in RCC development [61-63]. However, this signaling has been suggested to be targeted with drugs in many cancer types in addition to RCC, such as breast cancer, colorectal cancer, non-small cell lung cancer and ovarian cancer [64]. The tyrosine kinase receptor MET and its signaling reduces the response to VEGFR inhibitors, and is therefore involved in resistance in ccRCC [58].

Circulating levels of VEGF have been shown to be higher in RCC patients compared to non-cancer patients [63], not to mention being higher in those with metastatic

RCC [65]. One study has suggested that levels over 250 pg/ml are associated with a more aggressive disease [66]. VEGF at generally high concentrations in the blood may also function as a growth factor for RCC metastases.

Several papers have demonstrated that metastatic RCC patients with elevated levels of serum VEGF are more likely to respond to VEGF-targeted therapy [66, 67], including Fujita et al., who demonstrated an increased recurrence of clear cell RCC among patients with high levels of serum VEGF [68].

"The kidneys are not present for necessity in animals but have the function of perfecting the animal itself" Aristotle (384-322 BC).

1.7. Epidemiology of Renal Cell Cancer

Renal cell carcinoma (RCC) is the sixth most frequently diagnosed cancer in men and the 10th in women. It represent 5% and 3% of all cancers in men and women, respectively [69]. In 2019, the overall incidence of RCC in Norway was 7.7 % (3.4% and 1.7 %, for men and women, respectively). In 2018, the mortality rate was 2.3 % of overall cancer mortality. Between 1995 and 2019, median age at diagnosis decreased from 70 years to 67 years [70]. There is a large difference in the reported incidence of RCC worldwide. Developed countries have a significantly higher incidence (Figure 2).

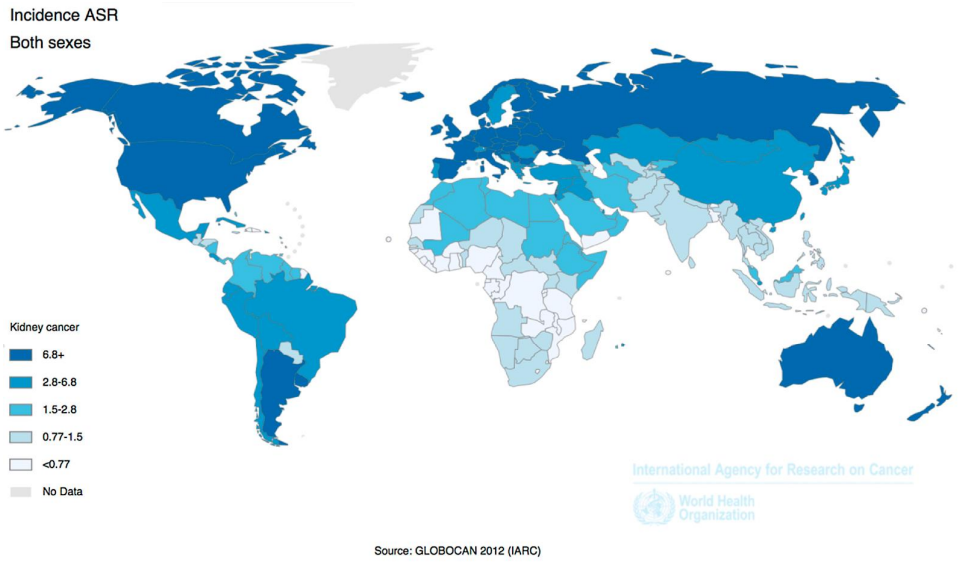

Figure 2: Worldwide incidence rate (age-standardized) of RCC for both sexes. Numbers are expressed per 100,000 [71].

In recent years, the incidence of RCC has been rising and this is largely as a result of the increase in the incidental detection on imaging performed for other purposes

[72]. Nevertheless, this increase has not caused an increase in mortality, as shown in Figure 3. Indeed, over the past two decades, there has been an improvement in the 5-year survival rate from 50-74% [69]. This improvement in survival is considered multifactorial and can be explained to an extent by stage migration, an increasing incidence of clinical stage T1, and that T1 tumors are getting smaller (3.6 cm in 2003 vs 4.1 cm in 1993) [73]. Cases of primary metastatic RCC have been stable but the percentage has gone down because of the increasing incidence of RCC. Other reasons for the improved survival is the advancement in both surgical techniques and medical therapies [74].

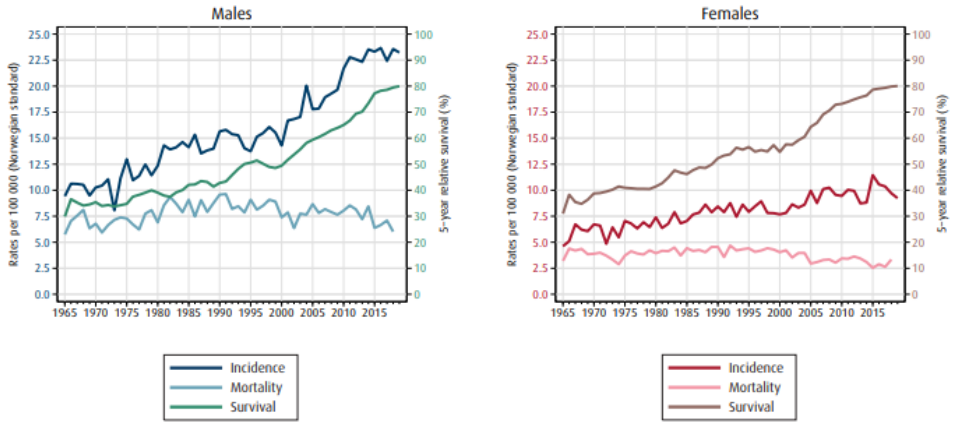

Figure 3: Kidney cancer trends in incidence and mortality rates and 5-year relative survival proportion in Norway, from Cancer in Norway 2019 [70].

1.8. Risk and genetic factors

RCC has a few well-documented risk factors including increasing age, obesity, hypertension, kidney disease and smoking [75]. Type 2 diabetes has been linked to an increased incidence of RCC in women (HR 1.6; 95% CI 1.19-2.17) [76]. In the same study by Joh et al, the authors found that women with obesity, hypertension and type 2 diabetes had four times the risk of developing RCC compared to healthy

women. In the Vitamin and Lifestyle (VITAL) study, there was a gender difference in univariate analysis, though not in multivariate analysis [75].

The difference in incidence observed in developed countries is partly caused by Westernization and the associated increase in the metabolic syndrome, which consists of obesity, hypertension and type 2 diabetes [76].

Hereditary renal cell carcinoma is implicated in approximately 3% of all RCC cases and is usually inherited in an autosomal dominant manner [77]. There are a few syndromes, which predispose individuals to develop RCC. The most well studied is von Hippel-Lindau (VHL) syndrome, which carries an estimated 70% risk for developing clear cell RCC. The incidence of von Hippel-Lindau is 1/30,000 and it consists of retinal and central nervous haemangioblastomas, in addition to clear cell RCC [77].

Birth-Hogg-Dubé syndrome is a mutation in the FLCN gene, however, the underlying process is not fully understood. The syndrome leads to fibrofolliculomas, lung cysts, pneumothorax and RCC. The risk of developing RCC is 25%. The hybrid chromophobe is the most common histological finding [77].

Hereditary leiomyomatosisis has an estimated 15% risk of RCC, papillary type 2. The incidence for this is 1/200 and it is caused by mutation in the FH gene. Patients with this mutation have a more aggressive cancer and typically develop metastasis disease at a young age (mean age 41 years) even when the tumors are small.

Patients with these mutations should undergo annual surveillance in the form MRI or ultrasound. The treatment strategy for patients with VHL and Birth-Hogg-Dubé syndrome is to treat tumors that are 3 cm or larger. However, this is not the case for hereditary leiomyomatosis whereby current guideliens recommend treatment for tumors of all sizes [77].

Other rare syndromes include succinate dehydrogenase-related RCC, hereditary papillary RCC, hereditary BAP1-associated RCC and constitutional chromosome 3 translocation [77].

1.9. Diagnostic work up

Historically, diagnosis of kidney cancer relied on clinical findings with the the classical triad of macroscopic haematuria, flank pain and an abdominal mass. A disseminated disease could be suspected in cases of systemic stigmata such weight loss, fever, night sweats and malaise. In the modern era, the majority of patients are diagnosed incidentally due to imaging for other clinical reasons. O'Conner at al. reported findings from 3,001 consecutive CT colonographic examinations and found a >1 cm renal lesion in 14.4% of the cases [78]. At our institution, 47% of patients diagnosed with RCC had presented with the abovementioned symptoms. 42% of these cases had reported macroscopic haematuria while 37% had systemic symptoms of the cases [79].

CT is an essential component of the pre-operative work-up, both in regard to ruling out metastasis and to treatment planning. There are many scoring systems used to evaluate kidney tumors. R.E.N.A.L score is composed of the **r**adius of the tumor, if it is **e**ndophytic/exophytic, the **n**earness to the sinus or collecting duct, **a**nterior or posterior and **l**ocation relative to the polar line [80]. Another one is PADUA (preoperative aspects and dimensions used for an anatomical), which is used for the classification of tumors in patients who are candidates for partial nephrectomy. Tumors are allocated points based on size, polar location, exophytic/endophytic status, relation to the renal rim, involvement of the renal sinus and urinary collecting system and anterior/posterior location. A higher score indicates the patient will be technically demanding to operate on compared to a patient with a lower score [81]. Other scoring systems like the C-Index and Renal tumor contact surface area are not as widely used as R.E.N.A.L. and PADUA [82, 83].

Currently there are no tumor markers specific for RCC, which are routinely used in the diagnostic work-up. Thus, there is a need for both diagnostic and prognostic markers in renal cell cancer. This could help avoid the overtreatment of small RCCs [84].

1.10. Classification/prognostic factors

1.10.1 Histopathological classification

In the last decade there have been significant changes in RCC classification, which includes the 2012 International Society of Urological Pathology (ISUP) Vancouver classification and the 2016 WHO classification [85]. RCC originates from the epithelium, and accounts for >90% of cancers occuring in the kidney [86].

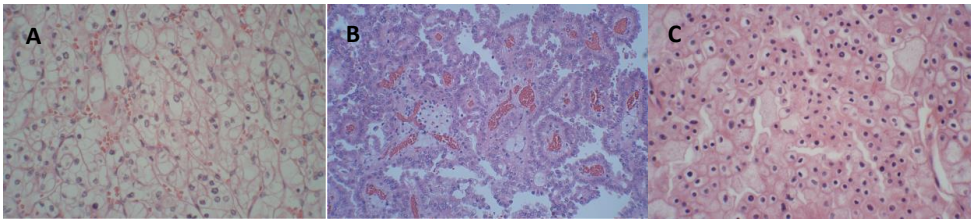

Figure 4: a) Clear cell RCC, b) Papillary RCC type I, c) Chromophobe RCC. (By courtesy of Dr. Leif Bostad)

1.10.1.1 Clear cell renal cell carcinoma (ccRCC)

Clear cell RCC is the most common subtype (approximately 70%) [87]. As aforementioned, there is a loss of function in the VHL gene, which regulates angiogenesis, glycolysis and apoptosis. As a result, ccRCCs are highly vascularized as well as rich in lipids and glycogens [86].

Macroscopically, these tumors appear yellow with frequent hemorrhagic, cystic and necrotic areas, as well as being rich in clear cytoplasm surrounded by a vascular

network (Figure 4a). Traditionally, the grading system historically applied for ccRCC and papillary RCC is Fuhrman grading, which defines four nuclear grades (1-4) and a higher score correlates with increasing nuclear size, nucleolar prominence and irregularity [86]. This grading system has been revised and is now called the WHO/ISUP grading system [88].

1.10.1.2. Papillary renal cell carcinoma

Papillary renal cell carcinoma accounts for 15-20% of RCC [87].This subtype originates from the renal tubular epithelium and is typically found in patients with end-stage kidney disease or acquired cystic disease. Macroscopically, the tumor can have various colors from gray, yellow and dark brown, and usually with hemorrhaging. Microscopically, the tumor has a prominent pseudo-capsule, which is composed of papillae formed by fibro vascular cores that contain foamy macrophages and psammoma bodies (Figure 4b) [88].

Papillary renal cell carcinoma is divided into type 1, which has papillae covered by cells with nuclei in a single layer and type 2, which is characterized by nuclear pseudostratification [88].

1.10.1.3. Chromophobe renal cell carcinoma

Chromophobe renal cell carcinoma accounts for 5-7% of RCC and most tumors are sporadic. Macroscopically, the tumor is brown in color with a central scar. They are also usually bigger than the other subtypes (mean size 7 cm) [88].

Microscopically, there is predominance of large pale cells, with reticular cytoplasm and a prominent cell membrane (Figure 4c) [88]. In tumors ≤T2a, chromophobe renal cell carcinoma is at a low risk of developing metastasis [89] the prognosis is excellent [90].

1.10.1.3. Other types

Collecting duct carcinoma is rare (1-2% of RCC) and arises from the collecting ducts of Bellini. Renal medullary carcinoma is a rare but aggressive form of RCC and further subtyoes do exist and account for less than 1% of RCC [88].

1.10.2. TNM - classification and stage

The tumor node metastasis classification used is from 2010, revised and updated in 2016 (Table 3).

T - Primary tumor	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor ≤7 cm, limited to the kidney
T1a	Tumor ≤ 4 cm
T1b	Tumor > 4 cm, but ≤ 7 cm
T2	Tumor > 7 cm, limited to the kidney
T2a	Tumor > 7 cm, but ≤ 10 cm
T2b	Tumor > 10 cm
T3	Tumor extends into major veins or surrounding tissue, but not to the adrenal gland or beyond Gerota fascia
T3a	Grows into the renal vein or its branches, or invades perirenal and/or renal sinus fat
T3b	Tumor invades the vena cava below the diaphragm
T3c	Tumor invades the vena cava above the diaphragm, or invades its wall
T4	Tumor invades beyond Gerota fascia
N - Regional lymph nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No lymph node metastasis
N1	Lymph node metastasis
M- Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis

Table 3: TNM classification from 2016 American Joint Committee on Cancer [91, 92].

Stage is a prognostic marker in RCC and is composed of the elements displayed in Table 4.

Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1-2	N1	M0
	T3	Any N	M0
Stage IV	T4	Any N	M0
	Any T	Any N	M1

Table 4: Stage grouping according to Union Internationale contre le Cancer /American Joint Committee on Cancer [91].

1.10.3. Prognostics scores and nomograms

The TNM staging system is the most utilized tool for prognostic information where higher stage, lymph node and metastatic correspond to worse CSS [93]. The nomograms employed incorporate a multitude of measurements such as factors affected by systemic inflammation (e.g., thrombocytosis, neutrophil/lymphocyte ratio, monocyte/lymphocyte ratio and platelet/lymphocyte ratio). Other commonly used measurements include hypercalcemia, elevated CRP, erythrocyte sediment ratio, ALP, LHD and lowered hemoglobin are predictive of survival.

1.10.3.1 Preoperative nomograms for non-metastatic RCC

In 2009, Karakiewicz et al published a nomogram based on 2474 patients who underwent radical or partial nephrectomy between 1984 and 2006. It includes age,

gender, clinical stage, presence of metastases, tumor size and symptoms. In patients without metastasis at time of surgery, this model predicts a CSS of 91 % at 1 year after surgery, 84 % at 2 years, 75 % at 3 years and 75% at 5 years [94].

Raj et al established a nomogram, which predicts metastatic free survival. The score is based on age, gender, radiological size, symptoms, evidence of necrosis and lymph nodes on CT (Figure 5)[95].

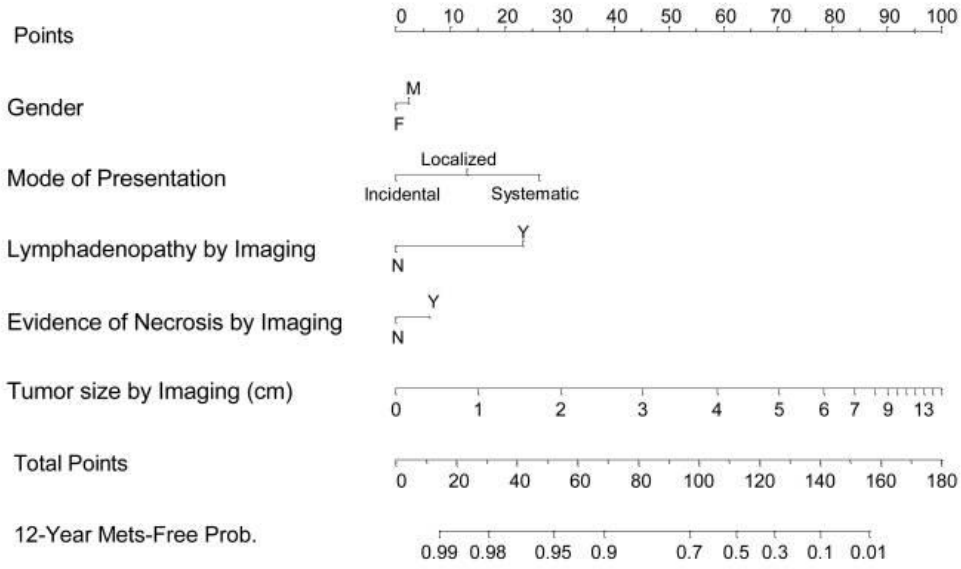

Figure 5: Preoperative nomogram by Raj et al [95].

1.10.3.2 Postoperative nomogram for radically treated local disease

There have been many nomograms developed to stratify patients into risk groups and allow a tailored follow up schedule to be delivered accordingly. The most common is the University of California Los Angeles Integrated Staging System, which is based on stage, grade and ECOG [96, 97]. SSIGN (Stage, size, grade and necrosis) is based on stage, tumor size, nuclear grade and tumor necrosis [98]. The Leibovich score includes tumor stage, regional lymph node status, tumor size, nuclear grade and

tumor necrosis. Patients are divided into three groups to estimate the risk for developing metastasis [99]. The Leibovich score has been validated at our institution [100]. The updated Leibovich scoring system now distinguishes between different histological types. Kattan is a nomogram based on patients' symptoms, histology subtype, tumor size and stage. Each variable carries weighted score and a higher end score correlates to a poorer recurrence free survival [101]. In developing these nomograms, many factors were assessed. Those, which became part of the final version held a predictive value for survival and progression.

1.10.3.3 Prognostic nomogram for metastatic disease

Patients with metastatic disease are classified into different risk groups and the treatment is tailored accordingly. The International Metastatic RCC Database Consortium criteria (Heng criteria) and the Memorial Sloan Kettering Cancer Center score (MSKCC or Motzer score) are widely used and both have undergone external validation [102, 103]. Both scoring systems include ECOG >1, > 1 year from diagnosis, anaemia and hypercalcemia. Neutrophilia and thrombocytosis are included in the Database Consortium criteria and LDH forms part of the MSKCC. Both systems have three groups: favorable (no factors), intermediate (one or two factors) and poor (more than three factors) [104]. These systems correlate with a concordance index of 0.657 [102].

	Heng	MSKCC
ECOG	>1	Not included
Time from diagnosis to metastasis	< 1 year	< 1 year
Hemoglobin level	<normal	<normal
Serum calcium	>normal	> 10 mg/dL
Neutrophil count	>normal	Not included
Platelet count	>normal	Not included
LHD	Not included	>1.5 upper limit of normal

Table 5: Comparison between International Metastatic RCC Database Consortium Criteria (Heng) and Memorial Sloan Kettering Cancer Center Score. Interpretation: No risk factor- Good risk; 1-2 risk factors- Intermediate risk; >3 risk factors- high risk.

1.11. Treatment

1.11.1. Surgery

Surgery is the gold standard treatment for localized RCC. In the past two decades we have witnessed a revolution in surgical techniques for this disease process. Historically, radical nephrectomy was the mainstay intervention while partial nephrectomy was only indicated in selected cases. Contemporary treatment options are more diverse and individualized as discussed below.

1.11.1.1 Partial nephrectomy

European Guidelines recommend partial nephrectomy whenever feasible for tumors ≤ 7 cm, as long as one can maintain negative surgical margins, functional preservation and minimal complications [105]. This method provides a CSS equal to radical nephrectomy, but preserves renal tissue and thereby reduces the risk of developing

metabolic issues or hypertension [106]. Candidates for partial nephrectomy are selected by tumor size, tumor localization and comorbidity status. Solitary functioning kidney and/or reduced kidney function represent strong indications for partial nephrectomy in order to reduce the risk of developing end stage kidney disease (ESKD) [107].

There is an increasing use of scoring systems such as R.E.N.A.L score and PADUA, which have been previously mentioned. Higher scores indicate greater tumor complexity and therefore performing partial nephrectomy will be demanding [80, 81]. With reference to partial nephrectomy, these scoring systems have shown a predictive value in regard to surgical complications including bleeding or damage to the adjacent organs [80, 81, 83]. While partial nephrectomy was previously performed using an open approach, the first laparoscopic procedure of this kind was reported in 1994 [108]. Following the introduction of laparoscopic radical nephrectomy, the application of open partial nephrectomy initially declined, however, it has been increasing since 2000 [109]. A major contributing factor to this shift has been the introduction of the robot-assisted technique in 2003. The latter enables greater instrumental flexibility compared to a purely laparoscopic method. Furthermore, improvements in surgical technique have allowed carefully selected T2 tumor cases to also be possible candidates for partial nephrectomy [110, 111].

1.11.1.2 Radical nephrectomy

Radical nephrectomy is preformed when partial nephrectomy is not feasible. The indications for radical nephrectomy in localized disease are central tumors, tumors in non- functioning kidneys or minimal kidney tissue to spare and large tumors, which are not suited for partial nephrectomy. Radical nephrectomy can be performed using both a minimally invasive and an open technique. The former results in shorter hospital stay, reduced analgesic requirement and a shorter convalescence period compared to the open technique. However, the latter does carry the advantage of

shorter operations time [112]. At 2 years of follow up, Health related quality of life is reported to be similar in both groups [113]. In addition to this, radical nephrectomy is indicated in locally advanced disease, e.g., with renal vein involvement or caval thrombosis and in the presence of lymph node metastasis. In most centers this is done using an open technique, however, in specialized centers there is a developing experience with a robot-assisted technique [114, 115].

Outcomes after RCC surgery are also related to volume of both the surgeon and the center [116]. In a Norwegian study, one there was a significant difference between low volume hospitals (<20 cases/year) and high-volume hospitals (≥40 cases/years) in regard to use of minimally invasive technique and partial nephrectomy. Low volume hospitals performed fewer partial nephrectomies and more open surgeries compared to high volume centers. Low volume centers also reported a higher 30-day mortality [116].

1.11.1.3 Cytoreductive nephrectomy

Cytoreductive radical nephrectomy was established in the era of interferon and interleukin therapy, where it was shown to render a survival benefit of 3-6 months [117]. After the introduction of targeted therapy as first-line therapy for metastatic RCC, the role of cytoreductive surgery is now under question [118].

The CARMENA study is a non-inferiority study, which randomized metastatic RCC patients into two groups; those operated with cytoreductive nephrectomy followed by sunitinib and those only receiving sunitinib. There was no difference in OS between these groups [118]. The results from SURTIME revealed that cytoreductive nephrectomy followed by sunitinib did not affect progression free survival [119].

In metastatic patients, with low metastatic burden and therefore not in immediate need for systemic treatment, cytoreductive nephrectomy has demonstrated an OS advantage and is still recommended [120].

There is ongoing study evaluating the feasibility of cytoreductive surgery in metastatic RCC and the results are awaited on the effect of immune checkpoint inhibitors in this setting.

Cytoreductive surgery can serve as a palliative option in patients with severe bleeding, pain and paraneoplastic syndrome. In some cases embolization and radiation are treatment strategies which are used for palliation and local control.

1.11.1.4 Local therapy of metastasis

Local metastasectomy is recommended in European Guidelines when complete resection is achievable because of an increased OS and CSS [93]. In bone metastasis one can also utilize radiotherapy, stereotactic radiation in pulmonary metastasis and stereotactic radiosurgery in the brain [93]. Advanced treatments strategies should be discussed in a Multidisciplinary meeting in the setting of a specialized center.

1.11.1.5 Adjuvant therapy

Many regimes have been attempted in an adjuvant setting, but currently there is no universal consensus regarding which should be recommended as the standard of care. The S-TRAC trial has been the one that is most promising, in which patients were randomized (≥ pT3) to sunitinib vs. placebo, while in the treatment group DFS was 6.8 years compared to 5.6 years in the placebo group. The ASSURE study did not show any difference between placebo and sorafenib, but they included all RCC subtypes, not only ccRCC as S-TRAC, and included ≥pT1b. PROTECT (paxopanib) and ATLAS (axitinib) studies have shown no gain in the adjuvant setting compared to the placebo [121]. There are ongoing studies on adjuvant immunotherapy that have not published and are anticipated to be reported in 2021-22 (Trial nr. NCT03024996).

1.11.2. Ablation

The most commonly used ablative techniques are cryoablation and radiofrequency ablation. Their application is suitable for small tumors (< 4 cm) and deliver a CSS comparable to surgery in this setting [122]. Typical candidates for these ablative techniques are elderly patients and those with a poor performance status [105]. According to American Guidelines however, these techniques are recommended for tumors < 3 cm [123]. Radiofrequency ablation carries a shorter procedural time compared to cryoablation. Both techniques do not require general anesthesia, with cryoablation being more favorable for larger tumors than radiofrequency ablation. Cryoablation is also associated with a higher risk of bleeding [124]. The size limits are being pushed for ablative techniques, such as in surgery, as new studies arising on T1b tumors reveal that both methods have the same CSS and complication rate. Cryoablation is superior in regard to primary success rate, as more patients needed more than one treatment session in the radiofrequency ablation group [125]. Follow-up on patients can be difficult because separating a local recurrence from post ablative damage is challenging. Novel techniques are in constant development, such as microwave ablation, irreversible electroporation, stereotactic ablative radiotherapy and high-intensity ultrasound [124, 126].

1.11.3. Observation

In the context of an ageing population worldwide and the inexorable rise of small renal mass diagnoses, the relevance of active surveillance is becoming more prominent. Small renal masses are solid, contrast enhancing tumors on CT, which are <4 cm and account for 66% of renal tumors [127]. The majority of small renal masses grow slowly (0.25 cm/year) and almost 30% do not grow at all [128]. Moreover, studies have shown that between 20-30% of small renal masses are benign [129, 130].

Active surveillance is considered a safe option in comorbid patients with tumors < 4 cm [131-135]. By using active surveillance, it seems that one does not limit treatment options for the patients that need intervention [134, 135]. Patients with small renal masses and cardiovascular risks are more likely to die of causes other than RCC [136]. There is no international consensus regarding whether a biopsy should be performed prior to inclusion into active surveillance, or how the follow-up schedule should be tailored. In the US and Canada, follow-up consists of CT/MRI every three months for the first year, every six months for the following two years and annually thereafter [123, 129, 137]. The triggers for leaving active surveillance are tumor growth >0.5 cm/year, tumor size >4 cm (3 cm in USA) or changes in patient factors. The EAU Guidelines have no clear follow-up schedule or triggers to move out of active surveillance [93]. Active surveillance is also becoming an option in tumors <1 cm in otherwise healthy individuals [130].

1.11.4. Systemic treatment

A quarter of patients have metastatic disease at diagnosis, whereas one-third of those who undergo radical treatment go on to develop metastasis [138]. Over the past 20 years, there has been a marked change in systemic therapy strategies for metastatic RCC - from high dose interleukin 2 and interferon, which have been used since the 1990s, to further immunotherapy and targeted therapy [86].

A

	Standard of care	Alternative in patients who can not receive or tolerate immune checkpoint inhibitors
IMDC favourable risk	Nivolumab/cabozantinib [1b] Pembrolizumab/axitinib [1b]	Sunitinib* [1b] Pazopanib* [1b]
IMDC intermediate and poor risk	Nivolumab/cabozantinib [1b] Pembrolizumab/axitinib [1b] Nivolumab/ipilimumab [1b]	Cabozantinib* [2a] Sunitinib*[1b] Pazopanib* [1b]

B

	Standard of care	Alternative
Prior IO	Any VEGF-targeted therapy that has not been used previoously in combination with IO [4]	
Prior TKI	Nivolumab [1b] Cabozantinib [1b]	Axitinib [2b]

IMDC = The International Metastatic Renal Cell Carcinoma Database Consortium; IO = immunotherapy; TKI = tyrosine kinase inhibitors; VEGF = vascular endothelial growth factor.
[1b] = based on one randomised controlled phase III trial.
[2b] = subgroup analysis of a randomised controlled phase III trial.
[4] = expert opinion.

Table 6: Treatment strategy for metastatic RCC, EAU Guidelines. A) First line and second line [139]; B) Later line therapy [93].

1.11.4.1. Immunotherapy

Immunotherapy has been applied in the setting of metastatic RCC for several decades and research has fueled its continued evolution. The first regime applied was IL-2 therapy. IL-2 is a growth factor necessary for T-cell growth and activation, with exogenous IL-2 modulating the immune response [140]. High-dose IL-2 delivered a benefit to relatively healthy patients with a favorable disease biology (clear cell, with no papillary or granular features). Some patients did achieve complete remission, which lasted for decades [141]. The response rate was originally 15% (37/255), in which 17 patients had a complete response and 20 a partial response, while 60% of those had a more than 90% reduction in tumor burden [142]. High-dose IL-2 has severe toxicity, primarily capillary leak syndrome, which causes oliguria, hypoxemia, edema, hypotension and tachyarrhythmia. More general side effects are fever, nausea, diarrhea and sepsis [143].

The response rate today is 25%, which is largely due to stricter patient selection for IL-2 therapy (Memorial Sloan Kettering Cancer Center intermediate risk profile, 96% ccRCC, and 99% have had a prior nephrectomy) [144]. When considering HD IL-2 as a second or third line of therapy, it is recommended to wait 8-12 weeks before initiating HD IL-2 because of cardiac toxicity [145].

Another regime is interferon α has an anti-proliferative and immune stimulatory activity [141]. It has been used as a monotherapy before the era of targeted therapies. In the initial clinical trials, interferon α was used in the control group [146, 147], though it is not used much in current clinical practice. It is approved in combination with bevacizumab, in which the response rate was demonstrated to be higher than interferon α alone [141].

In the last couple of years there has been a new kind of immunotherapy in development, which is based on the programmed death-1(PD-1) and its ligand. Nivolumab is fully humanized IgG4 PD-1 inhibitor antibody, which blocks the interaction between PD-1 (on activated T cells) and PD-1 ligand (expressed on

immune cells and tumor cells) [148]. The response rate is 20-29%, with a prolonged OS up to 25 months [148-150]. The expression of PD-1 ligand on tumor cells gives a worse outcome, but does not predict a response to nivolumab [148]. Pembrolizumab is another PD-1 inhibitor, which is being studied in both RCC alone and in combination with other drugs [141].

Anti PD-1 L agent atezolimuzab increases anti-tumor activity, and has shown a RR of 22% in patients with a Fuhrman grade 4 and sarcomatoid features in phase 1 clinical trial, which is promising [151].

Ipilimumab is an antibody inhibiting the cytotoxic T lymphocyte-associated protein 4 (CTLA-4)[152]. It was used in the US after the CheckMate 214 trial, in which ipilimumab and nivolumab in combination yielded a better response and survival than sunitinib in treatment-naïve patients in both the intermediate- and poor risk group [153].

A combination of an immunotherapy-based regime seems to be an important addition to the treatment for metastatic RCC, both for treatment-naïve patients and those who have had prior treatment [152-154].

1.11.4.2. Targeted therapy

Owing to the vascular nature of RCC, several therapies have arisen, which take advantage of this. Tyrosine kinase inhibitors (TKI) inhibit VEGF receptor and platelet-derived growth factor receptor (PDGFR), both of which play a role in the pathogenesis of ccRCC [146].

The first TKI was Sunitinib, which gave a six-month longer survival in non-treated ccRCC patients than the standard of care interferon alpha published in 2007 (11 months vs. five months) [146]. In the following years, subsequent TKIs became available and first line alternatives include sorafenib, sunitinib and pazopanib. Second

line options are axitinib and cabozantinib [86]. Sunitinib and pazopanib are the preferred treatment options in patients with a favorable risk profile [155, 156]. Pazopanib is associated with less fatigue and a better quality of life than sunitinib. The former is therefore a preferred option among many clinicians [157]. The CABOSUN study showed that cabozantinib, which is not only a VEGFR inhibitor but also a MET and AXL inhibitor, significantly prolongs PFS compared to sunitinib in treatment-naïve patients [158, 159]. These findings have influenced current guidelines to recommend cabozantinib as a first-line drug.

Bevacizumab is a VEGF monoclonal antibody, which is used for metastatic RCC. It has shown the effect on PFS [160]. Today, it is mainly used in the second or third line and is often combined with temsirolimus or an interferon [161].

Temsirolimus is an inhibitor of the mammalian target of rapamycin (mTOR) kinase, which is involved in the growth and proliferation of cells, and the response of cells to hypoxic stress. The disruption of mTOR signaling reduces angiogenesis, which is clinically relevant in RCC [147]. Temsirolimus increases OS in m metastatic RCC by 11 months, though using interferon alpha in combination does not increase OS [147].

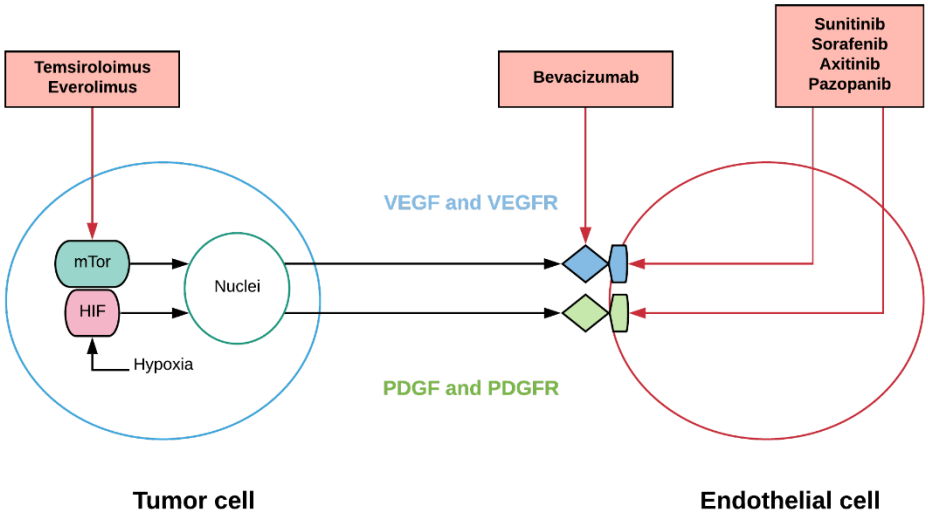

Figure 6: Overview of targeted therapy. Red arrows mean inhibition.

1.11.4.3. Combination therapy

There are many ongoing studies investigating combination therapies and sequencing. CheckMate 9ER randomized treatment-naïve metastatic ccRCC patients in two groups, nivolumab/cabozantinib and sunitinib. The nivolumab/cabozantinib group had a longer PFS (16.6 months vs. 8.3 months), and OS was also significantly higher compared to those who received sunitinib (p=0.001). The difference was found independently of the International Metastatic RCC Database Consortium [162].

The Keynote 426 compares pembrolizumab/axitinib versus sunitinib in treatment in naïve ccRCC patients; the patients who were treated with pembrolizumab/axitinib had a longer OS (p<0.001) and PFS (p<0.0001) among all risk groups. In the favorable risk group, OS was similar to sunitinib alone [163, 164].

Consequently, the results of ongoing studies may change the recommendations and sequencing in the near future.

Most studies are done on ccRCC, so there has therefore been no consensus on treatment strategies concerning metastatic non-ccRCC. The National Comprehensive Cancer Network recommends a clinical trial or sunitinib as a preferred choice, and cabozantinib and everolimus as another recommended choice [156].

1.11.4.4. Chemotherapy

RCC is primarily chemoresistant, but for tumors with predominant sarcomatoid features gemcitabine and doxorubicin have been shown to have an effect [165, 166]. For collecting duct or medullary subtypes, a partial response has been seen with carboplatin/gemcitabine, carboplatin/paclitaxel or cisplatin/gemcitabine [167, 168].

1.12. Survival

The survival rate in Norway for RCC has increased over the recent decades, Table 7 shows a 5-year relative survival [70]. The numbers in Norway are similar to the US, where all stages had a 5-year relative survival of 74, a localized 93, a regional 67 and a distant 12, as those numbers include cancer in the renal pelvis [69].

Stage	1995-1999		2000-2004		2005-2009		2010-2014		2014-2019	
Total	48.5	57.3	58.2	60.6	63.9	72.9	73.4	76.4	79.9	80.1
Localized	75.5	82.1	84.4	85.3	85.5	91.0	90.5	91.1	93.0	93.0
Regional	53.7	50.9	52.8	50.1	60.3	50.0	60.3	61.0	68.6	63.1
Distant	6.7	13.6	6.6	12.4	11.2	17.6	12.7	11.5	20.5	20.7
Unknown	45.0	56.5	65.0	62.3	72.9	75.9	45.5	51.1	63.4	40.8

Table 7: Five-year relative survival in Norway, by stage, gender (female in red) and period of diagnosis, from Cancer in Norway 2019.

1.13. Follow-up

The main reason for follow-up is to identify post-operative complications, monitor renal function and identify local recurrence or distant recurrence and the development of metastatic disease. It is imperative to identify these events and instigate a treatment plan as early as possible. Nonetheless, there is no international consensus on how the follow-up should be tailored and in general, the evidence for different follow up regimes is of low quality [93, 139, 169].

As stated earlier, many nomograms have been developed to score patients into risk groups to guide the follow up schedule. The most commonly applied in clinical practice is the University of California Los Angeles Integrated Staging System (UISS), which is based on stage, grade and ECOG [96, 97]. It can be used for both local and metastatic disease. The 5-year survival for localized RCC is 92% for the low-risk group, 67% for the intermediate group and 44% for the high-risk group [96].

The follow-up is tailored according to the risk for progression, and is not the same for those in different risk groups. Lam et al. published a follow-up program, which used the UISS groups as risk stratification [170].

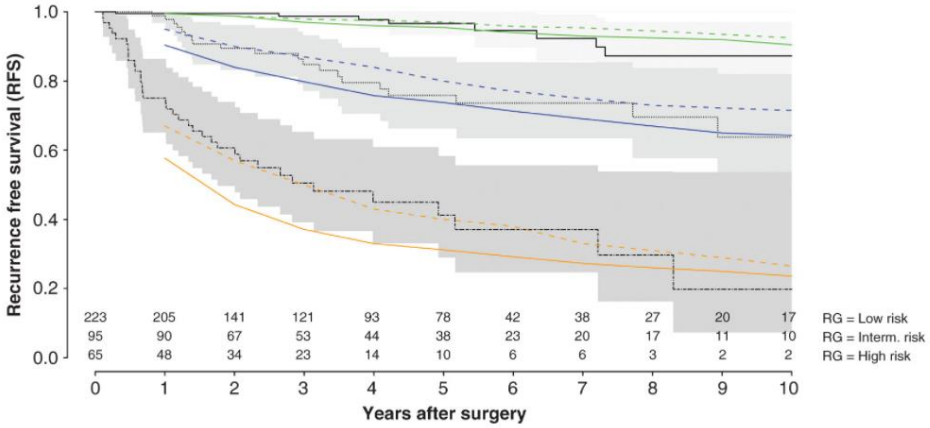

Figure 6: Kaplan-Meier for radically treated patients with ccRCC, the green, blue and yellow lines are survival estimate by the original Leibovich paper and black lines indicate the groups at our institution [100].

The follow-up consists of an annual chest CT for the low-risk groups, but every six months for the intermediate and high-risk groups. An abdominal CT is needed at 24 and 48 months for low risk, 12, 36 and 60 months for the intermediate group and every six months for the first two years for the high-risk group, and yearly thereafter. Every follow up assessment includes a history and clinical examination, in addition to laboratory studies (complete blood count, serum chemistries and a liver function test). The low-risk group does not need a follow-up over the five years.

Our institution has adapted this follow-up schedule and adjusted it for implantation in our department. Patients were scored using the Leibovich score (Table 8). The results showed that 65% of recurrences were diagnosed in the follow-up, whereas the 5-year recurrence was 0.98, 0.84 and 0.52 for the LR, IR and HR groups, which is acceptable [171].

Factor		Score
TNM	pT1a	0
	pT1b	2
	pT2	3
	PT3-4	4
Size	<10 cm	0
	> 10 cm	1
Regional lymph node status	pNx/pNO	0
	pN+	2
Fuhrman nuclear grade	Grade 1-2	0
	Grade 3	1
	Grade 4	3
Tumor necrosis	Not present	0
	Present	1

Table 8: Scoring system used at our clinic. 0-2 points- low risk for recurrence; 3-5- intermediate risk of recurrence; >6 point- high risk of recurrence [100, 172].

2. Aims of the thesis

2.1. General aim

The main aim of the thesis is to investigate whether cytokines and their soluble receptors in blood measured preoperatively can predict outcome in patients with renal cell carcinoma.

2.3. Specific aims

2.3.1 Paper I

The aim in this paper is to investigate whether the concentration of VEGF preoperatively can predict subtype, survival and recurrence in patients undergoing surgery for RCC.

2.3.2. Paper II

The primary aim is to investigate the effect of IL-6 family cytokines and their receptors on survival and recurrence in patients surgically treated for RCC. The secondary aim is to investigate if there is a difference in the immunohistochemistry between those patients with high IL-6 preoperatively compared to those with lower values.

2.3.3. Paper III

The aim here is to investigate the extended acute phase cytokine profile; IL-1/IL-6 family, TNF α and IL33R in patients with RCC and their effect on survival.

2.3.4. Paper IV

The primary aim is to map the serum levels of IL-1/IL-6 family cytokines, as well as relevant receptors from serum samples taken throughout treatments in patients with RCC. The secondary aim is to explore the possible interactions between these measurements, immunohistochemistry and intratumoral blood flow.

2. Material and Methods

3.1 For all papers

3.1.1 Patients

For **Papers I-III**, we identified 159 patients from a kidney cancer database at Haukeland University Hospital who were treated with partial, radical or a cyto-reductive nephrectomy between January 1, 2007 and March 31, 2010. All histological subtypes and stages were included. From the identified cases, patients with appropriate blood samples were included. Attrition analyses exhibited no difference with regard to descriptive statistics between individuals registered in the database who had blood samples bio-banked and those who did not.

All patients were followed to the time of the determination of the study, or the time of death, and information registered. The follow-up flow chart at Haukeland University Hospital is previously reported [171].

For **Paper IV**, patients with renal tumors planned for open surgery with partial or radical nephrectomy between April 2018 and June 2019 at Haukeland University Hospital were invited to participate in this prospective study.

All data collected for the study, including hemoglobin, C-reactive protein (CRP), comorbidities, American Society of Anesthesiologists (ASA) score and Eastern Cooperative Oncology Group-Performance Status (ECOG-PS), were stored in an electronic case report form.

3.1.2 Ethics

The studies were approved by the Regional Committee for Medical Research Ethics in Western Norway (78/05 and 2017/1757), whereas the database was approved by the

Norwegian Social Science Data Services. All patients signed consent forms at inclusion.

3.1.3. Tumor and laboratory assessment

Patients were histopathologically staged according to the 2009 TNM classification system, with the tumor histology graded based on the Fuhrman and ISUP criteria [173, 174].

Preoperative blood samples were drawn on the morning of the planned RCC operation, and serum was frozen at -80°C until analysis. Serum IL-6, GM-CSF, TNF-a, s-IL-33R and VEGF were detected using Luminex immune bead technology, and a high-sensitivity kit (Invitrogen/Biosource, Carlsbad, CA, USA). Gp130, IL-27, IL-31, IL-6Rα, OSM, IL-1Rα and CNTF were measured with the same method: a Human Premixed Multi-Analyte Kit from an R&D system and the latter through the use of the Milliplex map kit Human Pituitary Magnetic Bead Panel 1 (Millipore, Sigma-Aldrich, Oslo, Norway).

All analyses were performed strictly according to the manufacturer's instructions, while the levels were estimated by using a Luminex ©100™ (Luminex Corporation, Austin, TX, USA). All results are presented as the mean level and duplicate determinations.

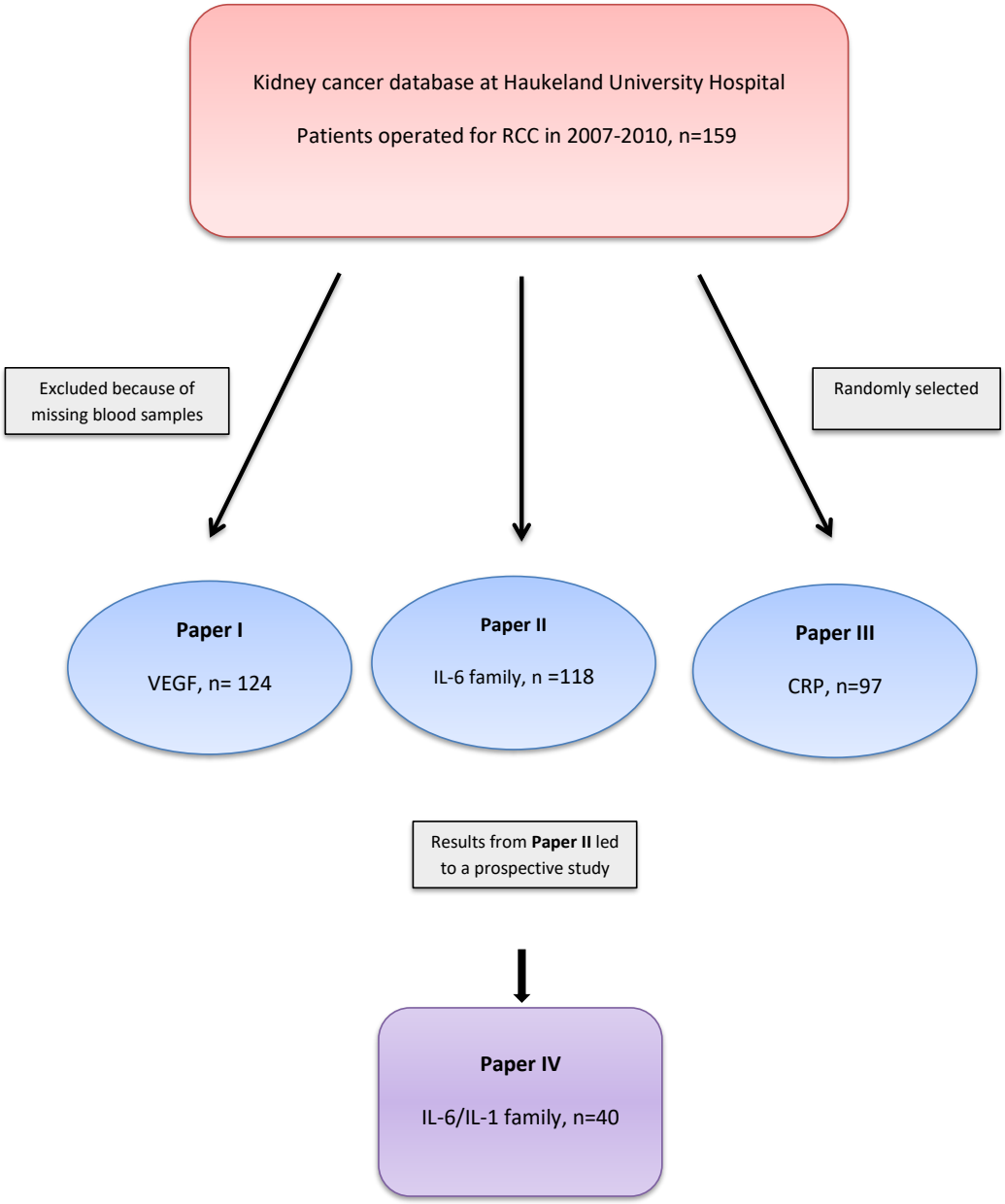

Figure 7: Study population and frame for the thesis

3.1.4. Immunohistological assessment for Papers II and IV

Tumor tissue from patients with the highest IL-6 serum levels (n=29) was investigated, with one representative block selected from each case. The selected slide contained both tumor tissue corresponding to the tumor ISUP grade, in addition to an area bordering on- and comprising kidney parenchyma (interphase zone). An experienced uropathologist classified all the RCCs based on hematoxylin and eosin-stained sections.

An immunohistochemistry (IHC) was performed using the automated benchmark ultra-system (Ventana-Diagnostics Roche). Four-micrometer sections from the formalin-fixed paraffin embedded tissue blocks were de-parafinized and rehydrated, while antigen retrieval was carried out by conditioning the cells in a TRIS-based buffer (CC1, Ventana) and heating. After endogenous peroxidase blocking, the slides were incubated with the primary antibodies. Detection was performed by using OptiView® (OV) and UltraView ® DAB detection kits (Ventana Medical Systems), with Hematoxylin used as a counterstain. Human spleen and lymph node sections were used as positive controls, while for negative controls, primary antibodies were omitted.

The entire tumor area in the slide was examined, and the subjective impression of density and the number of positive cells were scored semi-quantitatively and subjectively. The proportion of IL-6 and IL6R-positive tumor cells were scored 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+). For CD3, CD68 and FOXP3, 1+ means slight and scattered infiltration, 2+ moderate infiltration and 3+ the dense infiltration of positive cells in more than 50% of the area.

3.2 Study specific for each study

3.2.1. Paper I

There were 124 patients with appropriate blood samples, with the majority being male (93 patients, or 75%). The mean age was 63 years (median 64, range 19-85) and the mean tumor size 5.3 cm (median 4.0, range 1.2-18 cm). Moreover, a radical nephrectomy was done in 85 (69%) of the patients.

Follow-up was until death or to October 31, 2014 (at least four and a half years). The mean observation time was 73 months (median 71, range 55-93 months). Eighteen patients died from RCC, whereas 13 patients died from other causes. Twelve patients (11%), presumed to be radically treated for RCC, developed metastases during the follow-up period.

3.2.2. Papers II and III

In **Paper 2,** we had 159 consecutive patients treated with partial nephrectomy, a radical nephrectomy or a cyto-reductive nephrectomy at our institution between January 1, 2007 and March 31, 2010. All histological subtypes and stages were included. For IL-6 analyses, 118 patients with appropriate blood samples were available, while for the other cytokine analyses 97 patients were available. Most patients were male (n=88 (75 %)), the mean age was 63 years (median 64, IQR: 55-73) and the mean tumor size was 6.3 cm (median 5.3, interquartile range IQR: 3.7-8.7). A radical nephrectomy was performed in 66% (n=75) of the patients.

All patients were followed up according to the follow-up flow chart at Haukeland University Hospital, which is based on a Leibovich score (stage, lymph nodes, tumor size, nuclear grade and tumor necrosis) that has been previously reported [171]. The mean observation time was 99 (median: 105, IQR: 95-120) months for **Paper II** and for **Paper III** a median observation time of 100 months, with a range of 4-120 months. During the observation period, 20 patients died from RCC, while 19 patients died

from other causes. A total of 14 patients (12%), presumed to be radically treated, developed metastases during the follow-up period.

3.2.3 Paper IV

Preoperative blood samples were collected from a peripheral vein on the morning of surgery (Blood Sample-1: BS-1). During surgery, a second sample (BS-2) was taken from the renal vein (RV) as early as feasibly possible. This took place before a major dissection of the kidney and before clamping. Simultaneously, another sample was collected from a peripheral vein in the arm (BS-3). The last sample (BS-4) was collected at the first post-treatment assessment (4-6 weeks after surgery).

Immunohistological assessment

From **Paper II**, we retrieved immunohistochemistry (IHC) data from ccRCC patients (n=25) samples with high preoperative IL-6 levels (≥8 pg/ml). All but one in the present study had low preoperative IL-6 values (IL-6 < 8 pg/ml). Thus, for a comparison of IHC findings we analyzed two groups: low (IL-6 < 8 pg/ml); n=24 and high (IL-6 ≥ 8 pg/ml); n=26.

Imaging assessment

The majority of ccRCC patients (22 of 25) were investigated using a CT protocol, which consisted of an unenhanced acquisition, an early arterial enhancement phase (Bolus-tracking 150 HU in Aorta + 15 sec), a nephrogram phase (+100 sec) and an excretory phase (10 min). The tumor complexity was scored with a PADUA score [81]. For the remaining three patients, unenhanced acquisitions were not available. The attenuation of lesions was measured by identifying the most enhancing

homogenous area of the tumor. Furthermore, the region of interest within the homogenous area was maximized to obtain more reliable enhancement measures. The CE was split into four groups (Group 1: <20 HU, Group 2: 20-80 HU, Group 3: 81-149, and Group 4: ≥150).

3.3 Statistical analyses

For all **Papers (I-IV)**, descriptive analyses were performed for the patients and tumor characteristics. Comparisons between groups were performed with cross-tables and an exact Chi-square test, Mann-Whitney U-test and T-test for categorical, ordinal and continuous data, respectively. Correlations between variables were calculated using Kendall`s τ, Spearman's rho and Pearson`s r.

For **Papers I-III,** we did the multiple logistic regression models in a backward Likelihood Ratio test manner without a pre-selection of the variables.
Kaplan-Meier analyses were used to estimate disease-specific survival (DSS), recurrence-free survival (RFS) and overall survival (OS). For a survival comparison between different groups, a Log Rank Test was used.
A Cox proportional hazard model was used to determine DSS and RFS predictions after adjusting for other variables affecting survival in univariate analyses.

In **Paper II**, ROC curves were used to calculate the predictive value, sensitivity and specificity of IL-6/IL-27 as to recurrence and tumor diameter.

Bioinformatical analyses in **Paper III** were performed using j-Express (MolMine AS, Bergen, Norway).

In **Paper IV,** we applied the non-parametric Wilcoxon test with Bonferroni correction. Kappa analyses were used for interobserver correlations.
To predict IL-6 increase in the renal vein, we utilized general linear regression modeling.

In all the papers, we did the calculations using the IBM® SPSS® Statistics software (Release 22.0, 23.0, 24.0, 25.0 and 26.0). Boxplots in **Paper IV** were made using R version 4.0.4 (www.r-project.org), utilizing the packages {foreign}, {plyr} and {ggplot2}**.**

4. Summary of Results

4.1 Paper I

Patients with a high VEGF (above the median) and older patients were more likely to have ccRCC. The presence of multiple tumors in the same kidney displayed a trend towards being non-ccRCC.

By comparing the different quartiles of VEGF levels, Kaplan-Meier estimates showed that patients with a higher VEGF had inferior CSS rates than those with lower values (p=0.001). After merging the two upper and two lower quartiles, the difference in CSS was still significant (p=0.002). A high VEGF (OR 4.56, p=0.017) had a significant predictive value for CSS in a multivariate analysis, together with stage and nuclear grade.

In multivariate analyses, a high serum VEGF, stage and nuclear grade predicted recurrence among patients presumed to be radically treated OR= 4.37 (p=0.03), OR= 5.02 (p=0.011) and OR= 6.57 (p=0.008), respectively.

4.2. Paper II

IL-6 levels predicted recurrence, both by Kaplan-Meier survival analysis (p=0.001) and utilizing a Cox multivariate regression analysis, with age, gender and tumor size additionally included as co-variates (HR=7.13, CI: 2.23-22.8; p=0.001). IL-27 showed a significant prediction of recurrence, analyzed by Kaplan-Meier analysis (p=0.026) and multivariate Cox regression analysis, with co-variates being age, gender and tumor size (HR=6.89; CI: 1.56-30.4; p=0.011).

If the patients were grouped by tumor size (>7 cm vs. >= 7 cm) and studied by Kaplan-Meier analyses, both IL-6 (p=0.014) and IL-27 (p=0.001) predicted a recurrence among patients with large tumors.

IL-6 predicted DSS in both a Kaplan-Meier analysis ($p<0.001$) and multivariate regression analysis, including gender, age and tumor size (HR=4.82; CI: 1.96-11.86; p=0.001). IL- 6 values predicted OS (p=0.001).

By immunohistochemistry, we have determined the level of CD3, FoxP3, CD68, IL-6 and IL-6R-positive cells in tumors from patients with high IL-6 serum values. Patients with a high IL-6 exhibited a strong expression of IL-6 in endothelial- and vascular smooth muscle cells. Moreover, the level of intra-tumoral CD3-positive cells predicted survival.

4.3 Paper III

The preoperative CRP levels demonstrated the strongest association with tumor stage (i.e. diameter; Kendall's τ 0.315) and the presence of necrosis in the tumor (Kendall's τ 0.332. The CRP levels correlated to IL-6 levels (Kendall's τ 0.301, $p<0.001$), whereas no significant correlation with CRP was seen for the IL-1 subfamily mediators IL-33Rα or IL-1RA.

The IL-33Rα was significantly higher for patients with metastases compared to the patients with non-metastatic disease (Wilcoxon's rank sum test, p=0.017). The IL-33Rα levels for patients with large tumors (i.e. a diameter> 7 cm) differed from patients with metastatic disease (p= 0.038), but not from the patients with non-metastatic disease and small tumors. This difference was not seen in IL-1Rα. IL33Rα showed a prognostic value for CSS in ccRCC patients in a multivariate analysis with a Leibovich score (p=0.020).

The serum profile of IL-6 family cytokines in patients with RCC was exposed to hierarchical cluster analysis. There were two main clusters, and they were further divided into one subset with low IL-6/CNTF levels, and one with relatively high levels of the two cytokines. Based on these results, we classified the patients into two main subsets referred to as $CNTF^{high}IL\text{-}6^{high}$ and $CNTF^{low}IL\text{-}6^{low}$. We used Kaplan-Meier

analyses to compare the associations between IL-33Rα levels and cancer-related death (metastases or relapse) for the $CNTF^{low}IL\text{-}6^{low}$ and $CNTF^{high}IL\text{-}6^{high}$. Patients were classified into quartiles based on their IL-33Rα levels. The three lowest quartiles were classified together and compared to the patients with a higher quartile, with a significantly lower CSS for those with high IL-33Rα ($p<0.001$).

A hierarchical cluster analysis, including IL-6 family cytokines (IL-6, IL-6Rα, gp130, IL27, IL27, IL31, CNTF and OSM), two IL-1 cytokine family mediators (IL-1RA and IL-33Rα) and TNFα exhibited two main clusters. A Kaplan-Meier survival analysis comparing these two main subsets shows a significant difference between the two groups in regard to disease-specific survival ($p=0.004$). The two subsets showed highly significant differences in their IL-6, IL-33Rα and TNFα levels, $p<0.001$, $p=0.001$ and $p=0.006$, respectively.

4.4 Paper IV

Figure 8 shows the measurements of cytokines across all samples. For patients with ccRCC the IL-6 values in the RV (BS-2) were significantly higher than the samples taken preoperatively (BS-1) ($p=0.005$ and at postoperative control (BS-4) ($p=0.032$). The preoperative samples (BS-1) were not significantly different from the postoperative control samples (BS-4) ($p=1.0$) (Fig. 7). The median concentration of IL-6 in the RV was 1.97 (IQR: 1.01-37) times higher than in the preoperative samples (BS-2/BS-1). For the ccRCC patients, during surgery the mean ratio between RV and peripheral IL-6 levels (BS-2/BS-3) with confidence intervals was significantly higher

The measurements for the receptors IL-33R, gp130, IL-1Rα and IL-6Rα in the ccRCC group were relatively stable.

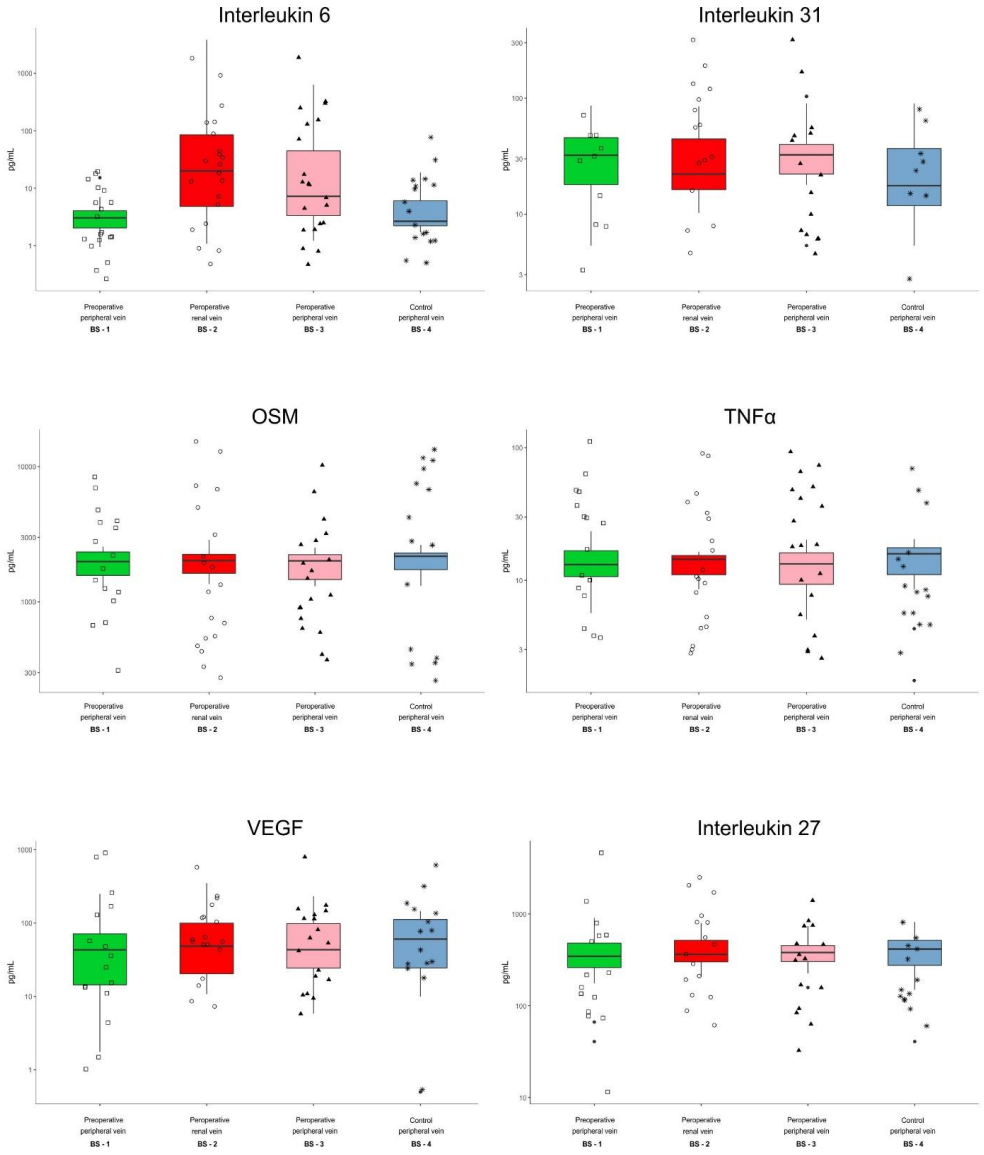

Figure 8: Measurements of cytokines in all samples taken in **Paper 4**

Regarding IL-6, none of the patients revealed an expression in tumor lymphocytes, and only one in interphase zone lymphocytes in IHC analysis. On the other hand, 23/24 (96%) were IL-6 positive in tumor cells and 20/23 (87%) in the vasculature. The expression of IL-6R in tumor cells was seen in 23/24 (96%) of the studied patients.

Comparing ccRCC patients with low IL-6 and those with high, there was a difference between them concerning the expression of IL-6 in tumor cells ($p<0.001$). Furthermore, there was a much higher expression of IL-6R in tumor cells ($p<0.001$) and FoxP3 in tumor lymphocytes in those with a higher pre-operative IL-6 ($p=0.039$).

FoxP3 in the interphase zone lymphocytes correlated to s-IL-6 intraoperatively (BS-2 and BS-3, $p=0.01$ and $p=0.042$, respectively). s-IL-6 preoperatively (BS-1) and at control (BS-4) correlated with IL-6 tumor lymphocytes, $p=0.011$, and $p=0.034$, respectively. Preoperatively, IL-6 (BS-1) correlates with IL-6 in tumor cells ($p=0.018$) and IL-6R in tumor cells ($p=0.013$).

Comparing CE and the IL-6 values, there was a significant correlation with both IL-6 samples taken during surgery (BS-2 and BS-3 with a p-value <0.01 and $p<0.05$, respectively). No significant correlation was found between IL-6 changes and IHC, nor between IHC and CE. In a linear regression model, only a higher CE remained an independent predictor of increased levels of IL-6 in the RV ($p<0.001$) with an explained variance (r^2) of 0.595.

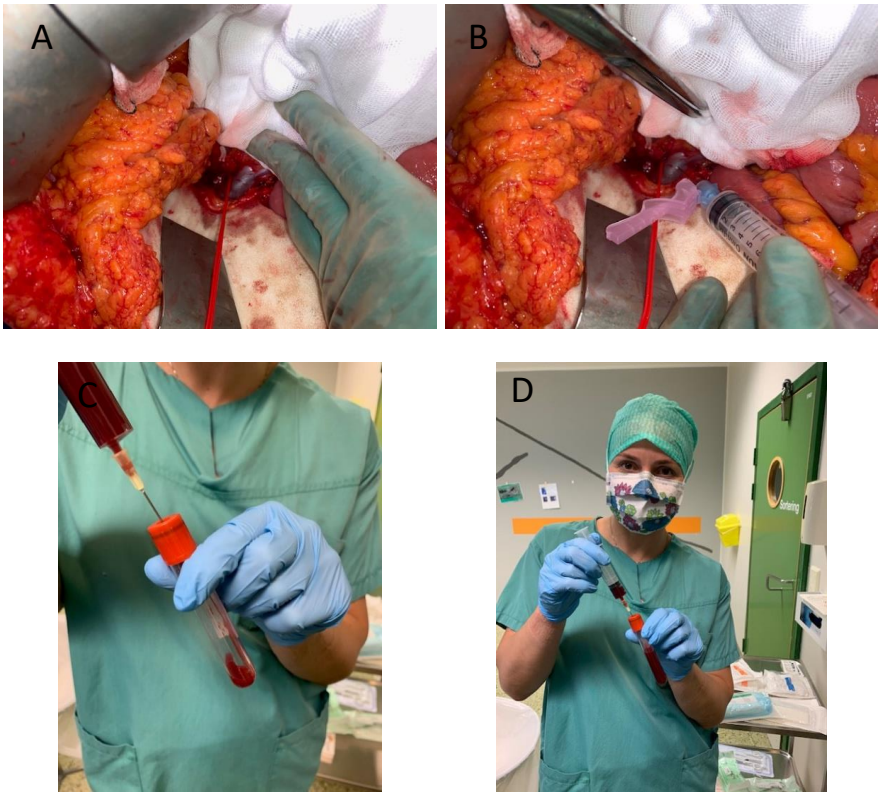

Figure 9: Procedure for attaining a blood sample; A) The renal vein is dissected and isolated B) Blood is drawn from the renal vein; C) The serum put in a sample glass; D) A happy investigator.

5. Discussion

RCC represents one of the major inflammatory related carcinomas [175], with cytokines playing a big role in inflammation, as well as in cancer development and metastases. The IL-6 family of cytokines and their receptors are therefore of interest in RCC. Most publications have written about IL-6, and some few on IL-27. In this thesis, one has focused on IL-6 family cytokines and their receptors, IL-1 family cytokines and their receptors, in addition to VEGF and CRP.

5.1 Recurrence

In a disease where one-third of patients who are presumed to be radically treated develop metastasis later in life, there is a great need for reliable markers to predict recurrence.

In **Paper I,** patients with high preoperative levels of serum VEGF were shown to be at a higher risk for recurrence after presumably being radically treated with RCC. This result is supported by Fujita et al., who demonstrated an increased recurrence of ccRCC among patients with high levels of serum VEGF [68].

In **Paper II,** IL-6 levels predicted recurrence, as did IL-27. When analyzed together, IL-6 was significant. In bigger tumors (≥ 7 cm), the prediction for IL-27 was also significant. Nevertheless, other IL-6 family cytokines, VEGF and IL-1 family cytokines did not have this predictive value. The discrepancy between the two papers in regards to VEGF and recurrence is explained by longer follow-up in the second paper and that each event can give statistical significance when there are few recurrences. In **Paper II** we analyzed the data without dividing the group into quartiles which was done in **Paper I**.

To the best of our knowledge, we are the first to show that IL-27 is predictive for recurrence in RCC. The data on IL-27 and RCC is sparse. IL-6 has been predictive of

recurrence in another Scandinavian study by Kallio et al., in which 34 of 91 patients experienced recurrence and had higher levels of IL-6 (Median 4.9 vs. 1.4 pg/ml, p=0.003) [176].

It would be interesting to collaborate with our Finnish colleagues, and do a prospective study on those patients with localized disease who were radically treated, and with a high IL-6, to see whether these patients received a benefit from TKI in an adjuvant setting.

5.2 Survival

In **Paper I,** we have shown that RCC patients with higher levels of serum VEGF had a worse prognosis in a dose-dependent manner, which has also been demonstrated in earlier studies [66, 177]. Jacobsen et al. found this to only be valid in male patients [177]. Negrier et al. were able to show that a high VEGF was an independent prognostic marker for survival in metastatic patients; however, this was only the case in a univariate analysis [178]. Some studies have reported that VEGF is higher in patients with RCC than in the normal population [63, 179], as well as being higher in those with metastatic disease [65]. VEGF is also a prognostic marker in patients with metastasis, as shown in the TARGET trial [180]. To the best of our knowledge, we are the first to show that VEGF serum levels in the same cohort predict type, recurrence and survival, thereby further strengthening the argument that VEGF is a key element of RCC biology. VEGF blockage by TKI and Bevacizumab are used in a metastatic setting, although TKI is no longer a first line of therapy after PD1 treatment was shown to yield better results. One might argue that highly selected patients with high pre-operative VEGF could respond better than patients with lower values.

In **Paper II,** the cytokine levels best predicted survival among patients with large tumors. These findings support that a high cytokine value points to a biologically aggressive tumor, more than a low differentiated tumor [34, 181, 182].

Patients with high levels of IL-6, but only with a clinically localized disease, were more likely to die from their RCC, and also had an increased risk of dying of any cause compared to those with low IL-6 serum values. Our findings are in agreement with those of Ljungberg et al. [183], Hrab et al. [55] and Blay et al. [35]. In regard to other urological malignancy high preoperative levels of IL-6 and IL-6R, a decreased CSS in patients assumed to be radically treated with cystectomy for bladder cancer and the IL-6R/IL-6 ratio, is a predictor of aggressive prostate cancer in patients treated with radical prostatectomy [184, 185].

Soluble IL-6Rα may bind to IL-6, and secondarily bind to gp130 receptors on the surface of cells, in this way stimulating pro-inflammatory functions through trans-activation. On the other hand, trans-activation is mostly immune inhibitory [186]. We have not shown negative prognostic effects of an increased serum IL-6Rα among RCC patients, hence supporting that IL-6 promotes inflammation in RCC tumors as a mechanism of IL-6-driven carcinogenesis. Soluble gp130 binds the soluble IL-6/IL6-Rα complex [187], and presumably acts as an IL-6/IL-6Rα decoy receptor [186]. We have demonstrated a negative prognostic value of an increased gp130 in the serum of patients with larger tumors, which is the opposite of what should be expected. Even so, gp130 is present in most cells [186], and the increased serum-soluble gp130 may be caused by generally increased tumor cellular turnover, which then basically drives the worse prognosis.

The combined effect of soluble IL-6Rα, gp130 and IL-6 as to prognosis seems to be complex. Regarding small tumors, the results are as expected, but concerning larger tumors s-gp130 levels paint another picture more consistent with that reflected by s-gp130 levels, e.g., cellular proliferation. Furthermore, the similar survival prediction of IL-6 and IL-27 suggests that this association is limited to cells actually carrying the IL-6 receptor on the surface, as no soluble IL-27 receptor has so far been recognized.

In the case of IL-6, we have studied the soluble receptors IL-6Rα and soluble gp130 levels. The decoy receptor gp130 had a decreased concentration versus no significant

change regarding the trans-activating IL-6Rα. Thus, it is supported that both the IL-6 classical- and trans-activation will be strengthened through these soluble receptors, with an increased serum IL-6 as part of the RCC pathophysiology. Regarding the IL-1 family cytokines and receptors, we have shown that s-IL-33R concentrations were increased in the RV. IL-33R is considered as a decoy receptor. Yet, most published studies on soluble (decoy) receptors indicate a worse cancer prognosis with such increased concentrations. This could be explained by the cellular turnover of tumors, but this needs to be studied in more detail. We have shown a considerable presence of T lymphocytes, both within the tumor and the interphase. In contrast, fewer lymphocytes were FoxP3-positive, hence suggesting few T regulatory cells. Interestingly, the presence of IL-6R on lymphocytes was more abundant with higher IL-6 serum levels, thereby suggesting that IL-6 may also inhibit T lymphocyte function though classical activation.

Serum CRP is a known prognostic factor for patients with RCC [54, 188], although CRP is only one marker of the acute phase reaction [189]. In **Paper III,** we studied an acute-phase cytokine profile (IL-6 and IL-1 cytokine family together with their receptor, as well as TNFα) in relation to survival in patients with RCC. In addition to the known prognostic value of IL-6 and CRP, we found that high IL-33Rα levels gave adverse prognosis. Several studies suggest that the IL-33/IL-33R axis could be important in tumorigenesis by exerting a direct effect on tumor cells [190], indirectly through stromal cells [191] or through changing tumor angiogenesis [192]. These prognostic findings are supported by other studies [193], as well as that IL-33R is a systemic marker of inflammation [194].

5.3 Levels of cytokines through sampling

The remarkably constant level of the measured cytokines and cytokine receptors from the pre-treatment samples to the six-week post-treatment samples was unexpected, but adds substantial validity to one-sample studies regarding (RCC)

cancer. Still, a scientific understanding of the half-life of human cytokines in blood is lacking. The elimination half-life for IL-6 is approximately 15 hours and 12 hours for rats and mice, respectively [53]. In humans, the elimination half-life is supposed to be approximately 13 hours [195]. To the best of our knowledge, there are no previous studies investigating the elimination half-life of IL-6 in RCC patients. This study supports a relatively long elimination half-life (5-15 hours) in humans because of the measured stability of the cytokine concentrations. Furthermore, the stability of many of the different cytokine concentrations throughout treatment suggests a "thermostat" that regulates cytokine concentrations, with the liver being a possible candidate for this [196].

Our results have demonstrated that serum concentrations of IL-6 increased during surgery. IL-6 is a cytokine produced by many cells as a response to stimuli [25]. Physical exercise, such as long-distance walking, has been shown to increase IL-6 up to 10 times over 24 hours [197]. It is therefore likely that a physical trauma such as open surgery may increase the general level of IL-6, both during surgery and immediately afterwards. We found a 3:1 ratio between IL-6 samples collected from the RV compared to preoperatively for all patients and 2:1 for ccRCC. This is lower than the 10:1 ratio that Blay et al. previously published in a series of three patients [198]. However, based on our intraoperative measurements, which show a significant difference between the samples from the renal vein and peripherally, the extrarenal production of IL-6 is probably not the entire explanation for this increase.

The concentrations of s-IL-Rα and s-gp130 measured in this study changed minimally. This supports that the hypothesis which measured IL-6 concentrations is functionally relevant, given that both IL-6 concentrations acting on the membrane-bound IL-6 receptor and the complex of IL-6/sIL-6Rα stimulated the relevant cell more. This is further supported by a minimal change in s-gp130 concentrations. The changed IL-6 levels therefore appear to be physiologically relevant.

5.4 Immunohistochemistry and blood flow

In **Paper II**, the IHC have also shown that among patients with a high IL-6, a surprisingly high expression of IL-6 was found in vascular cells, i.e., endothelial and smooth muscle cells, thereby suggesting that these cells produce IL-6. Endothelial cells are presumably stimulated by VEGF from the tumor [57], with this representing a possible loop in which the tumor may become autocrine-stimulated.

In **Paper IV,** we wanted to see if these results were also applicable for patients with a varying IL-6 before surgery, and we hypothesize that a substantial part of the increase in IL-6 is due to production within the tumor cells and/or from the tumor vasculature. The IHC data from **Paper IV** demonstrates the general expression of IL-6/s-IL-6R in tumor cells and IL-6 in vasculature as evidence of tumor IL-6 synthesis, which confirms our results from **Paper II**. When comparing patients with high versus low preoperative serum levels of IL-6, the former were shown to have both a higher density of IL-6 and a higher expression of IL-6R in tumor cells, which supports the theory that the tumor as a source for circulating serum IL-6. Moreover, the CE is an indicator of vascularization and blood flow through the tumor. The larger increase in IL-6 values in the RV among those with a higher tumor CE also indicates that RCC tumors are associated with IL-6 production. Overall, our results are in accordance with a hypothesis that RCC tumor cells secrete IL-6, and likely stimulate the vascular cells to do the same.

Fu et al. [199] have shown that the expression on tumor cells of IL-6/IL-6R worsens the prognosis. We have verified that both the IL-6 and IL-6R may be found on cancer cells from RCC patients with a high IL-6. Hence, it is supported that IL-6 may act directly on the tumor with a subsequently worse RCC prognosis, both in an autocrine and paracrine manner [200].

Lamb et al. investigated the prognostic potential of a tumor-produced IL-6 without finding a significant relationship between IL-6 in tumor and survival [201]. In **Paper II,** we have shown that patients with a high serum IL-6 have a worse prognosis

compared to those with a lower value. IL-6 in serum in these patients is partly caused by tumor IL-6 and tumor-associated IL-6 production (from vasculature), thus having an effect on survival.

IL-6 is thought to upregulate the production of hepatic and the intratumoral production of CRP [12]. Johnson et al. showed that patients with a localized RCC and a high density of CRP expression in the tumor had a 27-fold increased risk of overall mortality compared to those with a low CRP expression [13]. In these patients, CRP expression in the tumor exhibited a better survival prediction than serum CRP. The tumor surrounding the epithelial cell is also capable of producing CRP [12].

These results and ours show that IL-6 and CRP are produced in both RCC tumors and in the surrounding tissue and the rate of this production can also be predictive for survival.

5.5 Effect on follow-up and treatment

The follow-up for localized RCC has mainly been established on the known survival predictors included in the Leibovich scores. Leibovich is a composite score, including tumor size, pathological T and N stage, Fuhrman nuclear grading and histological necrosis [202]. The Leibovich score divides the patients into three group: a low- , intermediate- and high risk of recurrence. In this thesis, both the IL-6 and IL-27 recurrence prediction adjusted by Leibovich score still predicted survival.

In **Paper II,** we applied 8 pg/ml IL-6 levels as a cut-off between high and low IL-6 values, with 29 patients having high IL-6 values. Six out of nine patients with a detectable metastasis at diagnosis had high IL-6 values, as had seven of 14 individuals who subsequently developed RCC metastases. Several other patients presumably had other specific causes of their increased IL-6. Of those patients with a low IL-6 who died, or developed recurrent RCC disease (n=10), only one had a RCC tumor with a diameter <7 cm at diagnosis. The IL-6 values may therefore be utilized at the

individual level in order to sort patients with both a high and low risk of dying because of RCC disease.

Furthermore, ROC analyses suggested that a high IL-27 and IL-6 score predicted a recurrence with both a high sensitivity and specificity, especially as measured in patients with larger tumors. Consequently, we have demonstrated that IL-6 and IL-27 may be utilized as biomarkers in order to identify both a high- and low-risk recurrence of RCC patients at the time of diagnosis.

IL-6 taken preoperatively could be used together with Leibovich to simplify the follow-up with patients who are radically treated. Patients with an intermediate risk of recurrence by Leibovich score, and those with a low IL-6, could be followed up as low risk and those with high values as high risk. Doing that would reduce the use of imaging in controls and cause the patients less stress.

Patients with high IL-6/IL-27 values at diagnosis may be good candidates for adjuvant treatment with, e.g., VEGF inhibitors [160], as well as with anti-IL-6 therapy such as Siltuximab [203]. The Siltuximab (sIL-6) agent has shown promising results in phase I/II studies for metastatic RCC [203]. It is even possible that a combined blockage of IL-6/IL-27/VEGF would have achieved better results. The results of this thesis also demonstrate the need for future clinical studies of therapies investigating the blockage of gp130 pathways, i.e., bazedoxifene, which blocks the p-STAT3 inhibitor and in studied in colon cancer [204], and also in combination with other blockers like VEGF-TKIs [205] in order to prolong survival in patients with RCC [206]. Nonetheless, it should be borne in mind that babies born with a defective gp130 receptor may suffer from extended Stüve-Wiedemann syndrome, which is a serious, often lethal syndrome [207]. Thus, blocking gp130 may have serious side effects, making such treatment impossible. Our results also add to knowledge inspiring T cell boosting therapy to be further developed. In any case, the role of IL-27 biology in RCC should be studied judged against the background that new templates for biological therapy in RCC therapy are urgently needed [208].

As stated earlier, adjuvant therapy has not been particularly successful. It would be of interest to give those with high levels of preoperative IL-6 adjuvant therapy, as one might obtain a significant survival difference between the treatment and control group. This added selection might not only give survival benefits, but possibly spare non-responders of side effects.

Combination therapy for metastatic RCC is increasing, and there are many ways that can be done. Table 8 shows an overview of medications and their different mechanisms of function, which indicates that in order to increase survival different combinations between different classes might be the best way to succeed.

Medicine	**Signal pathway**							
	IL-2	**Interferon**	**IL-6**	**VEGF**	**m TOR**	**PDGFR**	**PD-1**	**gp130**
IL-2	**x**							
Interferon		**x**						
Siltuximab			**x**					
Bevacizumab				**x**				
Temsirolimus					**x**			
TKI				**x**		**x**		
Immunotherapy							**x**	
Bazedoxifene								**x**

Table 8: Overview of the mechanism of function of systemic treatment

6. Strength and Limitations

6.1 Strength

Despite being small studies, we found no selection bias in the study population with regard to tumor- and patient characteristics when we compared it to the complete material from our institution. In addition we have studied many cytokines together.

Paper IV is the first study to investigate the levels of IL-6- and IL-1-family cytokines in consecutive samples from the same cancer patients before, during and after surgery. Its strength is that each patient serves as their own control. We were able to study individual sample values, and therefore examine trends on an individual basis at multiple points in time. The intraoperative RV samples add considerable value to these findings.

6.2 Limitations

The present studies have limitations, insofar as being a relatively small and single-center study. Therefore, the analyses, especially on the sub-group level showing negative result, must be interpreted with caution.

There were few deaths and recurrences in our studies, so each event can have significant statistical consequences.

Paper IV is a small pilot study where there were few patients; as a result, there is a selection bias because we only included patients who had open surgery. This was because it is technically easier to attain blood from the renal vein in open surgery. In all laparoscopic surgery you need a long tube that is connected to a needle inside the patients and a syringe outside. In the abdomen, there is an increased pressure that makes the tube collapse, and the friction is quite high when one extracts blood with a small needle and a long tube with low radius. However, the surgical trauma itself

might be a confounder that complicates the understanding of the changes in IL-6 measurements. There is also a gender imbalance with more men (4:1) than the usual 1.5:1 ratio known from other cohorts [209].

7. Conclusion

- Tumor stage and a high level of serum-VEGF were predictors for an increased risk for recurrence and a cancer-specific death. Furthermore, serum-VEGF may be used to determine the subtype of RCC preoperatively.
- IL-6 and IL-27 have a predictive ability of recurrence and disease-specific survival in otherwise radically treated RCC patients. We believe that patients with a high IL-6 and IL-27 will be good candidates on which to base a biological therapy of RCC. Finally, both these cytokines hold promise for being important in relation to risk stratification regarding RCC prognosis, and thereby a need for treatment.
- The acute-phase protein CRP is a known prognostic factor for RCC. The acute-phase cytokine profile differs between RCC patients, with most cytokine serum markers included in our study showing no association with serum CRP levels. Based on the difference in the overall acute-phase cytokine profile, we classified RCC patients into two main subsets that differed significantly with regard to prognosis. This suggests the possible prognostic impact of an extended acute-phase cytokine profile.
- Serum levels of IL-6 increased during surgery. Intraoperative IL-6 and s-IL-33R values were higher in the RV compared to the periphery, which suggests secretion from the tumor or the tumor microenvironment itself. Supportive of this is an almost general expression of IL-6/s-IL-6Rα in tumor cells and IL-6 in vasculature in the RCC tumor microenvironment. Other studied cytokines were remarkably stable across all measurements.

8. Future Perspectives

One future perspective is to see whether one could make IL-6 a standard blood sample in the work-up in patients with renal cell carcinoma. Patients with low IL-6 and small tumors could be candidate to active surveillance and those with high levels should get radical treatment. After radical treatment IL-6 could be of value to help stratify patients into which control group they should enter. Those patients with an intermediate risk of recurrence by Leibovich would be split into those with a high preoperative IL-6, and therefore controlled as high risk with the rest as low risk. By reducing the control groups from three to two, one could save patients for controls (CT scans with big radiation) without it having a consequence for their health, hence reducing the use of resources both economic and human.

Patients with a high IL-6 are more likely to get a recurrence and die from RCC. Adjuvant treatment is not a standard treatment for RCC patients because one has not shown a significantly better survival in the studies that have been published. One future perspective would be to select patients with a high preoperative IL-6, VEGF, IL-27 and IL-33Rα to receive adjuvant therapy in hope of better survival. That would require a large randomized clinical trial.

As patients with high VEGF and IL-6 have worse prognosis one might think these patients would benefit from combinations treatment with IL-6 and VEGF blockage with Sitluximab and TKI or Becacizumab. This selection has not been done in studies published today. There might be a survival benefit for the patients but not least for the patients that today get treatment they do not respond to but get side effects that reduces their quality of life.

In modern medicine we aim to personalize treatment and by using these markers we are able to get a step closer to that aim.

Another point of interest is to take fusion MR to be able to evaluate the blood flow in the tumor and see if it correlates to the IL-6 in serum before surgery, as we know that patients with a high IL-6 have a high density of IL-6 in vasculature.

There is of interest to get a bigger sample size to see if the results stay the same or if new knowledge will arise. We take blood samples from all patients undergoing RCC surgery (not only the years in these thesis) in our clinic and thus we have many samples to be analyzed.

IL-6 has been studies in many urological cancers like bladder cancer and prostate cancer. It would be of interest to see if IL-27 and IL-33Rα have a predictive value in these cancer types as well as in other forms of cancer.

9. Reference

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Paper I

Paper II

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ORIGINAL ARTICLE

Serum levels of the IL-6 family of cytokines predict prognosis in renal cell carcinoma (RCC)

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Abstract

Purpose An improved understanding of RCC immunology should shed further light on RCC tumor biology. Our objective was to study to what extent serum levels of the IL-6 family of cytokines at diagnosis were relevant to survival.

Methods A total of 118 consecutively patients with RCC, in which the tumor was surgically removed at Haukeland University Hospital during the period from 2007 to 2010, were included. The patients were followed-up for 10 years. The morning before surgery blood was sampled and serum frozen, with levels of IL-6, IL-27, IL-31, OSM, CNTF, IL-6Rα and gp130 determined.

Results Among patients with the highest quartile of IL-6 (> 8 pg/ml) ($n = 29$), six of nine who had metastasis at diagnosis had such high IL-6 values. Among presumed radically treated patients, a high IL-6 and IL-27 strongly predicted recurrence. In particular, the predictions among patients with large (diameter > 7 cm) tumors were excellent regarding both IL-6 and IL-27 values. High gp130 serum levels predicted an overall survival (OS) among RCC patients with large tumors. Patients with a high IL-6 exhibited a strong expression of IL-6 in endothelial- and vascular smooth muscle cells. Moreover, the level of intra-tumoral CD3-positive cells predicted survival.

Conclusions IL-6 and IL-27 seem to play a role in RCC biology. IL-6 enables the pinpointing of metastatic condition at diagnosis, as well as together with IL-27, the predicting of survival and recurrence. Endothelial cells and vascular smooth muscle cells are both suggested as important sources of IL-6.

Keywords IL-6 · IL-27 · gp130 · Survival · Recurrence · Renal cell carcinoma

Introduction

Cancer diseases are major global killers of humans [1]; thus, there is an urgent need to better understand these diseases. It is generally accepted that carcinomas are caused by somatic DNA mutations with a consequent dysregulation of the affected cells. Furthermore, it has been known that carcinomas are not only built by actual carcinoma cells, but also, e.g., by intra-tumor immune cells. Biological information from carcinomas is collected with a biopsy, or from a resected tumor, both of which are instant pictures of a long-term ongoing process. One important source of tumor biology knowledge is serum samples, of which tumor-associated secretory interleukins/cytokines contribute, with the study of this cytokine reservoir in cancer patients being the primary goal of this study. One primary validity criteria of all cancer studies is the association to prognosis, so we will therefore presently use survival as our readout variable.

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Renal cell cancer (RCC) is the ninth most common cancer in men and 14th most common cancer in women. In 2018, 175,098 deaths by RCC were estimated, making it the 14th most common cause of global cancer deaths [2]. RCC represents one of the major immunogenic carcinomas [3]. Over the last few years, biological therapy has gained importance as a treatment for metastatic RCC, mostly by VEGF blockage [4]. Recently, modern immune therapy has also been introduced [5].

IL-6 is a cytokine produced by, e.g., macrophages, Th2 cells, B cells, astrocytes, endothelial cells, adipocytes and some tumor cells [6]. IL-6 has been shown to promote tumor proliferation, metastases and symptoms of cachexia [6]. In a review paper, the IL-6 serum level at diagnosis was significantly correlated to survival in 82/101 series, comprising 9917 out of 11,583 patients with 23 different cancer types [7].

IL-6 regulates inflammation by two main pathways: The *classic* signaling, in which it binds to a membrane-bound IL-6α receptor expressed in only a few cell types and then secondarily to membrane-bound gp130 (signal transducing receptor glycoprotein 130 kDa) present in many cells [8]. The *trans* signaling IL-6 binds to membrane gp130 through a primary binding to serum IL-6Rα [9]. The *classical* signaling stimulates the regenerative and anti-inflammatory activity, whereas *trans* signaling has more general stimulatory effects [9]. When IL-6/IL-6Rα binds to gp130, three signaling pathways may be activated: JAK-STAT, Ras-ERK cascade or P13K-Akt signaling. Through all three ways of *trans* signaling, IL-6 promotes the growth of cancer cells, whereas STAT3 IL-6 also promotes tumor cells' ability to escape apoptosis [10]. On the other hand, soluble gp130 receptor serves as a decoy receptor that inhibits the function of IL-6/IL-6Rα complex [11].

Several other cytokines also share the use of gp130 subunit receptor. These cytokines are collectively named the IL-6 family of cytokines [8, 12], which has several members, including IL-11, IL-27, IL-31, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and cardiotrophin-like cytokine factor 1 (CLC) [13]. The receptor signaling complexes for IL-6 and IL-11 contain a gp130 homodimer, whereas other family members signal via a heterodimeric receptor complex containing gp130 [13].

IL-6 has been shown to be secreted from RCC cells exposed to hypoxia, and hypothesized to contribute to RCC invasion and the development of metastasis [14–16]. In RCC serum IL-6 levels have been associated with extended tumor stage, grade and metastatic progression [16].

Regarding other IL-6 family cytokines, Pu et al. [17] showed that two polymorphisms in the IL-27 gene were associated with an increased risk for RCC. IL-27 acts through a receptor consisting of IL-27Rα and gp130, which mediates signaling mostly through STAT1 and STAT3, though similarly to IL-6. IL27-Rα is present on B, T and NK cells, neutrophils, monocytes and mast cells, as well as in lower levels in macrophages, hepatocytes, keratinocytes and endothelial cells [18]. IL-27 has demonstrated antitumor activity in prostate cancer, multiple myeloma, non-small cell lung cancer and ovarian cancer cell lines [18]. In contrast, high serum levels of IL-27 in breast and gastroesophageal cancer are correlated with advanced stage [18].

Tumor diameter, measured by CT prior to surgery, is a strong indicator for survival [19]. Most RCC-caused deaths occur in patients with tumors > 7 cm in diameter. Hence, we have aimed in particular at studying large RCC tumors as to evidence for cytokine involvement.

It is also of interest to study tumor tissue, both for the source of secretion and as a potential target [20]. We have therefore studied the level of macrophages and T lymphocytes in and around tumors, as well as IL-6 and IL-6 receptor levels on endothelial cells, macrophages and T lymphocytes, both in and adjacent to the tumors in patients with a high serum IL-6.

In this study, we have aimed at investigating whether the IL-6 family of cytokine members and pertinent cytokines receptors levels, both in serum preoperatively and in tumor tissue, relate to RCC biology by studying the prognostic value of these cytokine/receptor levels at diagnosis.

Material and methods

Patients

From the kidney cancer database at Haukeland University Hospital, we identified 159 consecutive patients treated with nephron sparing surgery (NSS), a radical nephrectomy (RN) or a cyto-reductive nephrectomy at our institution between January 1, 2007 and March 31, 2010. All histological subtypes and stages were included. For IL-6 analyses, 118 patients with appropriate blood samples were available, while for the other cytokine analyses 97 patients were available. Attrition analyses revealed no difference in regard to descriptive statistics between individuals registered in the database who had blood samples bio-banked and those who did not. Most patients were male [$n = 88$ (75%)], the mean age was 63 years (median 64, IQR 55–73) and the mean tumor size was 6.3 cm (median 5.3, interquartile range IQR 3.7–8.7). A radical nephrectomy was performed in 66% ($n = 75$) of the patients.

All patients were followed-up to January 18, 2018/10 years or time of death, and the information registered. The follow-up flow chart at Haukeland University Hospital, which is based on Leibovich score (stage, lymph nodes, tumor size, nuclear grade and tumor necrosis) has been previously reported [21]. The mean observation time

was 99 (median 105, IQR 95–120) months. During the observation period, 20 patients died from RCC, while 19 patients died from other causes. A total of 14 patients (12%), presumed radically treated, developed metastases during the follow-up period. Our institutional Follow-up regime has been described in detail by our group [22]. The Regional Committee for Medical Research Ethics in Western Norway (78/05), the study and the Norwegian Social Science Data Services all approved the database. All patients signed informed consent forms at inclusion.

Tumor assessment

Patients were staged according to the 2009 TNM classification system, and the tumor histology was graded according to the Fuhrman nuclear criteria [23].

Laboratory cytokine assessment

Preoperative blood samples were drawn on the morning of the surgery, and serum was frozen at − 80/150 °C until analysis. Serum IL-6 was detected using the Luminex immune-bead technology and a high-sensitivity kit (Invitrogen/Biosource, Carlsbad, CA, USA). In short, antibody-coupled beads were incubated with serum and incubated with a biotinylated detection antibody, before finally being incubated with streptavidin–phycoerythrin. Samples were then read by the Luminex's laser-based fluorescent analytical test instrument Luminex® 100™ (Luminex Corporation Austin, TX, USA). Gp130, IL-27, IL-31, IL-6Rα, OSM, and CNTF measured with the same method: Human Premixed Multi-Analyte Kit from R&D system, and the latter by the use of the Milliplex map kit Human Pituitary Magnetic Bead Panel 1 (Millipore, Sigma-Aldrich, Oslo, Norway).

Immunohistological assessment

Tumor tissue from patients with the highest IL-6 serum levels ($n = 29$) was investigated, with one representative block selected from each case. The selected slide contained both tumor tissue corresponding to the tumor ISUP grade and an area bordering on and comprising kidney parenchyma (interphase zone). An experienced uropathologist classified all the RCCs based on hematoxylin and eosin-stained sections.

Immunohistochemistry was performed using the automated benchmark ultra-system (Ventana-Diagnostics Roche). Four-micrometer sections from the formalin-fixed paraffin embedded (FFPE) tissue blocks were de-paraffinized and rehydrated, while antigen retrieval was done by conditioning the cells in a TRIS-based buffer (CC1, Ventana) and heating. After endogenous peroxidase blocking, the slides were incubated with the primary antibodies. Detection was performed by OptiView® (OV) and UltraView® (UV) DAB detection kits (Ventana Medical Systems), with hematoxylin used as a counterstain. Human spleen and lymph node sections were used as positive controls, while for negative controls, primary antibodies were omitted (Supplementary Table 1).

The whole tumor area in the slide was examined and the subjective impression of density and number of positive cells were scored semi-quantitatively and subjectively. The proportion of IL-6 and IL6R-positive tumor cells were scored 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 +). For CD3, CD68 and FOXP3, 1 + means slight and scattered infiltration, 2 + moderate infiltration and 3 + the dense infiltration of positive cells in more than 50% of the area.

Statistical analysis

Comparisons between groups were performed with cross-tables and exact Chi-square test, Mann–Whitney *U* test and *T* test for categorical, ordinal and continuous data, respectively. A patient's serum levels of IL-6 ≥ 8 pg/ml (the uppermost quartile), and of IL-27 for the uppermost quartile, were defined as high. The multiple logistic regression models were performed in a backward likelihood ratio (LR) test manner without a pre-selection of the variables.

Kaplan–Meier analyses were used to estimate DSS and recurrence-free survival (RFS). For a survival comparison between different groups, a log rank test was used. A Cox proportional hazard model was used to determine DSS and RFS predictions after adjusting for other variables affecting survival in univariate analyses. Correlations between variables were calculated using Kendall analyses, while ROC curves were used to calculate predictive value, sensitivity and specificity of IL-6/IL-27 as to recurrence. For all statistical analyses, a p value of less than 0.05 was considered statistically significant, and calculations were performed using the IBM® SPSS® Statistics software (Release 24.0).

Results

IL-6 family cytokines versus tumor characteristics in patients presumed radically treated

The patients ($n = 109$) were divided into two groups, those with a low (IL-6 < 8 pg/ml) vs. high (IL-6 ≥ 8 pg/ml) IL-6 values at diagnosis. The groups did not differ in RCC subtype, tumor size, pathological stage, nuclear grade or other known predictive factors. Histological positive

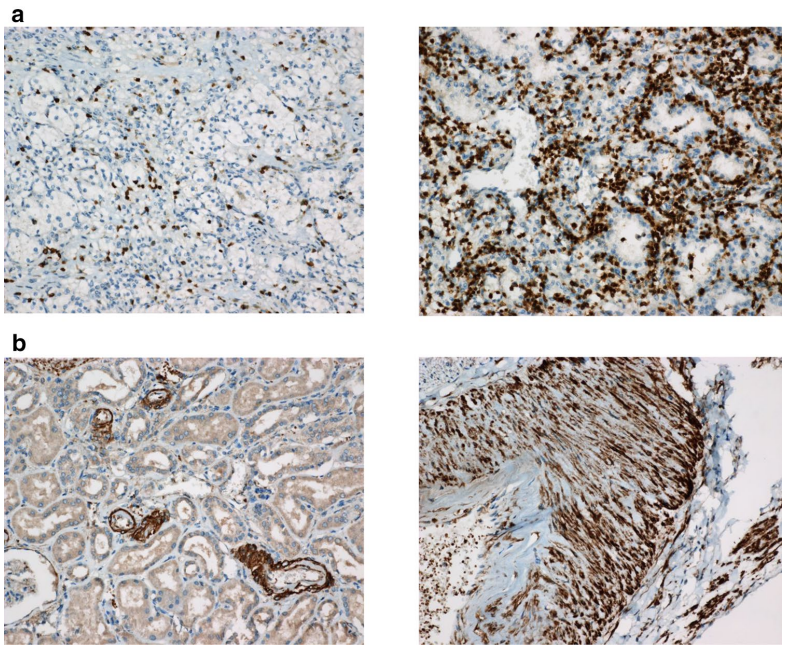

Fig. 1 **a** The panels show low (left) and high (right) scores with tumor area CD3 staining lymphocytes. **b** The panels show renal tissue outside the tumor: to the left small arteries showing IL-6 positivity, and to the right interlobular artery showing a strong IL-6 expression in medial smooth muscle cells

margins ($p = 0.05$) and pT stage ($p = 0.054$) differences were borderline differentiating between the patient groups (Supplementary Table 2). Immunohistochemistry was done in patients with high Il-6 in serum (Fig. 1).

IL-6 family cytokines and soluble receptors recurrence prediction

IL-6 levels predicted recurrence, both by Kaplan–Meier survival analysis ($p = 0.001$) (Fig. 2a) and utilizing a Cox multivariate regression analysis, with age, gender and tumor size additionally included as covariates (HR 7.13, CI 2.23–22.8; $p = 0.001$) (Table 1). IL-27 showed a significant prediction of recurrence, analyzed by Kaplan–Meier analysis ($p = 0.026$) (Fig. 2b) and multivariate Cox regression analysis, with covariates being age, gender and tumor size (HR 6.89; CI 1.56–30.4; $p = 0.011$) (Table 1).

If both IL-6 and IL-27 were included to one Cox multivariate regression analysis, the recurrence of those presumably cured was predicted by IL-6 ($p = 0.004$), but not regarding IL-27 ($p = 0.082$) (Table 2).

If the patients were grouped by tumor size (±7 cm) and studied by Kaplan–Meier analyses, both IL-6 ($p = 0.014$) (Fig. 2c) and IL-27 ($p = 0.001$) (Fig. 2d) predicted recurrence among patients with large tumors (diameter > 7.0 cm). Regarding gp130 levels there was not a statistic significance ($p = 0.082$) (Fig. 2e).

IL-6 family cytokines and soluble receptors vs. DSS

IL-6 predicted DSS in both Kaplan–Meier analysis ($p < 0.001$) (Fig. 3a) and multivariate regression analysis, including gender, age and tumor size (HR 4.82; CI 1.96–11.86; $p = 0.001$) (Table 1). In regard to IL-27, there was a borderline DSS prediction in the Kaplan–Meier analysis ($p = 0.052$) (Fig. 3b).

If both IL-6 and IL-27 were included in one Cox multivariate regression analysis for DSS, only IL-6 levels were predicted (HR 20.7; CI 2.6–44.4; $p = 0.001$) (Table 2).

IL-6, gp130 and IL-6Rα were included to one DSS multivariate analysis that also included gender, age and tumor size in one Cox regression survival model. Subsequently, IL-6 ($p < 0.001$) and IL-6Rα ($p = 0.02$), but not gp130, showed survival prediction (Table 2).

If analyzed by tumor size, patients with a tumor diameter from a 4 to 7 cm IL-6 level predicted survival by Kaplan–Meier analysis ($p = 0.001$) (Fig. 3c). The same was the case with large tumors (tumor diameter > 7.0 cm) ($p = 0.02$) (Fig. 3g). When IL-27 levels were analyzed by size, it was determined that a survival prediction was found among the patients with large tumors (diameter > 7 cm) ($p = 0.025$) (Fig. 3d). Including only tumors > 7 cm, s-gp130 levels exhibited no survival prediction ($p = 0.09$)

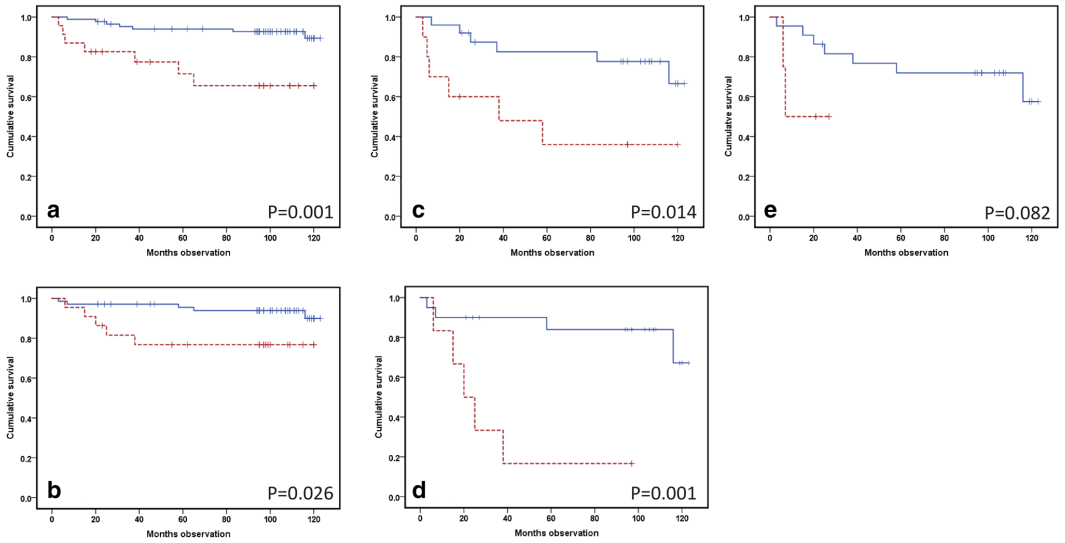

Fig. 2 Kaplan–Meier recurrence curves from IL-6 and some family members, as analyzed by Luminex in the serum of assumed radically treated renal cell carcinoma (RCC) patients, sampled prior to surgery. The blue line represents a low value, whereas the red dotted line indicates a high value. Differences between the groups are examined in log-rank tests and presented with *p* values. **a** IL-6 recurrence prediction among 109 RCC patients (low (< 8 pg/ml): $n = 86$ and high (≥ 8 pg/ml): $n = 23$). **b** IL-27 prediction of recurrence in 91 RCC patients (low: $n = 69$ and high: $n = 22$). **c**–**e** Recurrence prediction of IL-6, IL-27, and gp130 in patients with large (> 7 cm) RCC tumors (low: three lower quartiles)/high: highest quartile). **c** IL-6: $n = 35$ (25/10). **d** IL-27: $n = 26$ (20/6). **e** gp130: $n = 26$ (22/4)

Table 1 Recurrence and survival predictions from IL-6 and IL-27 in Cox regression analyses

	Univariate				Multivariate including age, gender and tumor size			
	HR	95% CI		*p* value	HR	95% CI		*p* value
		Lower	Upper			Lower	Upper	
Recurrence in presumed cured patients								
IL-6, $n = 109$	4.99	1.74	14.3	0.003	7.13	2.23	22.8	0.001
IL-27, $n = 91$	3.77	1.08	13.2	0.038	6.89	1.56	30.4	0.011
Disease-specific survival in all included patients								
IL-6, $n = 118$	4.97	2.06	12.0	< 0.001	4.82	1.96	11.9	0.001
IL-27, $n = 97$	2.82	0.95	8.40	0.062	3.02	0.94	9.64	0.063
Overall survival of all included patients								
IL-6, $n = 118$	2.81	1.46	5.40	0.002	2.99	1.54	5.81	0.001
IL-27, $n = 97$	2.05	0.94	4.50	0.072	1.98	0.86	4.57	0.108

HR hazard ratio, *CI* confidence interval

(Fig. 3e). The same was the case with the soluble sIL-6Rα levels ($p = 0.08$) (Fig. 3f).

IL-6 family cytokines and soluble receptors vs. overall survival (OS) with all patients included

In Kaplan–Meier analysis, IL-6 values predicted OS ($p = 0.001$) (Fig. 4a). In a Cox multivariate survival analysis, including the gender, age and tumor size of the patient, a significant survival prediction was still determined (HR 2.99; CI 1.5–5.81; $p = 0.002$) (Table 1). IL-27 showed no survival prediction with a Kaplan–Meier approach ($p = 0.066$) (Fig. 4b). Regarding Cox multivariate regression analysis (HR 1.98; CI 0.86–4.57; $p = 0.11$) (Table 1), OS was not predicted. The model was tested and was stable for HR with regard to the IL-6 and IL-27 groups.

Table 2 Outcome predictions from combined IL-6 and family cytokine members in Cox regression analyses

	Multivariate (only cytokine/receptor combined)				Multivariate including age, gender and tumor size			
	HR	95% CI		*p* value	HR	95% CI		*p* value
		Lower	Upper			Lower	Upper	
Recurrence in radically treated patients ($n = 91$)								
IL-6	6.64	1.80	24.4	0.004	25.5	3.04	213.4	0.003
IL-27	3.10	0.87	11.1	0.082	1.54	0.20	11.7	0.675
Disease-specific survival ($n = 97$)								
IL-6	7.47	2.26	24.7	0.001	10.8	2.62	44.4	0.003
IL-27	1.98	0.65	6.00	0.227	0.85	0.22	3.30	0.813
IL-6	10.1	3.07	33.4	< 0.001	20.7	5.25	81.4	< 0.001
IL-6Rα	0.17	0.021	1.31	0.089	0.068	0.007	0.66	0.020
gp130	1.11	0.24	5.12	0.889	2.64	0.45	15.4	0.281

HR hazard ratio, *CI* confidence interval

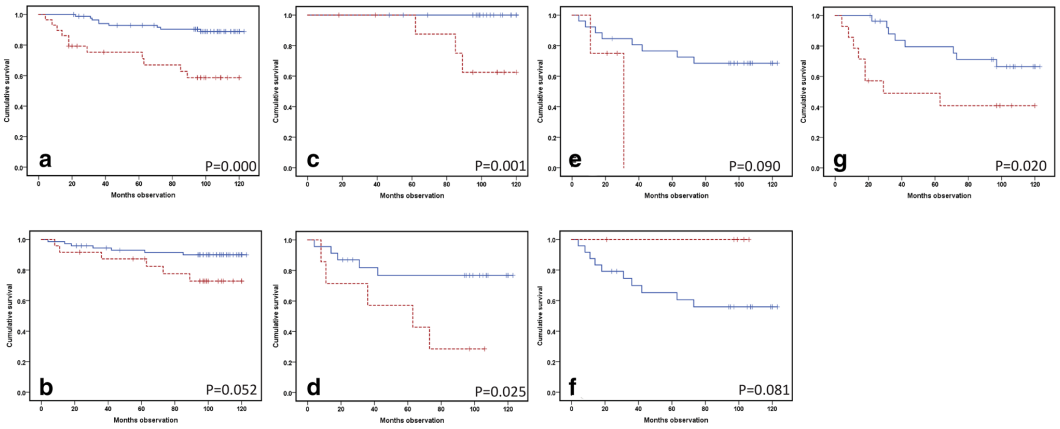

Fig. 3 Kaplan–Meier curves demonstrating disease-specific survival (DSS) prediction from IL-6, as well as related cytokines and receptors in pre-treatment RCC blood samples collected before surgical treatment. Analytes were measured simultaneously by Luminex technology. Low values are shown with a blue continuous line, and high values in red dotted lines. In addition, the graphs comprise *p* values from log-rank tests. **a** IL-6 DSS prediction among 118 RCC patients (low (< 8 pg/ml): $n = 89$ and high (≥ 8 pg/ml): $n = 29$). **b** IL-27 prediction of DSS in 97 RCC patients (low: $n = 73$ and high: $n = 24$). **c** and **g** DSS prediction from IL-6 in medium-sized (4.1–7 cm, $n = 37$) and large (> 7 cm, $n = 42$) RCC tumors. The highest quartile is denoted by high ($n = 10/14$), and the remaining values low ($n = 27/23$). **d**–**f** IL-27, gp130 and IL6R alpha prediction of DSS in patients harboring a large (> 7 cm) RCC tumor ($n = 30$). Quartiled analytes as above (high: $n = 7/4/6$ and low: $n = 23/26/24$)

If the patients were grouped by tumor size, the IL-6 values in particular predicted survival among patients with medium-sized tumors (tumor diameter from 4 to 7 cm) ($p = 0.018$) (Fig. 4c), but not statistically significant ($p = 0.063$) among large tumors (Fig. 4e). If gp130 levels were studied in patients with large tumors only, a high gp130 level predicted a lower survival ($p = 0.001$) (Fig. 4d).

Outcome dependent on IL-6 levels at the individual patient level

We detected IL-6 > 8 pg/ml in 29 patients: six of those with metastasis at the time of diagnosis, with seven of the remaining 23 patients presumed radically treated having had a subsequent RCC recurrence. Of those patients with a low IL-6 who died, or developed recurrent RCC disease ($n = 10$), only one had a RCC tumor < 7 cm at diagnosis. Five of the 10 patients with a high IL-6 who were still alive and without disease recurrence at the study closure,

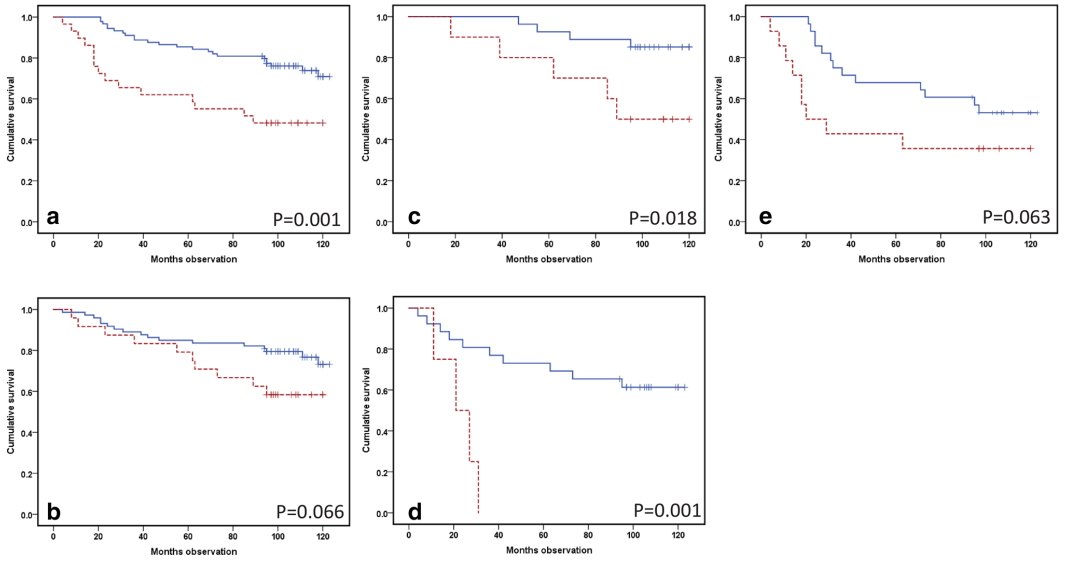

Fig. 4 Overall survival (OS) Kaplan–Meier curve predictions from IL-6 and the family molecules IL-27 and gp130 by Luminex in serum collected before the surgical treatment of RCC patients. The blue continuous line visualizes low values (lower quartiles), with the high values in the red dotted line. Log-rank test p values are included in the separate windows. **a** IL-6 OS prediction among 118 RCC patients (low (<8 pg/ml): $n=89$ and high (≥8 pg/ml): $n=29$). **b** IL-27 prediction of OS in 97 RCC patients (low: $n=73$ and high: $n=24$). **c** OS prediction from IL-6 in medium-sized [(4.1–7 cm, $n=37$), $n=42$] RCCs. Twenty-seven patients were designated as low, with 10 patients having a value within the highest quartile. **d** Prediction of OS from quartiled gp130 in RCC patients with a tumor size exceeding 7 cm (low: $n=26$ and high: $n=4$). **e** IL-6 OS prediction in large (>7 cm, $n=42$) RCC tumors; 28 high value patients and 14 with a low value

Table 3 Description of immunohistochemical analyses, staining assessment and numbers

	−=0.0	±=0.25	±=0.5	+=1.0	+(+)=1.5	++=2.0	++(+)=2.5	+++=3.0
CD3-positive tumor lymphocytes	1	0	5	11	4	2	4	1
CD3-positive lymphocytes in interphase zone	3	0	7	10	2	4	1	1
CD68-positive cells in tumor	0	0	3	7	7	6	5	0
CD68-positive interphase zone cells	14	1	5	5	1	2	0	0
FoxP3 in tumor lymphocytes[a]	9	14	2	2	0	0	0	0
FoxP3 in interphase zone lymphocytes[a]	9	8	6	3	0	1	0	0
FoxP3 in tumor cells[a]	25	2	0	0	0	0	0	0
IL6 in tumor lymphocytes	18	2	4	4	0	0	0	0
IL6 in interphase zone lymphocytes	16	10	2	0	0	0	0	0
IL6 in tumor cells	5	8	6	4	2	3	0	0
IL6 in vasculature	1	2	1	3	2	6	1	12
IL6 receptor in tumor lymphocytes	5	13	6	1	0	3	0	0
IL6R in interphase zone lymphocytes	3	12	6	5	0	2	0	0
IL6R in tumor cells	1	10	6	7	4	0	0	0

Patient samples ($n=28$) were scored in a semi-quantitative fashion, reviewed by an expert in pathology (LB) and further transformed into numeric values for statistical analyses according to the following: +++ =3, ++(+)=2.5, ++=2, +(+)=1.5, +=1, ±=0.5, =0.25, and − =0.0

[a]$n=27$

had either a second primary cancer or an autoimmune disease at diagnosis.

Outcome by ROC analyses

Both tumor diameter and IL-6 values predicted DSS and recurrence. According to IL-6 for recurrence, estimated areas under the curve (AUC) were 0.723 ± 0.075 ($p = 0.007$) and 0.692 ± 0.074 ($p = 0.020$), employing presumed radically treated or all patients, respectively. Regarding IL-27, the corresponding AUC results were 0.762 ± 0.080 ($p = 0.007$) and 0.757 ± 0.079 ($p = 0.008$), respectively (Fig. 5). Including only presumed radically treated patients and with large tumors (diameter > 7 cm), the AUC were 0.908 ± 0.069 ($p = 0.001$) in the case of IL-27, and 0.707 ± 0.098 ($p = 0.048$) in the case of IL-6 (Fig. 5).

IL-6 and IL-27 prediction of recurrence adjusted by Leibovich scores

IL-6 and IL-27 levels were studied by Cox regression adjusted by Leibovich scores. The results showed that both IL-6 ($p = 0.01$) and IL-27 ($p = 0.014$) still predicted survival following such an adjustment (Supplementary Table 3).

Patients with high serum IL-6: outcome compared to tumor and tumor border (interphase) tissue CD3, CD68, IL-6 and IL6R-positive cells determined by immunohistochemistry

By immunohistochemistry, we have determined the level of CD3, FoxP3, CD68, IL-6 and IL6R-positive cells in tumors from patients with high IL-6 serum values (Table 3). The following number of patients with at least a 10% (1 +) expression on markers denoting cell *characteristics* were found at least at 1 + levels: intra-tumor CD3 + lymphocytes: 22/28, interphase zone CD3 + lymphocytes: 18/28, intra-tumor CD68 + cells: 25/28, interphase zone CD68 + cells: 8/28, FoxP3 + intra-tumor lymphocytes: 2/27, FoxP3 + interphase zone lymphocytes: 4/27 and FoxP3 + tumor cells: 0/27.

Regarding the present IL-6 content of the various tumor-associated cells, the following were determined: intra-tumor lymphocytes: 4/28, interphase zone lymphocytes: 0/28, tumor cells 9/28 and most density was seen in vascular cells:

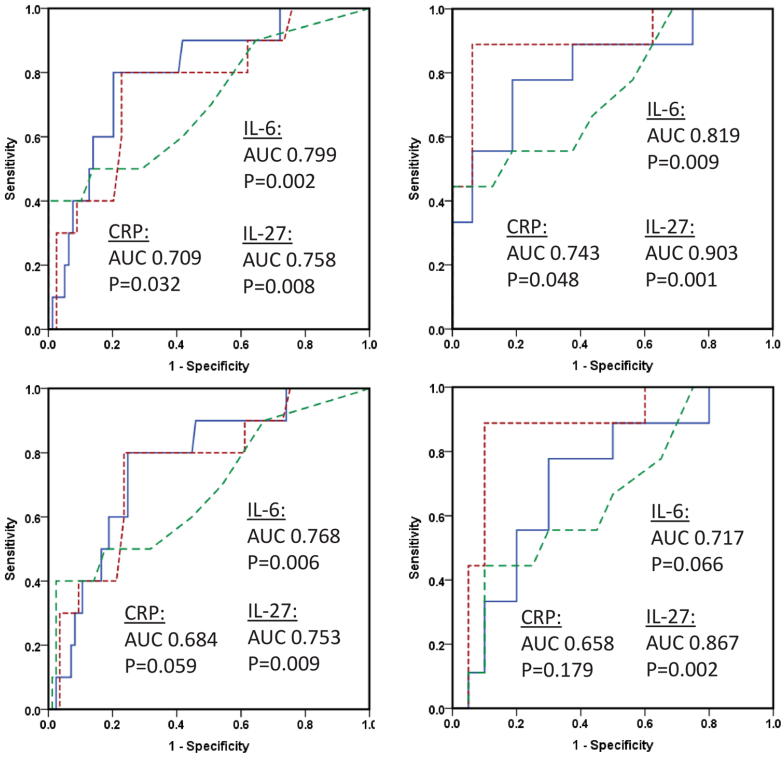

Fig. 5 ROC recurrence. Receiver operating characteristic (ROC) curves comparing recurrence prediction of IL-6, IL-27, and CRP in presumed radically treated (upper panel, $n = 89$) and all (lower panel, $n = 95$) RCC patients with such values, as analyzed in their serum ahead of surgical treatment. In both cases, 10 were regarded as positive. The right column shows results in patients with a tumor above 7 cm, of which nine had a positive annotation. The blue continuous line represents IL-6, the red dotted line is IL-27, and green semi-hatched line indicated by CRP

IL6 24/28. In the case of the IL6R, the following numbers were denoted: intra-tumor lymphocytes 4/28, interphase zone lymphocytes: 7/28 and tumor cells: 11/28.

The cellular derived measurement did not substantially correlate to tumor diameter or CRP levels. The various above-mentioned variables were also tested regarding prognostic value. In particular, the extent of T lymphocytes (CD3 + cells) infiltration in the tumors predicted survival. A high CD3 + value predicted a decreased survival. This was valid concerning recurrence ($p = 0.017$) and DSS ($p = 0.032$), but not for OS (Fig. 6).

Discussion

High IL-6 and IL-27 serum levels predicted a worse prognosis. Among clinically presumed radically treated patients, a high IL-6 and high IL-27 strongly predicted a recurrence in both univariate and multivariate analyses. IL-6 also predicted DSS and OS. Overall, the predictions among patients with large tumors (diameter > 7 cm) were excellent regarding both high IL-6 and IL-27 values. Of the soluble receptors studied, high gp130 serum levels predicted a worse OS among the RCC patients with large tumors.

The cytokine levels best predicted survival among patients with large tumors. These findings support that a high cytokine value points to a biologically aggressive tumor, more than a low differentiated tumor [8, 10, 24].

Patients with high levels of IL-6, but only with a clinically localized disease, were more likely to die from their RCC, and also had an increased risk of dying of any cause compared to those with low IL-6 serum values. Our findings are in agreement with those of Ljungberg et al. [25], Hrab et al. [26] and Blay et al. [27].

Increased IL-27 levels predicted recurrence and DSS, especially among patients with a tumor diameter > 7 cm. To the best of our knowledge, this has not been shown in other published studies. Only one study has thus far been published on IL-27 and RCC, which showed that patients with specific polymorphisms of IL-27 are more susceptible to RCC [17].

Soluble IL-6Rα may bind to IL-6, and secondarily bind to gp130 receptors on the surface of cells, in this way stimulating pro-inflammatory functions through *trans*-activation. On the other hand, *cis*-activation is mostly immune inhibitory [28]. We have not shown negative prognostic effects of increased serum IL-6Rα among RCC patients, supporting that IL-6 promotes inflammation in RCC tumors as a mechanism of IL-6-driven carcinogenesis. Soluble gp130 binds the soluble IL-6/IL-6Rα complex [29], and presumably acts as an IL-6/IL-6Rα decoy receptor [28]. We have demonstrated a negative prognostic value of increased gp130 in the serum of patients with larger tumors, which is the opposite of what should be expected. However, gp130 is present on most cells [28], and the increased serum soluble gp130 may be caused by generally increased tumor cellular turnover, which then basically drives the *worse* prognosis.

The combined effect of soluble IL-6Rα, gp130 and IL-6 as to prognosis seems to be complex. Regarding small tumors, the results are as expected, but concerning larger tumors s-gp130 levels paint another picture more consistent with that reflected by s-gp130 levels, e.g., cellular proliferation. Furthermore, the similar survival prediction of IL-6 and IL-27 suggests that this association is limited to cells actually carrying the IL-6 receptor on the surface, as no soluble IL-27 receptor has so far been recognized.

IL-6 and the IL6R may also be determined in tumor tissue [20]. Fu et al. [20] have shown that the expression on tumor cells of IL-6/IL6R worsens the prognosis. We have verified that both the IL-6 and IL6R may be found on cancer cells from RCC patients with high IL-6. Hence, it is supported that IL-6 may act directly on the tumor with a subsequent

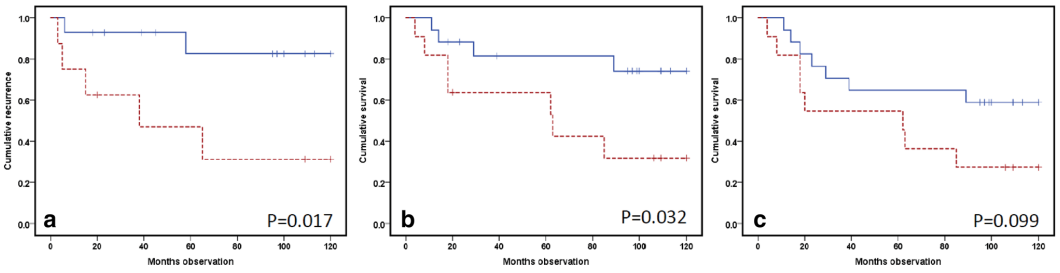

Fig. 6 Outcome predictions: **a** recurrence, **b** disease-specific survival, and **c** overall survival of total 28 RCC patients from immunohistochemical quantification of tumor CD3-positive T lymphocytes in surgical resection specimen. The expression levels were qualitatively scored by an experienced pathologist and further dichotomized by median value into high (All patients: $n = 11$/radically treated: $n = 8$) and low ($n = 17$ for all patients/$n = 14$ for radically treated). *p* values come from log-rank tests

worse RCC prognosis, both in an autocrine and paracrine manner [30].

We have also shown that among patients with a high IL-6, a surprisingly high expression of IL-6 was found in vascular cells, i.e., endothelial and smooth muscle cells, thereby suggesting that these cells produce IL-6. Endothelial cells are presumably stimulated by VEGF from the tumor [31], with this representing a possible loop where the tumor may become autocrine stimulated.

High IL-6 values in serum also signal a worse OS, and as such, IL-6 values are coupled with many serious diseases [32]. IL-6 is elevated in hypertension, as well as being associated with a higher incidence of future cardiovascular events and mortality [33]. This may partly explain the shown overall survival prediction.

Moreover, we have studied levels of T lymphocyte tumor infiltration and presence in the tumor periphery in a sub-group of patients selected by high IL-6 serum levels. A high T lymphocyte count predicted an increased recurrence and decreased survival. Nevertheless, T regulatory lymphocytes, i.e., Fox P3 lymphocytes, were not to any extent found within the tumor. This is in line with what has previously been shown in head and neck squamous cell carcinomas [34], namely that survival prediction in solid tumors is likely dependent on several immune-related dimensions, like presently one associated with general inflammation through IL-6, and another associated with specific immunity though T lymphocytes [35].

RCC survival prediction is expected to be secondary to factors like the ones included in the Leibovich scores. Leibovich is a composite score, including tumor size, pathological T and N stage, Fuhrman nuclear grading and histological necrosis [36]. With the IL-6 and IL-27 recurrence prediction adjusted by the Leibovich score, both of these cytokine levels still predicted survival.

Clinically, the present results may be relevant. When applying 8 pg/ml IL-6 levels as a cut-off between high and low IL-6 values, 29 patients had high IL-6 values. Six out of nine patients with detectable metastasis at diagnosis had high IL-6 values, as had seven of 14 individuals who subsequently developed RCC metastases. *Several other* patients presumably had other specific causes of their increased IL-6. Of those patients with a low IL-6 who died, or developed recurrent RCC disease ($n = 10$) only one had a RCC tumor with a diameter < 7 cm at diagnosis. The IL-6 values may therefore be utilized at the individual level to sort patients with both a high and low risk of dying because of RCC disease.

Furthermore, ROC analyses suggested that a high IL-27 and IL-6 score predicted a recurrence with both a high sensitivity and specificity, especially as measured in patients with larger tumors. Thus, we have demonstrated that IL-6 and IL-27 may be utilized as biomarkers to identify both a high- and low-risk recurrence of RCC patients at the time of diagnosis.

Patients with high IL-6/IL-27 values at diagnosis may be good candidates for adjuvant treatment with, e.g., VEGF inhibitors [37], as well as with anti-IL-6 therapy such as Siltuximab [38]. The agent Siltuximab (αIL-6) has shown promising results in phase I/II studies for metastatic RCC [38]. It is even possible that a combined blockage of IL-6/IL-27/VEGF would have achieved better results. The results of our study also demonstrate the need for future clinical studies of therapies investigating blockage of gp130 pathways, i.e., bazedoxifene, which blocks p-STAT3 inhibitor [39], and also combined with other blockers like VEGF-TKIs [40] to prolong survival in patients with RCC [41]. However, it should be borne in mind that babies born with a defect gp130 receptor may suffer from extended Stüve-Wiedemann syndrome, which is a serious, often lethal syndrome [42]. Thus, to block gp130 may have serious side effects, making such treatment impossible. Our results also add to knowledge inspiring T cell boosting therapy to be further developed. In any case, the role of IL-27 biology in RCC should be studied judged against the background that new templates for biological therapy in RCC therapy are urgently needed [43].

This study includes a limited number of patients. Therefore, the analyses, especially on the sub-group level showing negative results, must be interpreted with caution. We have measured the cytokines and soluble receptor levels just once. In particular, cytokines in the blood may have a short half-life [44], as a broader picture could have been painted with additional measuring points.

Conclusions

IL-6 and IL-27 have been shown to have a role in RCC biology through the predictive ability of recurrence and disease-specific survival in otherwise radically treated RCC patients. We believe that patients with a high IL-6 and IL-27 will be good candidates on which to base a biological therapy of RCC. Finally, both these cytokines hold promise for being important in relation to risk stratification regarding RCC prognosis, and thereby a need for treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

Ethics approval The study was approved by the Regional Committee for Medical Research Ethics in Western Norway (78/05), and the database was approved by the Norwegian Social Science Data Services.

Consent to participate All patients were given oral and written information about the study, and they gave written informed consent.

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Supplementary Table 1: Immunohistochemistry

Antibody	Source	Epitope retrieved	Dilution	Incubation time (min)	Detection kit
CD3 (A0452)	DAKO	CC1, 36 min	1:100	32	UV
CD68 (KP1,M0814)	DAKO	CC1, 64 min	1:5000	32	UV
FOXP3 (560044, clone:259D/C7)	BD Biosciences	CC1, 64 min	1:20	32	UV
IL6 (ab 9324)	Abcam	CC1, 48 min	1:200	120	OV
IL6R (ab 128008)	Abcam	CC1, 48 min	1:800	32	OV

Supplementary Table 2: Comparison of tumor characteristics between IL-6 low and high groups (cut-off 8 pg/ml) in those assumed to be radically treated.

Variable	All patients (n=109)(%)	IL-6 low (n= 86)(%)	IL-6 high (n=23)(%)	p-value
RCC subtypes				
Clear Cell	83(76)	64(74)	19(83)	0.43
Papillary	14(13)	10(12)	4(17)	
Chromophobe	6(5.5)	6(7)	0(0)	
Multilocular cystic	5(4.5)	5(6)	0(0)	
Others/ Unclassified	1(1)	1(1)	0(0)	
Size in cm (range)	5.3(1.2-18)	5.0(1.2-18)	6.3(1.6--15)	0.10
pT – Stage[1]				
pT1a	52(48)	44(52)	8(35)	0.054
pT1b	26(24)	22(26)	4(17.5)	
pT2	17(16)	13(15)	4(17.5)	
pT3	10(9)	5(6)	5(21.5)	
pT4	3(3)	1(1)	2(8.5)	
Nuclear grade[2]				
G1-G2	62(57)	51(60)	11(48)	0.71
G3-G4	46(43)	34(40)	12(52)	
Tumor thrombi				
Present (n=109)	6(6)	5(6)	1(4)	0.78
Positive Margin Present (n=109)	3(3)	1(1)	2(9)	0.05
Histologic necrosis Present (n=109)	32(30)	23(27)	9(39)	0.25
Sarcomatoid components Present (n=109)	4(4)	3(3)	1(4)	0.85

[1]UICC TNM 2009 version

[2] 108

Supplementary Table 3: Cox multivariate regression among patients with recurrence (date) following presumed radical treatment dependent on IL-6 (N=108)/IL-27 (N=91) values adjusted by Leibovich score.

	Hazard ratio	*95% Confidence Interval*		*p-value*
		Lower	*Upper*	
IL-6	1.036	1.008	1.064	0.010
Leibovich group (low vs. intermediate)	7.073	1.367	36.60	0.020
Leibovich group (low vs. high)	27.72	5.554	138.3	<0.001
IL-27	1.002	1.000	1.004	0.014
Leibovich group (low vs intermediate)	32.20	1.681	616.6	0.021
Leibovich group (low vs high)	103.2	6.419	1658.0	0.001

Paper III

cancers

MDPI

Article

The Biological Context of C-Reactive Protein as a Prognostic Marker in Renal Cell Carcinoma: Studies on the Acute Phase Cytokine Profile

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Abstract: High serum levels of the acute phase protein C-reactive protein (CRP) are associated with an adverse prognosis in renal cancer. The acute phase reaction is cytokine-driven and includes a wide range of inflammatory mediators. This overall profile of the response depends on the inducing event and can also differ between patients. We investigated an extended acute phase cytokine profile for 97 renal cancer patients. Initial studies showed that the serum CRP levels had an expected prognostic association together with tumor size, stage, nuclear grading, and Leibovich score. Interleukin (IL)6 family cytokines, IL1 subfamily mediators, and tumor necrosis factor (TNF)α can all be drivers of the acute phase response. Initial studies suggested that serum IL33Rα (the soluble IL33 receptor α chain) levels were also associated with prognosis, although the impact of IL33Rα is dependent on the overall cytokine profile, including seven IL6 family members (IL6, IL6Rα, gp130, IL27, IL31, CNTF, and OSM), two IL1 subfamily members (IL1RA and IL33Rα), and TNFα. We identified a patient subset characterized by particularly high levels of IL6, IL33Rα, and TNFα alongside an adverse prognosis. Thus, the acute phase cytokine reaction differs between renal cancer patients, and differences in the acute phase cytokine profile are associated with prognosis.

Keywords: renal cell carcinoma; acute phase reaction; C-reactive protein; IL33Rα; IL1 family; IL6 family; tumor necrosis factor α

1. Introduction

Renal cell carcinoma is a common malignancy and among the most lethal genitourinary cancers [1]. Standard treatment involves partial or radical nephrectomy for local tumors, whereas targeted therapies can be considered for metastatic disease [2,3]. The systemic serum levels of several cytokines, especially Interleukin (IL)6 are associated with prognosis in several urogenital cancers [4–6], including renal cell carcinoma [7,8]. IL6 belongs to the IL6 cytokine family. These cytokines utilize glycoprotein 130 (gp130) for intracellular signal transduction [9]. IL6 along with other family members are regulators of the acute phase reaction and initiate intracellular signaling either through the cytokine binding to the complete

membrane receptor (classical signaling) or through the binding of the soluble cytokine-receptor complex to membrane-expressed gp130 (trans signaling) [9]. Thus, IL6 family cytokines form an interacting network of soluble mediators, including the cytokines themselves together with their membrane-bound and biologically active soluble receptor chains.

The acute phase reaction is a systemic response that accompanies acute and chronic inflammation. It is triggered by tissue damage and characterized by the altered serum levels of several inflammation-regulatory proteins, including C-reactive protein (CRP), and can be induced by IL6 family cytokines, as well as IL1β and tumor necrosis factor (TNF)α [10,11]. CRP binds a wide range of exogenous and endogenous ligands; these complexes bind to Fc or CD14/Toll like receptors (TLRs), thereby leading to a systemic plasma protein response involving several cytokines that reinforce the initial CRP-inducing cytokine response [11]. IL6 is important for the release of several acute phase proteins. The overall acute phase protein profile differs between various inducers, and other IL6 family cytokines have effects similar to IL6 [10,12].

IL1 α/β are members of the IL1 subfamily of the IL1 cytokine family and are important in the acute phase reaction together with the soluble IL1 receptor antagonist (RA) [13–16]. The release of IL1RA by hepatocytes as part of the acute phase reaction is at least partly regulated by IL6 [17].

IL33 is another member of the IL1 subfamily of cytokines, and the soluble IL33 receptor α chain (IL33Rα) should also be regarded as an acute phase protein [18–21]. IL33 binds to IL33Rα, which forms a dimer with the signal-initiating IL1RAcP co-receptor [22]. The same co-receptor is utilized by the IL1 receptor chain [22]. The soluble IL33 receptor IL33Rα (sIL33Rα) is a decoy receptor that shows altered systemic levels in several diseases [23,24] and is identical to the extracellular region of the membrane-bound (referred to as IL33Rα long or IL33RαL) chain, except for five additional amino acids [25–28]. A third IL33RαV variant, with another hydrophobic tail and lacking one extracellular domain, also exists [29]. IL33RαL is expressed by various cells, including epithelial, endothelial, and immunocompetent cells [25,30]; IL33RαV is expressed by certain epithelial and immune cells [25]; sIL33Rα is released by several cells, including kidney and immunocompetent cells [25]; and IL33 is expressed mainly by non-hematopoietic cells [30]. Downstream receptor signaling involves MyD88 and several of its downstream pathways that ultimately target NF-κβ and AP-1 [30], but IL33 can also bind to chromatin or directly inactivate NF-κβ [30–33]. Its final effect seems to be the stimulation of renal carcinogenesis [27].

Tumor diameter [34] and preoperative serum CRP levels are independent prognostic parameters in non-metastatic renal cell carcinoma; CRP thus serves as a marker of the acute phase reaction [35]. The aim of the present study was to characterize the heterogeneity of the cytokine-driven acute phase reaction (i.e., the biological context of CRP) in patients with renal cell carcinoma by investigating an extended pre-therapy acute phase cytokine profile that includes seven IL6 family members, IL1 subfamily members (IL33Rα and IL1β/IL1RA), and TNFα.

2. Results

2.1. Clinical, Biological, and Prognostic Characteristics of the Renal Cancer Patients

During a defined time period, 154 patients were surgically treated for renal cancer. They all gave their written informed consent but due to practical or technical reasons, a preoperative serum sample could be collected only for 118 patients. These 118 patients included 9 with metastatic and 109 with local disease. Our hospital is responsible for the treatment of all renal cancer patients for a defined geographical area, and our patient cohort represents all diagnosed patients from a defined time period. The characteristics of the whole patient cohort and for the patients only with non-metastatic disease are presented in Table 1, whereas the characteristics of the patients who could not be sampled preoperatively due to practical or technical reasons are given in Table S1.

Table 1. Clinical and tumor characteristics of patients with renal cell carcinoma scheduled for surgery during the period 2007–2010; the table presents the results for all 118 patients for whom a preoperative serum sample was available (unless otherwise specified) and for the patients with local renal cancer disease (i.e., without metastases). The results are presented as the median and interquartile (if specified) range for continuous variables, except for long-term survival. Categorical data are expressed as numbers with a percentage (or in specified cases interquartile range) in parenthesis.

Parameter	All Patients (n = 118)	Patients without Metastases (n = 109)
Age in years at diagnosis (interquartile range)	63.8 (55.1–2.5)	63.9 (55.4–73.5)
Gender; male/female	88 (74.6)/30 (25.4)	80 (73.4)/29 (26.6)
Charlson Comorbidity Index (interquartile range)	1 (0–2)	1 (0–2)
ASA score (interquartile range)	2 (2–2)	2 (2–2)
Surgical treatment		
Radical nephrectomy	80 (67.8)	71 (65.1)
Partial nephrectomy	38 (32.2)	38 (34.9)
Peripheral blood levels		
B-Hemoglobin (g/dL, n = 100/91)	14.2 (8.8–17.3)	14.0 (8.8–17.3)
Erythrocyte sedimentation rate (mm, n = 92/84)	13 (2–129)	14 (2–129)
S-creatinine (μM, n = 100/91)	76.5 (45–725)	77.0 (45–725)
S-calcium (mM, n = 99/90)	2.40 (1.96–3.00)	2.40 (1.96–3.00)
S-alkaline phosphatase (U/L, n = 96/87)	81 (45–527)	81 (45–527)
S-CRP (mg/L, n = 116/107)	3 (1–220)	3 (1–112)
Tumor size (cm) [1]	5.3 (1.9–17.5)	4.9 (1.9–16.8)
≤7.0	76 (64.4)	74 (67.9)
>7.0	42 (35.6)	35 (32.1)
Histology		
Subtype		
Clear cell	91 (77.1)	83 (76.1)
Papillary	14 (11.9)	14 (12.8)
Chromophobe	6 (5.1)	6 (5.5)
Multilocular cystic	5 (4.2)	5 (4.6)
Others/unclassified	2 (1.7)	1 (0.9)
Nuclear grade		
G1-G2	62 (52.5)	62 (56.9)
G3-G4	55 (46.6)	46 (42.2)
Unknown	1 (0.9)	1 (0.8)
Detectable metastases at the time of diagnosis [2]	9 (7.6)	Not relevant
Observation time (months) [3]	100 (4–120)	103 (11–120)
Long-term overall survival (mean, standard error) [4]	96.5 (3.5)	101.7 (3.3)
Long-term recurrence-free survival (mean, standard error) [4]	106.0 (3.0)	112.3 (2.3)

[1] Tumor size was measured on CT scans. The complete tumor-node-metastasis (TNM) staging of the patients included in the present cytokine study is given in Table S2 [36,37]. All patients with metastases had tumor diameters > 4 cm. [2] Clinical examination together with CT scans of the abdomen and chest were used to classify patients as with or without metastases. [3] Patients were observed from the time of diagnosis until death or until November 2018. [4] Median survival was not reached.

The whole patient cohort included nine patients with detectable metastatic disease at the time of diagnosis. The 109 patients with non-metastatic disease included 80 surviving patients, 11 patients who died from relapsed cancer, and 18 patients who died from other causes. The IL33Rα levels were determined for 96 patients and the other nine cytokines for 97 patients (one additional patient); six patients with metastatic disease were included for all the mediators. These 97 patients comprise of all patients who were sampled during the study period without additional selection. Our cohort included 70 survivors, six patients who died from their metastatic cancer disease detected at the time of diagnosis, 7 additional patients who also died from their renal cell carcinoma, and 14 patients who died from other causes.

We compared the clinical and biological parameters listed in Table 1 for potentially cured patients (i.e., no detectable metastases at the time of diagnosis) with those of cancer-free survivors and patients who later died from relapse/metastases. These last two groups differed significantly from the cancer-free survivors with regard to their serum CRP levels ($p = 0.003$), frequency of large tumors at the time of diagnosis ($p < 0.001$), and frequency of Fuhrman G3-G4 nuclear grading ($p = 0.001$). All these parameters are regarded as prognostic factors for renal cancer patients, and these differences are, therefore, expected [38–42]. Thus, these patient characteristics show that our cohort of renal cancer patients can be regarded as representative. The patients included in our cytokine studies were randomly selected from the 118 patients in the cohort.

2.2. The CRP Levels in Renal Cancer Patients; Strongest Associations with Tumor Characteristics, Weak Associations with Comorbidity, and Only Associated with IL6 among the Ten Cytokine Mediators

The acute phase reaction can be initiated by inflammation and tissue damage, but epidemiological studies have also demonstrated that the CRP levels in elderly individuals can be associated with frailty or comorbidity, i.e., they can be a part of the aging process [43–46]. We thus investigated whether the CRP level at the time of diagnosis was significantly associated with clinical characteristics, tumor characteristics, comorbidity scores, or cytokine serum levels (Table S3). The preoperative CRP levels showed the strongest associations with tumor stage (i.e., diameter; Kendall's τ 0.315) and the presence of necrosis in the tumor (Kendall's τ 0.332). The Eastern Cooperative Oncology Group (ECOG) performance status showed an association of borderline significance, whereas the Charlson comorbidity index and the American Society of Anesthesiologists (ASA) physical classification score showed no associations. Thus, the CRP level mainly reflected the characteristics of the malignant disease among the patients.

Preoperative serum CRP levels showed a correlation with IL6 levels (Kendall's τ 0.301, $p < 0.001$), whereas no significant correlation with CRP was seen for the IL1 subfamily mediators IL33Rα (Kendall's τ 0.173) or IL1RA (Kendall's τ 0.246). For the other IL6 family members and TNFα, the Kendall's τ value was generally lower (usually < 0.10) and/or associated with p-values > 0.10. IL6 is regarded as a major driver of the acute phase reaction [10], and an association between the CRP and IL6 levels is, therefore, not unexpected. The systemic IL1β levels were generally low with minor variations and undetectable levels in several patients; the detection of low IL1β levels is consistent with previous studies of cancer patients [47]. Thus, high IL6 levels are an additional phenotypic characteristic of the acute phase reaction for renal cancer patients with high CRP levels, whereas variation in the other acute phase cytokine mediators is not reflected by CRP in renal cancer patients.

2.3. Serum Levels of the IL1 Subfamily Mediators IL33Rα and IL1RA Show No Significant Correlation; Only IL33Rα Is Increased in Metastatic Disease, and Only IL33Rα is Associated with Survival

The preoperative serum levels of IL33Rα and IL1RA did not show any significant correlation. We also classified the patient subset with non-metastatic disease into three groups based on their IL33Rα/IL1RA levels: (i) both with levels above the corresponding median; (ii) only one of the mediators having a level above the median; and (iii) both levels below the corresponding median. These three patient subsets did not differ in their overall survival. Finally, neither the IL33Rα nor IL1RA serum levels showed any significant associations with ECOG performance status, ASA score, Charlson comorbidity index, tumor size, or Fuhrman nuclear grading.

The IL33Rα levels were significantly higher for patients with metastases ($n = 6$, median level 29,130 pg/mL, range 23,520–162,569 pg/mL) compared to the patients with non-metastatic disease ($n = 90$, median 22,656 pg/mL, range 7053–75,572 pg/mL, Wilcoxon's rank sum test, $p = 0.017$). We also classified our patients with non-metastatic disease based on their tumor stage. The IL33Rα levels for patients with large tumors (i.e., diameters > 7 cm) differed significantly from patients with metastatic disease ($p = 0.038$) but not from the patients with non-metastatic disease and small tumors (Figure 1). In contrast, the IL1RA levels for patients with metastatic disease ($n = 6$, median 802 pg/mL, range

335–1607 pg/mL) did not differ significantly from those of patients without metastases (n = 91, median 684 pg/mL, range 281–2711 pg/mL), and IL1RA also did not differ between patients with non-metastatic disease and those with metastatic disease or between those with small versus large tumors without metastases. Thus, IL33Rα and IL1RA belong to the same IL1 cytokine subfamily and should both be regarded as acute phase mediators. Nevertheless, these two mediators differ in metastatic versus non-metastatic disease and thereby contribute to the heterogeneity of the acute phase cytokine reaction in renal cancer patients.

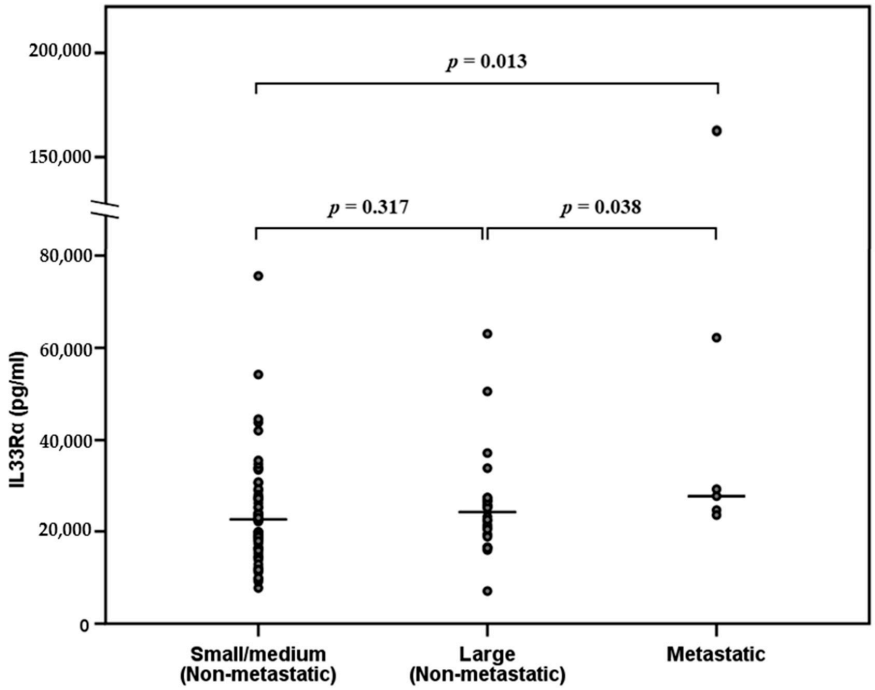

Figure 1. Preoperative IL33Rα serum levels in patients with renal cell carcinoma; a comparison of patients with small tumors (≤7 cm in diameter) with no metastases, with large tumors (>7 cm) and no metastases, and metastatic disease.

We thus investigated the association between survival and the IL33Rα level, CRP level, Leibovich score, tumor size, Fuhrman's nuclear grading, ASA score, and age via univariate Cox prediction analyses. We then examined the death from renal cancer and overall survival for the patients who were classified as radically treated after surgery (Table 2). IL33Rα showed an association of borderline significance with cancer-related death, whereas highly significant associations were observed for the tumor characteristics and CRP levels. For overall survival, significant associations were seen for the tumor characteristics, serum CRP, and patient age, whereas IL33Rα did not reach significance. Finally, IL1RA showed no significant associations with cancer-related death or overall survival in the Kaplan–Meier or Cox analyses.

The Leibovich score is used for the prognostic evaluation of patients with renal cancer [38,40,42,48]. We, therefore, investigated the IL33Rα levels and Leibovich score using a multivariate analysis for patients with a clear-cell subtype of kidney malignancy. We had relatively few cancer-related deaths in our cohort, and for this reason, we included only these two parameters. Moreover, the Leibovich score was chosen because it includes several prognostic parameters, and the IL33Rα level remained significant when corrected for the Leibovich score (Table 3).

Table 2. Univariate Cox survival predictions in radically treated renal cell carcinoma patients using serum IL33Rα and CRP, as well as the included clinico-histopathological parameters. Values are given as the hazard ratio (95% confidence interval). The whole patient cohort included 109 patients, but the IL33Rα levels were analyzed for 90 randomly selected patients. Other values that differ from $n = 109$ are specified.

Variable	Disease-Specific Survival		Overall Survival	
IL33Rα (ng/mL), $n = 90$	1.05 (1.00–1.09)	$p = 0.034$	1.02 (0.99–1.06)	$p = 0.178$
CRP (mg/L), $n = 107$	1.03 (1.01–1.04)	$p = 0.011$	1.02 (1.01–1.04)	$p < 0.001$
Age	1.05 (0.99–1.11)	$p = 0.083$	1.07 (1.03–1.11)	$p = 0.001$
ASA score	1.43 (0.49–4.19)	$p = 0.510$	1.38 (0.71–2.68)	$p = 0.342$
Tumor size	3.40 (1.58–7.31)	$p = 0.002$	1.66 (1.15–2.39)	$p = 0.006$
Pathological TNM stage	4.53 (2.44–8.44)	$p < 0.001$	2.13 (1.43–3.18)	$p < 0.001$
Fuhrman nuclear grading, $n = 108$	2.51 (1.26–4.98)	$p = 0.009$	1.61 (1.22–2.11)	$p = 0.001$
Leibovich score, $n = 82$ *	4.03 (1.81–8.97)	$p = 0.001$	1.91 (1.19–3.08)	$p = 0.007$

* Patients with clear-cell renal cancer; value missing for one patient.

Table 3. The impact of IL33Rα for progression in 67 patients randomly selected out of 83 patients with clear-cell renal cell carcinoma assumed to be radically treated; a multivariate analysis including IL33Rα together with the Leibovich score. The results are presented as the hazard ratio (95% confidence interval) and *p*-values.

Variable	Progression-Free Survival	
IL33Rα (ng/mL)	1.07 (1.01–1.14)	$p = 0.020$
Leibovich, intermediate risk (score 3–5) *	26.9 (2.1–352.0)	$p = 0.012$
Leibovich, high risk (score ≥ 6) *	49.5 (4.3–576.0)	$p = 0.002$
Leibovich, overall	-	$p = 0.008$

* Compared to patients in the low-risk Leibovich group (score ≤ 2), with the maximum score being 11 [38].

2.4. The IL6 Cytokine Family Profile Identifies Patient Subsets That Differ in the Prognostic Impact of IL33Rα, Whereas the Impact of IL1RA/TNFα Does Not Differ

We investigated the serum levels of the IL6 family cytokines IL6, IL27, IL31, CNTF, and OSM, together with the soluble receptor components gp130 and IL6Rα. These IL6 family mediators form an interaction network through their overlapping receptor binding (with gp130 as a common signal-initiating receptor chain), common downstream intracellular signaling, and the potential for both classical and trans signaling (i.e., binding of the soluble receptor/ligand complex to membrane-expressed gp130) for several of these cytokines [9]. The overall results were investigated by hierarchical clustering analysis (Figure 2). CNTF and IL6 had the widest variation ranges among the included mediators. This analysis identified two main patient subsets that did not differ with regard to the serum levels of the IL6 family mediators, IL1 subfamily mediators, TNFα, or CRP. Finally, the number of patients dying from renal cancer (i.e., patients with metastases at diagnosis or later relapse) or dying from other causes did not differ between the two main patient clusters.

Each of these two main clusters was further divided into two subclusters characterized mainly by differences in their IL6 and CNTF levels, as indicated to the right in Figure 2. Patients included in the two sub-clusters are characterized by low or relatively low levels of IL6 and/or CNTF (Figure 2 right part, indicated by the blue color in the figure and referred to as $IL6^{low}CNTF^{low}$ patients). We first compared the soluble mediator levels for the $IL6^{high}CNTF^{high}$ and $IL6^{low}CNTF^{low}$ patients (Table S4). The systemic IL1RA levels were significantly higher for the $IL6^{low}CNTF^{low}$ patients (median 736 pg/mL, range 371–2710, Wilcoxon's test, $p = 0.027$) compared to the $IL6^{high}CNTF^{high}$ patients (656 pg/mL, range 280–1493). The systemic levels of CRP, TNFα, and other IL1 subfamily or IL6 family mediators did not differ significantly between these two patient subsets. Lastly, the number of patients dying

from renal cancer (i.e., patients with metastases at diagnosis or later relapses) or dying from other causes also did not differ between the IL6^{high}CNTF^{high} and IL6^{low}CNTF^{low} patients.

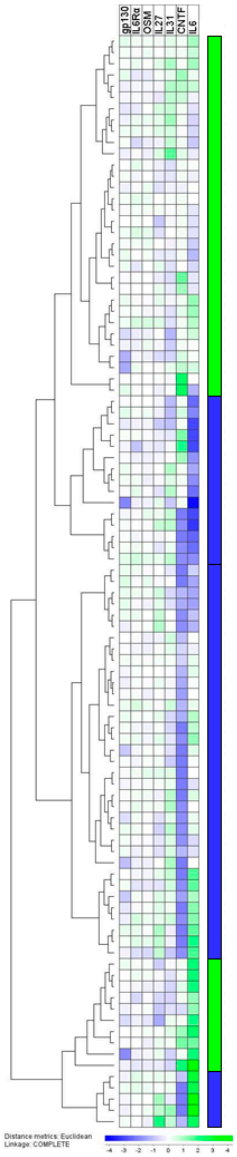

Figure 2. (see page 7). The serum profile of IL6 family cytokines in patients with renal cancer: a hierarchical cluster analysis. This analysis included the seven soluble mediators, IL6, IL6Rα, gp130, IL27, IL31, CNTF, and OSM. Cytokine/receptor is indicated at the top of the figure, and the patient clustering is shown to the left. This analysis created two main clusters (an upper large and a small lower cluster), and each of these two main clusters were further divided into one subset with low IL6/CNTF levels and one with relatively high levels of the two cytokines. Based on these results, we classified the patients into two main subsets referred to as CNTF^{high}IL6^{high} (see the right part, indicated by a green color) and CNTF^{low}IL6^{low} (right part, blue color).

We used Kaplan–Meier analyses to compare the associations between IL33Rα levels and cancer-related death (metastases or relapse) for the $IL6^{low}CNTF^{low}$ and $IL6^{high}CNTF^{high}$ patients (see Figure 2). Patients were classified into quartiles based on the IL33Rα variation range. Patients in the three lower quartiles showed a similarly low mortality for both the $IL6^{low}CNTF^{low}$ and $IL6^{high}CNTF^{high}$ subsets and were, therefore, classified together and compared with the patients in the highest quartile. The results are presented in Figure 3. A significant association between prognosis and IL33Rα levels was only observed for the $IL6^{low}CNTF^{low}$ patients, whereas such an association was not detected for the $IL6^{high}CNTF^{high}$ patient subset. Thus, the prognostic impact of a single acute phase mediator (i.e., IL33Rα) may differ between patient subsets identified by the acute phase cytokine profile (i.e., the IL6 family profile). This prognostic impact only for certain patients may also explain why IL33Rα levels did not differ when comparing the survivors and non-survivors in our whole study population (Figure S1).

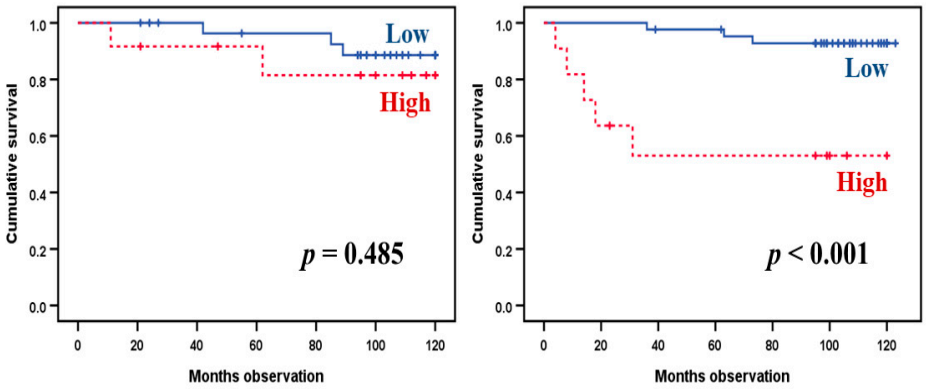

Figure 3. Comparison of kidney cancer-related death for the patient subsets identified in the hierarchical clustering analysis based on IL6 family mediators. As indicated in Figure 2, the 97 patients could be sub-classified into the two main subsets referred to as (left) $IL6^{high}CNTF^{high}$ and (right) $IL6^{low}CNTF^{low}$ subsets. The patients were classified into quartiles based on their IL33Rα serum levels, and we compared the survival of patients classified in the highest versus the three lowest IL33Rα quartiles. The $IL6^{high}CNTF^{high}$ (left) and $IL6^{low}CNT^{low}$ patients (right) were analyzed separately. The *p*-values are indicated in the figure images.

2.5. The Prognostic Impact of an Extended Acute Phase Cytokine Profile for Renal Cancer Patients

Our IL6 family cytokine profiling (Figure 2) clearly illustrates that the acute phase reaction in patients with renal cell carcinoma possessed heterogeneity that was only partly reflected in the CRP level. To further investigate the prognostic impacts of these differences on the acute phase profile, we performed a hierarchical clustering analysis based on TNFα, two IL1 subfamily mediators (IL1β was not used due to undetectable levels in many patients and only minor variations between patients), and the seven IL6 family members. Our present and previous studies suggest that IL33Rα is associated with the acute phase reaction. Moreover, previous studies have shown that the nine other mediators are involved in the regulation of the acute phase response (see Section 1). The results of this clustering analysis are shown in Figure 4. After this analysis, two main patient subsets were identifiable.

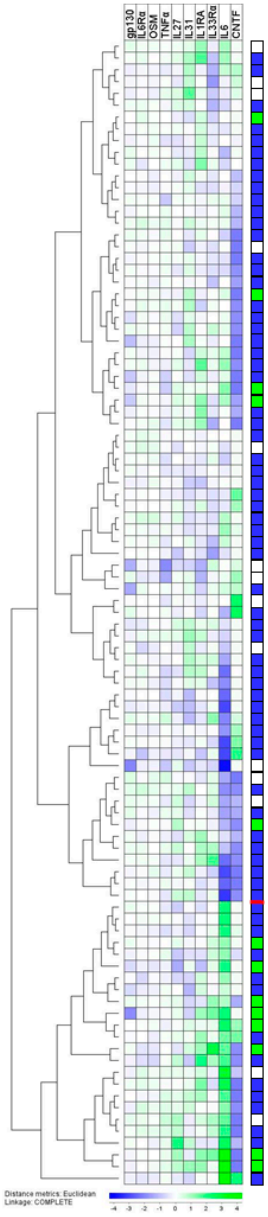

Figure 4. (see page 10). The serum profile of the acute phase cytokines in patients with renal cancer; a hierarchical cluster analysis including IL6 family cytokines (IL6, IL6Rα, gp130, IL27, IL31, CNTF, and OSM), two IL1 cytokine family mediators (IL1RA and IL33Rα), and TNFα. The mediators are indicated at the top of the figure, and the patient clustering is shown to the left. The survival of individual patients is summarized in the right part of the figure and shows patients still alive (blue), dead from renal cancer disease (green), and dead from other causes (white).

We performed a Kaplan–Meier survival analysis comparing the two main subsets identified in Figure 4. This analysis is presented in Figure 5. As shown, the two patient clusters differed in their

disease-specific survival. As expected from the results presented in Figure 4, the two main patient clusters did not differ in their overall survival, indicating that most of the patients (14 patients) died from other cases, and only 13 patients (six with metastases at the time of diagnosis) died from their malignant disease.

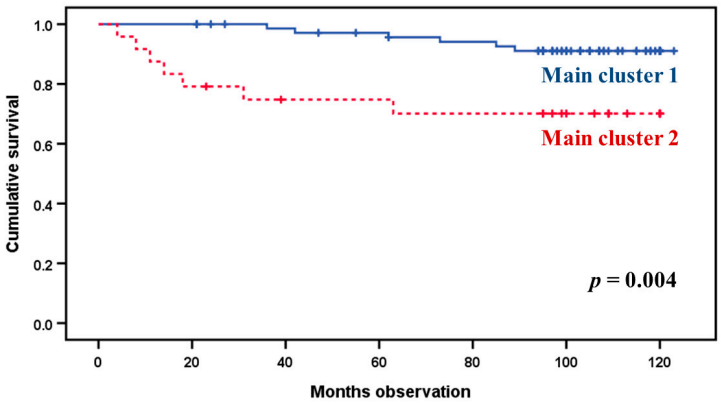

Figure 5. Comparison of cancer-related death for patients in the two main clusters identified in the hierarchical clustering analysis based on the systemic levels of 10 cytokine mediators (see Figure 4; upper main cluster 1, lower main cluster 2). All 97 patients were included in this comparison. A Kaplan–Meier analysis was performed, and the *p*-value for this comparison is indicated in the figure.

We ultimately compared the serum levels of all acute phase proteins, including the CRP levels, between the two main subsets identified (Table 4). The two subsets showed highly significant differences in their IL6, IL33Rα, and TNFα levels, whereas their IL1RA and CRP levels showed differences of only borderline significance. The IL6 and IL33Rα differences remained significant even after Bonferroni corrections. Thus, the two main patient clusters were mainly determined by the levels of the three mediators, and this sub-classification, therefore, was determined by acute phase characteristics that are only partly reflected in the serum CRP levels.

Table 4. The serum mediator levels in patients with renal cancer; a comparison of the two main patient subsets identified in the unsupervised hierarchical cluster analysis based on the seven IL6 family members (gp130, IL6Rα, IL6, IL27, IL31, OSM, and CNTF), two IL1 subfamily members (IL1RA and IL33Rα), and TNFα. The results are presented as the median level and variation range. The table presents the levels of the IL6 family members included in the clustering analysis together with the levels of IL1RA, IL33Rα, TNFα, and CRP.

Mediator (Concentration)	Upper Main Cluster (n = 73)	Lower Main Cluster (n = 24)	p-Value
gp130 (pg/mL)	92,745 (22,606–121,962)	88,475 (24,351–108,820)	0.332
IL6 Rα (pg/mL)	34,382 (17,789–48,588)	34,057 (22,510–46,610)	0.536
IL6 (pg/mL)	2.9 (0.0–16.3)	↑ 12.1 (0.5–73.2)	<0.001
IL27 (pg/mL)	673 (254–1173)	795 (367–2738)	0.188
IL31 (pg/mL)	196 (87–584)	160 (83–410)	0.058
OSM (pg/mL)	5789 (4500–7911)	5636 (3827–7003)	0.347
CNTF (pg/mL)	454 (98–2555)	274 (98–1961)	0.548
IL33Rα (pg/mL), n = 72/24	21,842 (7053–75,572)	↑ 26,652 (15,853–162,569)	0.001
IL1RA (pg/mL)	670 (281–2237)	↑ 876 (488–2711)	0.044
TNFα (pg/mL)	24.5 (6.8–37.2)	↑ 27.8 (18.1–37.9)	0.006
CRP (mg/L), n = 71/24	3 (1–19)	↑ 5 (1–220)	0.021

3. Discussion

The serum CRP level is a generally accepted prognostic factor for patients with renal cell carcinoma [41,49]. However, CRP is only one of several acute phase proteins, and the systemic serum profiles of acute phase cytokines (i.e., potential drivers of the acute phase reaction) seem to differ between patients and may also depend on the cause of the acute phase reaction [10]. In this context we investigated the acute phase cytokine profiles among a large group of patients with renal cancer admitted for surgical treatment.

As described above, our original cohort of 118 patients (109 without metastases) represents an unselected group of patients, i.e., the patients were derived from a defined geographical area during a defined time period and included all patients that could be sampled before surgery. The 97 patients included in our present study were randomly selected from this cohort. For this reason, we regarded our patients to be representative. Only a minority of the patients had advanced disease, while a majority of the patients had stage T1 tumors and tumor-node-metastasis (TNM) stage I (see Table S2). As expected, the cancer-free survival was high, but due to the high median age, several patients died from causes other than their cancer.

We cannot exclude the possibility that inflammaging (i.e., inflammation associated with aging, see [50]) or other chronic inflammatory diseases contributed to the observed acute phase reaction. However, we did not perform any additional selection of patients included in our present cytokine studies. Our present results should, therefore, be regarded as real-word data from a representative group of patients with renal cancer. We cannot exclude the possibility that the acute phase reaction in some of our patients may have been, at least partly, caused by inflammaging or nonmalignant chronic inflammatory diseases, but, despite this, we still detected a prognostic impact of the acute phase cytokine response when investigating our unselected patients. None of the survival analyses demonstrated different results with the inclusion of age, Charlson comorbidity index, ASA score, and ECOG performance status as co-variates.

Recent studies show that CRP is an important regulator in inflammation, but in clinical practice, it is used as a marker of both inflammation and the complex acute phase reaction [11]. The aim of the present study was to investigate the biological context of CRP (i.e., the acute cytokine network response and the acute phase reaction) in a representative cohort of patients with renal cancer. Our selection of mediators was based on previous studies showing that IL1, TNFα, and IL6 are important in the development of the acute phase reaction [10]. First, the IL1 cytokine family includes the IL1 subfamily [22] with the members IL1α/β and IL33, their receptors, and the antagonistic IL1RA. The IL1 and IL33 binding receptor chains co-localize with the same signal-initiating IL1RAcP co-receptor [22]. We, therefore, included IL1β together with its antagonist IL1RA in our present study [10,11,22]. In addition, we included the soluble IL33 decoy receptor IL33Rα because this biomarker should also be regarded as an acute phase protein (i.e., a systemic marker of inflammation) [18–21], but we did not include IL33 itself because it is produced by renal cancer cells and its local release is likely more important [51]. Second, we included TNFα, which is an acute phase cytokine and also important for the development of the acute phase reaction [10]. Finally, we investigated the levels of IL6 family members and soluble IL6 receptor components because IL6 is an important regulator of the acute phase reaction [10], and the systemic IL6 level also seems to have a prognostic impact on renal cancer [52–54]. We focused on the IL6 cytokine family profile because several such family members contribute to the regulation of the acute phase response. Their receptor binding partly overlaps, their intracellular signaling is similar, and several of them show both classical and trans signaling [9,10]. We then included IL6 family members that show systemic levels in a majority of immunocompetent and immunocompromised individuals [55,56].

We investigated a relatively large group of renal cancer patients that were randomly selected from a consecutive group of patients. Our patients should be regarded as representative in their clinical (e.g., age, performance status, and survival), biological (e.g., tumor and cancer cell characteristics), and prognostic parameters (e.g., Leibovich score, tumor characteristics, and CRP level). However, our

patients had a long follow-up time. For this reason, Fuhrman nuclear grading was used at the time of inclusion instead of the newly recommended system [41,57].

The prognostic impact of CRP shows that the acute phase reaction is important in renal cell carcinoma [39,58–60]. CRP is not only a marker but also a mediator with distinct biological functions [61–63]. However, the systemic acute phase reaction is a very complex response, and the aim of our present study was, therefore, to investigate the systemic levels of acute phase cytokines in renal cancer patients with a focus on the acute phase cytokine profiles, rather than those of single cytokines. IL6 family cytokines were included because they are important regulators of the acute phase reaction [10], but we investigated only IL6 family cytokines that usually show detectable serum levels [55,56]. IL1β/IL1RA and TNFα are important in the regulation of the acute phase response [10]. The inclusion of IL33Rα in our acute phase cytokine profile is justified by our present results, describing an association between IL33Rα levels and prognosis, and by those of previous studies showing that IL33Rα is an acute phase protein [18–21].

Studies on several malignancies (including renal cancer) suggest that the IL33/IL33Rα axis could be important in tumorigenesis through exerting direct effects on malignant cells [64] or indirectly through effects on stromal cells [65], including altering the regulation of tumor angiogenesis [66]. An association between serum IL33Rα levels and prognosis has been described for patients with hepatocellular carcinoma [67] and breast cancer [68]. A recent study also investigated serum IL33 levels and tumor IL33 expression via immunocytochemistry for renal cancer patients [27]. A high tumor expression of IL33 was then associated with advanced disease and an adverse prognosis; additional experimental studies showed that IL33 enhanced cancer cell growth and induced chemoresistance. A similar prognostic impact was described in another retrospective study that also assessed IL33 tumor expression via immunohistochemical staining [51]. However, yet another study described an adverse prognostic impact from the low renal cancer expression of IL33 at the mRNA level [26]. The use of different methodological approaches may explain this discrepancy. We also observed a possible prognostic impact of sIL33Rα independent of the Leibovich score, which is mainly based on tumor characteristics [38], but the low number of cancer-related deaths represents a limitation for the statistical analysis of patient survival in our present study. Finally, the immunoregulatory functions of IL33/IL33Rα may also be important for the effect of this axis on human malignancies, e.g., through induction of Treg cells or the inhibition of antigen presentation [26,33,69,70]. For these reasons and because of the similarities in downstream receptor signaling between IL1 and IL33, we included sIL33Rα in our acute phase cytokine profile together with IL1β and IL1RA. This was further supported by previous studies showing that IL33Rα is associated with prognosis in renal cancer and represents a systemic marker of inflammation [71].

We investigated IL6 family members that have detectable serum levels in most healthy individuals [55,56]. IL6 family cytokines have similarities in their receptor structures, with gp130 being the common signaling structure for all the receptors; in addition, some of the receptors can bind different IL6 family cytokines, and several family members are capable of both classical and trans signaling. For this reason, one should regard this family as the IL6 family network. We, therefore, focused on the IL6 family profile rather than on single family members. Even though several of these members seem to be involved in regulating the acute phase response, differences in the IL6 family profile could be used to identify patient subsets by hierarchical clustering analyses. However, the main subsets identified by hierarchical clustering based on IL6 family cytokines showed no association with CRP levels or patient survival (Figure 2). Finally, even though IL6 and CRP levels showed a significant correlation, the levels of these two cytokines did not differ when comparing the two main patient subsets (i.e., $IL6^{high}CNTF^{high}$ versus $IL6^{low}CNTF^{low}$ patients, see Figure 2). This is likely due to the impact of other IL6 family members (especially CNTF) in this IL6 family-based cluster analysis. This is also consistent with our observation that IL6 is the only cytokine biomarker presenting a significant correlation with CRP levels.

We ultimately performed a hierarchical clustering analysis based on TNFα, two IL1 subfamily members, and seven IL6 family members. Based on this overall acute phase cytokine profile, we identified two main subsets. These two subsets were not independent of the CRP level but differed significantly with regard to patient survival. The majority of patients dying from their malignant disease (metastatic disease at the time of diagnosis, later death from relapse) were included in a cluster characterized by especially higher levels of IL6, IL33Rα, and TNFα compared to the other main cluster, whereas IL1RA and CRP only showed differences with borderline significance. The IL6 and IL33Rα differences remained significant even after Bonferroni corrections. Thus, the overall clustering analysis based on an acute phase profile identified two main subsets. The patient survival differed between these two subsets, and this prognostic impact mainly reflected differences in IL6/IL33Rα/TNFα.

4. Materials and Methods

4.1. Patients

This retrospective biobank study was approved by the regional ethics committee (REK VEST 78/05) and the Norwegian Social Science Data Services; the study was conducted in accordance with the Declaration of Helsinki. Blood samples were collected after written informed consent from 118 consecutive patients with newly diagnosed renal cell carcinoma during the time period of 2007–2010 (median observation time 100 months, range 4–120 months). All patients were followed according to our risk-stratified follow-up program for surgically treated renal cell carcinoma [72]. The present study included 97 randomly selected patients from this cohort. The patient and tumor characteristics are presented in Table 1 and Table S2.

4.2. Analyses of CRP Levels

CRP levels were analyzed using the immunoturbidimetric method provided by Roche (Basel, Switzerland). During the entire period, the lower limit of detection for the serum CRP was 1 mg/L.

4.3. Blood Sampling and Cytokine Analyses

Peripheral venous blood samples were collected on the morning of the day of the planned renal cancer surgery. Samples were stored at room temperature for less than two hours before they were centrifuged. The serum was collected, aliquoted, and later stored frozen at −80 °C until being analyzed.

Samples derived from the 97 unselected patients were available for analyses. The samples were then thawed and centrifuged at 16,000× *g* for 4 min immediately before analysis. The IL6 levels were analyzed by a high-sensitivity ELISA kit (R&D Systems Europe Ltd., Abingdon, UK). Gp130, IL6Rα, IL27, IL31, OSM, IL1RA, and TNFα were determined using a Human Premixed Multi-Analyte Kit for Luminex technology (R&D Systems). IL33Rα was also determined using Luminex analyses (R&D Systems). A Human Pituitary Magnetic Bead Panel 1 was used to measure CNTF (EMD Millipore Corporation, Billerica, MA, USA). All analyses were performed strictly according to the manufacturer's instructions and the levels estimated by using a Luminex® 100™ (Luminex Corporation, Austin, TX, USA). All results are presented as the mean level of duplicate determinations.

One patient sample was included in all assays to evaluate the inter-platelet variation, but we did not detect any substantial differences between assays. The variation between duplicates was generally less than 10% of the mean concentration. Neither IL33Rα nor IL1RA levels showed any correlations with the sample storage time.

4.4. Statistical and Bioinformatical Analyses

The IBM® SPSS® Statistics software, version 25.0 (IBM Corp., Armonk, NY, USA), was utilized. A comparison of descriptive data was performed using cross-tables and an exact Chi-square test. A Mann–Whitney U test was used for a comparison between different groups, and Kendall's tau (τ)

was used for correlation analyses. Kaplan–Meier analyses were used for the percentage estimation of outcome prediction, including a Log-Rank test between groups. Cox proportional hazard models were also used for survival analyses. A p-value < 0.05 was regarded as statistically significant. Correction for multiple comparisons was done by Bonferroni. Bioinformatical analyses were performed using J-Express (MolMine AS, Bergen, Norway) [73]. All cytokine and receptor levels were normalized by their median values, naturally log-transformed, entered into a complete linkage, and used to generate hierarchical clustering. The distance measures were Euclidean.

5. Conclusions

The systemic levels of the acute phase protein CRP are a generally accepted prognostic factor for renal cell carcinoma. Our present study shows that the acute phase cytokine profile differs between renal cancer patients, and most cytokine serum markers included in our present study showed no association with serum CRP levels. Based on differences in the overall acute phase cytokine profile, we classified renal cancer patients into two main subsets that differed significantly with regard to prognosis. Our results suggest that the possible prognostic impact of an extended acute phase cytokine profile or acute phase proteins other than CRP depends on biological context and differs between patient subsets. The possible prognostic impact of the acute phase cytokine profiles should be further investigated for patients with renal cell carcinoma. However, the cancer-related patient death was relatively low in our patient cohort, and the possible prognostic impact of these phenotypic differences in the acute phase reaction has to be further investigated in larger patient cohorts.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/7/1961/s1, Table S1: attrition analysis—a comparison of preoperative clinical and biological characteristics for all renal cancer patients with and without available preoperative serum samples during the defined time period; Table S2: classification of the 118 patients with renal cancer—a presentation of staging; Table S3: serum CRP levels for patients with renal cell carcinoma: a summary of CRP correlation analyses; Table S4: unsupervised hierarchical cluster analysis based on the preoperative serum levels of IL6 family mediators—a comparison of two main subsets of renal cancer patients referred to as $CNTF^{high}IL6^{high}$ and $CNTF^{low}IL6^{low}$, respectively; Figure S1: serum levels of IL33Rα for patients with renal cell carcinoma: A comparison of survivors and non-survivors.

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SUPPLEMENTARY INFORMATION

The Biological Context of C-Reactive Protein as a Prognostic Marker in Renal Cell Carcinoma; Studies of the Acute Phase Cytokine Profile

Table S1. Attrition analysis – a comparison of preoperative clinical and biological characteristics for all renal cancer patients with and without available preoperative serum samples during the defined time period. The results are presented as mean ± standard error of the mean (continuous variables) or as the percentage of the patients.

PARAMETER	Available serum sample (*n* = 118)	No available sample (*n* = 36)	*p*-Value
Age at diagnosis-surgery (years)	62.7 ± 1.3	59.6 ± 2.4	0.252
Male gender	81%	67%	0.093
BMI at operation (kg/height in m^2)	27.2 ± 0.4	25.9 ± 0.6	0.145
Primary tumor size (cm)	6.3 ± 0.3	6.2 ± 0.6	0.804
Type of surgery			0.321
Radical nephrectomy	68%	58%	
Partial nephrectomy	32%	42%	
ASA score			0.581
1 and 2	81%	81%	
3 and 4	19%	19%	
Charlson comorbidity index			0.284
0-1	65%	67%	
≥2	35%	33%	
Stage			0.438
I-II	78%	78%	
III-IV	22%	22%	
Fuhrman nuclear grading			
1-2	53%	53%	1.000
3-4	47%	47%	
Histological necrosis			0.430
No necrosis	66%	58%	
Necrosis present	34%	42%	
Serum CRP level (mg/L)	11.7 ± 2.5*	12.7 ± 5.5**	0.852

* n = 117
** n = 33

Table S2. Classification of the 118 patients with renal cancer—a presentation of staging. The 97 patients included in our present cytokine studies were randomly selected from this population-based cohort. The table summarizes the tumor stadium and the TNM (tumor, nodal, metastasis) stage. The results are presented as the number of patients with the percentage in parenthesis, we present the results for all patients and the patients without metastases (see also Table 1; for additional details about the classification see 7th edition from 2010 in [36,37]).

	All patients (*n* = 118)	**Patients without metastases (*n* = 109)**
Staging of the renal tumor		
T1a (tumor only in the kidney, diameter ≤ 4 cm at its largest area)	54 (45.8)	54 (49.5)
T1b (tumor only in the kidney, diameter > 4 but ≤ 7 cm)	27 (22.9)	25 (22.9)
T2a (tumor only in the kidney, greatest dimension > 7 but ≤ 10 cm)	15 (12.7)	13 (11.9)
T2b (tumor only in the kidney, >10 cm)	4 (3.4)	4 (3.7)
T3a (growth outside the kidney but not beyond Gerota's fascia)	13 (11.0)	10 (9.2)
T3b (growth into the renal vein below the diaphagm)	1 (0.8)	-
T4 (tumor invades beyond Gerota's fascia, extension into the adrenal gland)	4 (3.4)	3 (2.8)
TNM stage		
I (tumor stage T1, no involvement of lymph nodes, no metastases)	79 (66.9)	79 (72.5)
II (tumor stage T2, no involvement of lymph nodes, no metastases)	17 (14.4)	17 (15.6)
III (Either tumor stage T3 with no involvement of lymph nodes and no metastases; or tumor stage T1-T3, but with lymph node involvement and no metastases)	10 (8.5)	10 (9.2)
IV (Either tumor stage T4 independent of the nodal status but no metastases; or metastases independent of the tumor and nodal status)	12 (10.2)	3 (2.8)

Table S3. Serum CRP levels for patients with renal cell carcinoma; a summary of CRP correlation analyses.

Parameter	Kendall's τ	*p*-Value
Age, *n* = 116	-0.018	0.788
ECOG classification, *n* = 116	**0.283**	<0.0005
ASA score, *n* = 116	0.215	0.006
Charlson comorbidity index, *n* = 116	-0.033	0.652
Fuhrman grade, *n* = 116	0.155	0.041
Tumor stage, *n* = 116	**0.315**	**<0.0005**
N stage, *n* = 116	-0.144	0.072
Histological necrosis, *n* = 116	**0.332**	**<0.0005**
IL33Rα, *n* = 94	0.173	0.019
IL1RA, *n* = 95	0.246	0.001
TNFα, *n* = 95	-0.027	0.716
IL6, *n* = 116	**0.301**	**<0.0005**
IL6Rα, *n* = 95	-0.039	0.596
gp130, *n* = 95	-0.041	0.573
IL27, *n* = 95	0.087	0.240
IL31, *n* = 95	0.008	0.911
OSM, *n* = 95	-0.034	0.649
CNTF, *n* = 95	-0.120	0.121

Table S4. Unsupervised hierarchical cluster analysis based on the preoperative serum levels of IL6 family mediators – a comparison of two main subsets of renal cancer patients referred to as $CNTF^{high}IL6^{high}$ and $CNTF^{low}IL6^{low}$, respectively. The cluster analysis identified two main patient clusters, and each of these clusters could be further divided into a sub-cluster with low IL6/CNTF levels or relatively high levels of these two cytokines. Based on these results we classified the patients into two main subsets referred to as $CNTF^{high}IL6^{high}$ and $CNTF^{low}IL6^{low}$, respectively. The table compares the cytokine levels for these two patient subsets. The results are presented as the median and range for each of the mediators together with the corresponding p-value (Mann-Whitney U test).

	$CNTF^{high}IL6^{high}$ (*n* = 42)	**$CNTF^{low}IL6^{low}$ (*n* = 55)**	***p*-Value**
gp130	91,032 (33,865-121,962)	91,380 (22,606-113,815)	0.754
IL6Rα	33,695 (22,510-48,588)	34,602 (17,789-46,610)	0.398
IL6	3.8 (0.4-16.3)	3.3 (0.0-73.2)	0.922
IL27	652 (254-1040)	710 (365-2738)	0.077
IL31	208 (83-584)	183 (87-410)	0.613
OSM	5817 (4500-7911)	5769 (3827-7023)	0.657
CNTF	490 (98-1281)	199 (98-2555)	0.178
CRP, *n* = 41 / 54	3 (1-48)	4 (1-220)	0.186
IL33Rα, *n* = 42 / 54	23,027 (7053-75,572)	23,156 (7731-162,569)	0.924
IL1RA	656 (281-1493)	736 (371-2711)	**0.027**
TNFα	24.2 (6.8-37.2)	26.2 (13.5-37.9)	**0.047**

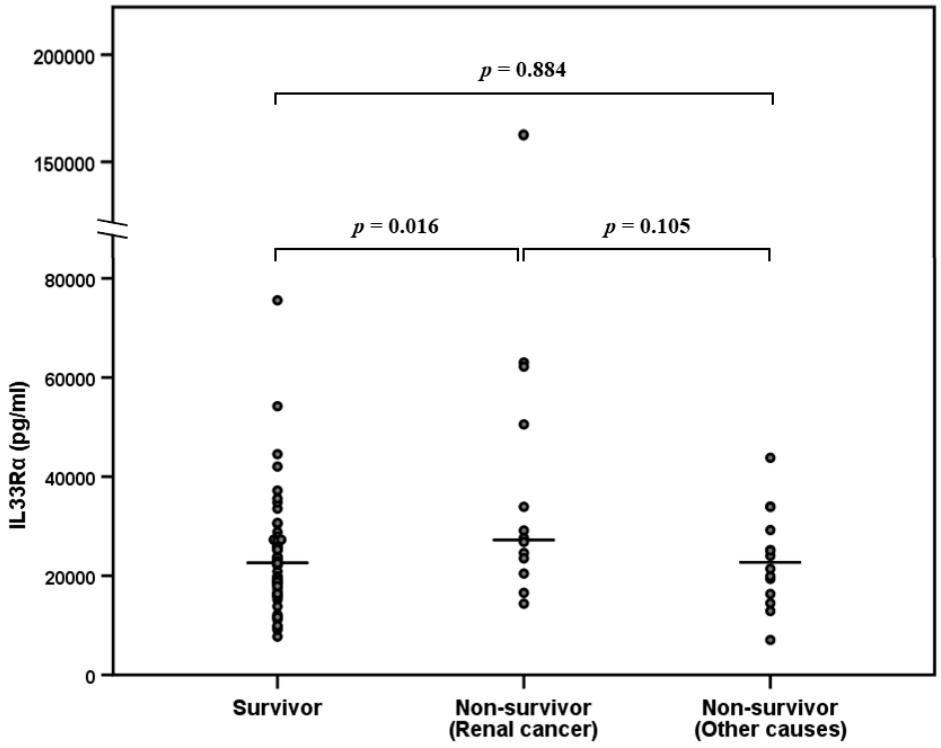

Figure S1. Serum levels of IL33Rα for patients with renal cell carcinoma; a comparison of survivors and non-survivors. The figure compares the levels for patients with cancer-free long-term survival (left); patients dying from their malignant disease (non-survivors, middle figure), i.e. patients with metastatic disease at the time of diagnosis and patients later dying for cancer progression/relapse; patients dying from other causes during the observation period (right, non-survivors other causes). The p-values from the statistical comparisons are indicated in the figure.

Paper IV

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The levels of IL-6 and soluble IL-33R are increased in the renal vein during surgery for clear cell renal cell carcinoma

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ABSTRACT

Purpose: The main aim was to map serum levels of IL-1/IL-6 family cytokines and relevant receptors from serum samples taken across treatment in patients with Renal Cell Carcinoma (RCC). Additionally, we explored the possible interactions between these measurements, immunohistochemistry and intratumoral blood flow.

Methods: We included 40 patients undergoing open surgery for renal tumors. Blood samples were collected before, during (taken simultaneously from a peripheral site and the renal vein (RV) before clamping) and after surgery. Samples were analyzed for IL-6, IL-27, IL-31, OSM, TNF-α, serum (s)-gp130, s-IL-6Rα, s-IL-33R, IL-1Rα and VEGF. All 35 RCC tumors were histologically subtyped as clear cell (CCRCC), papillary or chromophobe. Immunohistochemistry for the CCRCC group included expression of IL-6/IL-6R. Intratumoral blood flow was determined by calculating intratumoral contrast enhancement on preoperative computerized tomography (CT) imaging.

Results: In the CCRCC patients, the intraoperative RV concentration of IL-6 was significantly higher than in both the preoperative and postoperative samples ($p = 0.005$ and $p = 0.032$, respectively). Furthermore, the intraoperative ratio showed significantly higher levels of IL-6 in the RV than in the simultaneously drawn peripheral sample. Immunohistochemistry showed general expression of IL-6 (23/24) in both tumor cells and the vasculature (20/23). Moreover, s-IL-6R was expressed in tumor cells in 23/24 studied patients. Increased blood flow in the CCRCC tumors predicted increased IL-6 levels in the RV ($p < 0.001$). The other cytokines and receptors showed an overall stability across the measurements. However, the intraoperative ratios of IL-33R and gp130 showed significantly higher levels in the RV.

Conclusion: Serum levels of IL-6 increased during surgery. Intraoperative IL-6 and s-IL-33R values were higher in the RV compared to the periphery, suggesting secretion from the tumor or tumor microenvironment itself. Supportive of this is an almost general expression of IL-6/s-IL-6R in tumor cells and IL-6 in vasculature in the tumor microenvironment. Other studied cytokines/receptors were remarkably stable across all measurements.

Abbreviations: ASA, American Society of Anesthesiologist; BS, Blood sample; CHRCC, Chromophobe Renal Cell Carcinoma; CC, Clear cell; CE, Contrast enhancement; DSS, Disease specific survival; ECOG-PS, Eastern Cooperative Oncology Group-Performance Status; ISUP, International Society of Urological Pathology; IHC, Immunohistochemistry; MDT, Multidisciplinary Team; PRCC, Papillary Renal Cell Carcinoma; ROI, Region of Interest; RCC, Renal Cell Carcinoma; RV, Renal Vein

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1. Introduction

Renal cell carcinoma (RCC) is a complex disease with substantial mortality [1] where pathological tumors (pT)-stage and histological grade are the best studied prognostic markers [2]. Even though the treatment has improved, curative RCC treatment is still based mainly on surgery [3]. Therefore, there is an urgent need to learn more about the biology of the RCC in order to improve and extend treatment options.

Much has been learned from the study of the RCC cells from biopsies and RCC cell lines. Von Hippel-Lindau's research with genomic mutations generating RCC tumors represents one of the crucial breakthroughs in this area [4]. However, as with most other carcinomas [5], RCCs primarily originate from somatic mutations, i.e. proximal tubule cells turn malignant with subsequently broken growth regulation [5]. The roles of inflammation in cancer vary, but may be extensive [6]. The presence inflammation may stimulate cancer cells to escape apoptosis and grow uncontrollably, which allows the cancer cells to disseminate and deregulate tumor surveillance [6]. RCC represents one of the major inflammatory related carcinomas [7].

What often kills recurrent RCC patients is disseminated disease [2]. Malignant tumors seed tumor cells into the blood or lymphatic circulation in order and give rise to distant metastasis [8]. Such tumor cells need supportive cells in order to build metastases. The latter includes fibroblasts, vascular, and inflammatory cells [8]. A limiting step of metastasis formation is the tumor cell's ability to form such aggregates [9].

High levels of many inflammatory cytokines measured from blood at diagnosis, points to subsequent RCC metastasis formation [10]. The best evidence is found regarding interleukin (IL)-6, but other cytokines in the IL-6 and IL-1 families and associated receptors show the same ability [11]. IL-6 has also been shown to promote tumor proliferation, metastases and cachexia [12]. IL-6 is synthesized by monocytes, macrophages, Th2 cells, B cells, astrocytes, endothelial cells, adipocytes and some tumor cells [12]. IL-6 has two different ways to initiate cell signaling; classic and trans signaling. IL-6 stimulates classic signaling, whereby it binds to a membrane-bound IL-6 receptor expressed in only a few cells (hepatocytes, neutrophils, monocytes, macrophages and some lymphocytes) [13]. The alternative IL- 6 *trans*-signaling is more generalized, and binds membrane signal transducing receptor glycoprotein 130 kDa (gp130) through the sIL-6R. Thus, in short, IL-6 promotes general inflammation [14]. Soluble gp130 can bind to sIL-6 and prevent IL-6 binding to sIL-6R. As a result, it inhibits trans signaling and functions as a buffer [12]. S-gp130 is present in high serum concentrations and under normal circumstances, the concentration is double that of IL-6 [13]. All cytokines in the IL-6 family utilize glycoprotein 130 (gp130) for cellular membrane signal transduction [15]. Therefore, knowing how IL-6Ra and gp-130 change, will help better our understanding of the mechanisms behind the consequences of a changed s-IL-6.

Further members of the family include IL-11, IL-27, IL-31, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and cardiotrophin-like cytokine factor 1 (CLC) [16]. Both IL-6 and other IL-6 family cytokines (IL-27) and receptors (s-gp130) have also been predictive for RCC survival [11]. IL-1 family members also play a crucial role in innate immunity [17]. IL-33 is an IL-1 family member, and soluble ST2/IL-1 receptor ligand 1 is an IL-33 receptor [18]. s-IL-33R is a biomarker in cardiovascular disease and has a critical role in e.g., lung, liver and head and neck squamous cancer [19]. However, it has been shown that high serum levels of s-IL-33R at diagnosis predicts worse prognosis among RCC patients [20]. However, it is noteworthy that most of these studies rely on only one sample from each patient. An important question is therefore to what extent the cytokine concentrations vary depending on whether they are sampled before, during and/or after removal of the tumor. Therefore, the first aim of this study was to determine if there is a variation in plasma concentrations of members of IL-6, IL-1 family and VEGF cytokines as well as certain receptors when comparing measurements before, during and following RCC surgery.

Furthermore, in a previous study among clear cell RCC (CCRCC) patients [11], we demonstrated that patients with high IL-6 had a worse prognosis and a high expression of IL-6 on immunohistochemistry. Therefore, it is of interest to extend such studies to a more general CCRCC population. The second aim of this study is to explore whether there is a difference in immunohistochemistry between the CCRCC patients with high and low levels of IL-6 preoperatively.

Our previous study has demonstrated that IL-6 is found in endothelial cells within the CCRCC tumor [11]. Therefore, this raises the question whether different levels of vascularization and subsequently blood flow through the tumor are associated with measurable changes in cytokine levels. Accordingly, the third aim of the study was to use contrast enhancement (CE) on CT imaging as a proxy for blood flow in order to investigate whether there exists an association between flow through RCC tumors, immunohistochemistry and serum levels of inflammatory related cytokines.

2. Material and Methods

2.1. Inclusion and data collection

Patients with renal tumors planned for open surgery with partial or radical nephrectomy between April 2018 and June 2019 at Haukeland University Hospital (Bergen, Norway) were invited to participate in this prospective study. All patients followed standardized diagnostic work-up of our institution, which included routine blood tests and chest-CT, in addition to abdominal imaging. Pre-treatment image-guided tumor biopsies were taken when indicated (19 of 40 patients). Following a complete diagnostic evaluation, all these patients were given a recommendation for surgical treatment by the weekly multidisciplinary team (MDT) meeting.

All data collected for the study, including hemoglobin, C-reactive protein (CRP), comorbidities, American Society of Anesthesiologists (ASA) score and Eastern Cooperative Oncology Group-Performance Status (ECOG-PS) were stored in an electronic case report form. The Regional Committee for Medical Research Ethics in Western Norway approved the study (Approval No. 2017/1757). All patients signed an informed consent forms for this study.

2.2. Cytokine sampling and measurements

Preoperative blood samples were collected from a peripheral vein on the morning of surgery (Blood Sample-1: BS-1). During surgery, a second sample (BS-2) was taken from the renal vein (RV) as early as feasibly possible. This took place before major dissection of the kidney and before clamping. Simultaneously, another sample was collected from a peripheral vein in the arm (BS-3). The last sample (BS-4) was collected at the first post treatment assessment (4–6 weeks after surgery). For all samples, the blood was allowed to clot at room temperature before undergoing 15 min of centrifugation at 1000*g*. It was then stored at −80 °C. The kit used was Quantikine ® High Sensitivity ELISA - Human IL-6 by R&D systems, a bi-techno brand. In this method, a monoclonal antibody, specific for human IL-6, is pre-coated on a microplate. IL-6 in the samples is bound by the immobilized antibody. The samples are then washed four times with Wash buffer. After that, 200 µL Human IL-6 HS Conjugate is added to each well and incubated for one hour at room temperature. Then washing is repeated before 200 µL of Streptavidin Polymer-HRP (1X) is added to each well. The samples are then incubated for 30 min at room temperature. Washing is repeated before adding 200 µL of Substrate Solution to each well and incubating for 30 min. Finally, 50 µL of Stop Solution is added to each well and the

color should change from blue to yellow. If it turns green or the colors do not seem uniform, the individual must mix it more thoroughly by tapping gently on the plate. The density of the plate is optimized by using a microplate reader.

TNF-a, s-IL-33R and VEGF were detected using the Luminex immune-bead technology and a high-sensitivity kit (Invitrogen/Biosource, Carlsbad, CA, USA). Antibody-coupled beads were incubated with serum and incubated with a biotinylated detection antibody, before finally being incubated with streptavidin–phycoerythrin. Samples were then read by the Luminex's laser-based fluorescent analytical test instrument Luminex® 100™ (Luminex Corporation Austin, TX, USA). Gp130, IL-27, IL-31, IL-6Rα, and OSM, measured with the same method: Human Premixed Multi-Analyte Kit from R&D system, and the latter by the use of the Milliplex map kit Human Pituitary Magnetic Bead Panel 1 (Millipore, Sigma-Aldrich, Oslo, Norway).

2.3. Histopathological and immunohistological assessment

An experienced uropathologist (LB) reclassified all tumors using hematoxylin and eosin-stained (H&E) xsections. All tumors were staged according to the 2009 TNM classification system [21], subtyped into clear-cell (CCRCC), papillary (PRCC) or chromophobe (CHRCC) and graded according to International Society of Urological Pathology (ISUP) criteria [22,23]. Presence of necrosis and sarcomatoid components was registered. Each patient was allocated to a 3-tier risk group according to their Leibovich score [24]. Fig. 1 shows an example of tumor staging.

During the re-examination, one representative block was selected from each slide set. The selected slide contained both tumor tissue corresponding to the tumor nuclear grade and an area bordering on and comprising kidney parenchyma (interphase zone). Immunohistochemistry was performed using the automated benchmark ultra-system (Ventana-Diagnostics Roche). Four-micrometer sections from the formalin-fixed paraffin embedded (FFPE) tissue blocks were deparaffinized and rehydrated, while antigen retrieval was performed by conditioning the cells in a TRIS-based buffer (CC1, Ventana) and heating accordingly. After endogenous peroxidase blocking, the slides were incubated with the primary antibodies. Detection was performed by using OptiView® (OV) and UltraView ® (UV) DAB detection kits (Ventana Medical Systems), with Hematoxylin used as a counterstain. Human spleen and lymph node sections were used as positive controls, while for negative controls, primary antibodies were omitted (Supplementary Table 1).

The whole tumor area in the slide was examined and the subjective impression of density and number of positive cells were scored semi-quantitatively and subjectively. The proportion of IL-6 and IL6R-positive tumor cells were scored as “no positive tumor cells” (0), “less than 10% positive tumor cells “(±0.5)”, “10% positive tumor cells” (1+), “10–50% positive tumor cells” (2+), or “more than 50% positive tumor cells” (3+). For CD3, CD68 and FOXP3, 1+ means slight and scattered infiltration, 2+ moderate infiltration and 3+ the dense infiltration of positive cells in more than 50% of the area.

From a previously published study from our group [11], we retrieved immunohistochemistry (IHC)-data from CCRCC patients (n = 25) samples with high preoperative IL-6 levels (≥8 pg/ml). All but one in the present study had low preoperative IL-6 values (IL-6 < 8 pg/ml). Thus, for comparison of IHC findings between patients with low and high values of IL-6, we analysed two groups; low (IL-6 < 8 pg/ml); n = 24 and high (IL-6 ≥ 8 pg/ml); n = 26.

2.4. Imaging assessment

The majority of CCRCC patients (22 of 25) were investigated using a CT protocol which consisted of an unenhanced acquisition, an early arterial enhancement phase (Bolus-tracking 150 HU in Aorta + 15 sec), a nephrogram phase (+100 sec), and an excretory phase (10 min). The tumor complexity was scored with a PADUA score [25] by an uro-radiologist (LAR). For the remaining three patients, unenhanced acquisitions were not available. The attenuation of lesions was measured by identifying the most enhancing homogenous area of the tumor. Further, the region of interest (ROI) within the homogenous area was maximized to get more reliable enhancement measures. The CE was split into four groups (Group 1: <20 HU, Group 2: 20–80 HU, Group 3: 81–149, and Group 4: ≥150). A pilot of 5 cases, not a part of this study, was performed to harmonize the measurement of CE method between the observers (GG and KMH).

2.5. Statistical analysis

Descriptive analyses were performed for the patients and tumor characteristics. Given the data is not distributed normally, the non-parametric Wilcoxon test with Bonferroni correction was employed to compare paired samples and multiple measurement. The correlation was found using Pearson. Mann-Whitney *U* test was used for comparison of IHC between two groups. Kappa analyses were used for interobserver correlations. Kappa values should be interpreted as follows: 0–0.20 as none to slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement.

To create values for tumor contrast enhancement (ΔCE) for all CCRCC tumors, we assigned the median value for the three unenhanced acquisitions. Furthermore, we used the median value of preoperative IL-6 in three cases where preoperative measurements were unavailable due to hemolysis of the sample. To predict IL-6 increase in the RV, we utilized general linear regression modeling.

A p-value of less than 0.05 was considered statistically significant. Statistical calculations were performed using the IBM® SPSS® Statistics software (Release 26.0).

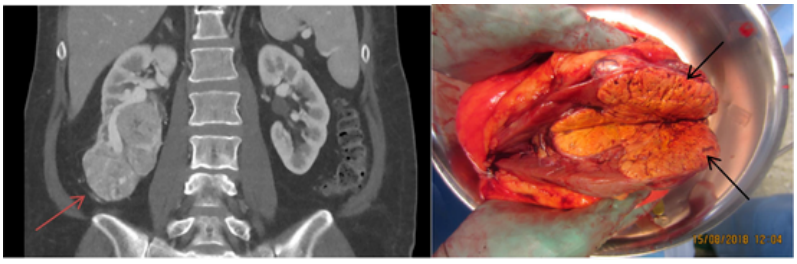

Fig. 1. CT-scan on the left and macroscopic presentation on the right of a patient treated with nephrectomy for a 12 cm (pT2b) tumor in the lower pole of right kidney, PADUA-score 13. Histopathological examination confirmed clear-cell RCC with ISUP nuclear grade 2. The arrows indicate the characteristic yellow cut surface that is the macroscopic hallmark of such a tumor.

3. Results

3.1. Tumor and patient's characteristics

Most patients had confirmed RCC (n = 35) and of these 25 had CCRCC, five had PRCC and five had CHRCC histology. Three patients had benign tumors and two patients had sarcomas. Partial nephrectomy was performed in 25 of 40 patients (62.5%), whereas a radical nephrectomy was performed in the remaining patients.

Overall, the mean size of the tumor on preoperative imaging was 5.2 cm (IQR 2.5–6.4) and the complexity of the tumors, as defined by PADUA-score, revealed a median value of 9 (IQR 8–11). Most of the patients had posterior tumors (68%). The overall male:female ratio was 4.7:1. Furthermore, 57% of the patients were in ASA-class 1–2 and 95% had performance status 0–1.

Table 1 and Table 2 shows patient and tumor related characteristics for the different histological types of RCC, respectively. Patients with PRCC were non-significantly older, while the contrast enhancement was higher in the CCRCC compared to the other RCC types ($p < 0.001$).

3.2. Cytokine levels

3.2.1. Variability in cytokine concentration across sampling

Fig. 2 shows the measurements of cytokines across all samples. For patients with CCRCC the IL-6 values in the RV (BS-2) were significantly higher than the samples taken preoperatively (BS-1) ($p = 0.005$ and at postoperative control (BS-4) ($p = 0.032$). The preoperative samples (BS-1) were not significantly different from the postoperative control samples (BS-4) ($p = 1.0$) (Fig. 2). The median concentration of IL-6 in the RV was 1.97 (IQR: 1.01–37) times higher than in the preoperative samples (BS-2/BS-1). For the CCRCC patients, during surgery, the mean ratio between RV and peripheral IL-6 levels (BS-2/BS-3) with confidence intervals, was significantly higher than the expected ratio of 1 (Fig. 3a). Tumor size did not affect measured concentrations of IL-6 in any of the samples (data not shown).

Similar analyses for IL-27, IL-31, OSM, TNFα, or VEGF in CCRCC patients did not identify any significant changes in the measured samples (Fig. 3a).

There were no significant differences in cytokine levels between CCRCC and PRCC/ CHRCC.

3.2.2. Stability of IL-1 and IL-6 family receptors across sampling

Fig. 4 shows all the measurements for the receptors IL-33R, gp130, IL-1Rα and IL-6Rα in the CCRCC group. Despite an overall impression of stability, there are a few differences, which reached statistical significance. For IL-33R, there was a significant difference intraoperatively (BS-2 vs. BS-3, $p = 0.041$). For gp130, the intraoperative peripheral BS-3 sample was significantly lower than both the sample taken pre- and postoperatively (BS-1 and BS-4, $p = 0.023$ and $p = 0.037$, respectively). IL-1Rα showed higher values in the RV compared to preoperatively (BS-2 vs. BS-1, $p = 0.008$). IL-6Rα demonstrated no significant differences across the measurements.

Table 1
Demographic and clinical characteristics of the 35 patients with renal cell carcinoma.

	Clear Cell RCC (n = 25)	Papillary RCC (n = 5)	Chromophobe RCC (n = 5)
Age (years) (Mean, Median (IQR))	63, 65 (57–74)	72, 72 (70–75)	62, 66 (48–74)
Gender (n, (%))			
Males	24 (96)	3 (60)	1 (20)
Females	1 (4)	2 (40)	4 (80)
ASA-Class[a] (n, (%))			
1–2	13 (52)	0	4 (80)
3–4	12 (48)	5 (100)	1 (20)
ECOG-PS[b] 0–1 present (n, (%))			
0–1	23 (92)	5 (100)	5 (100)
2+	2 (8)	0	0
GFR[c] (μmol/L) (Mean, Median (IQR))	79, 85 (73–93)	75, 76 (59–90)	92, 99 (75–105)
Operative method (n, (%))			
Partial nephrectomy	14 (56)	3 (60)	4 (80)
Radical nephrectomy	11 (44)	2 (40)	1 (20)

Data for 5 patients with other histopathological entities (sarcomas (n = 2) and benign lesions (n = 3)) are not presented.
[a] ASA- American Society of Anesthesiologists (ASA) score.
[b] ECOG-PS- Eastern Collaborative Oncology Group Performance Status.
[c] GFR-Glomerular filtration rate calculated using CKD-EPI Creatinine Equation 2009, IQR-Interquartile range, RCC- Renal cell Carcinoma.

Table 2
Histopathological and radiological characteristics of the 35 patients with renal cell carcinoma.

	Clear Cell RCC (n = 25)	Papillary RCC (n = 5)	Chromophobe RCC (n = 5)
Tumor size (cm)(Mean, Median (IQR))	4.3, 3.3 (2.4–5.3)	7.6, 3.4 (2.9–14.5)	5.0, 3.3 (2.5–8.5)
ISUP-grade[a] (n, (%))			
1	4 (16)	0	n/a
2	19 (76)	5 (100)	
3	2 (8)	0	
4	0	0	
Sarcomatoid component present (n, (%))	0	0	1 (20)
Necrosis present (n, (%))	1 (4)	1 (20)	
pT-stage (n, (%))			
1a	17 (68)	3 (60)	3 (60)
1b	4 (16)	0	1 (20)
2a	1 (4)	1 (20)	1 (20)
2b	2 (8)	1 (20)	0
3a	1 (4)	0	0
PADUA[b]-Score (median (IQR))	10 (7.5–11.5)	8 (7–11)	9 (9–11)
Contrast enhancement (HU) (Mean, Median (IQR))	111, 106 (70–131)	44, 42 (32–58)	86, 64 (56–127)
Leibovich[c]-score (median (IQR))	0 (0–2)	2 (1–4.5)	0 (0–3.5)

HU-Hounsfield Units, IQR-Interquartile range, RCC- Renal cell Carcinoma, pT-Stage – Pathological T-stage according to UICC 2010 version of the TNM classification.
Data for 5 patients with other histopathological entities (sarcomas (n = 2) and benign lesions (n = 3)) are not presented, n/a – ISUP nuclear grading is not applicable to chromophobe RCC
[a] ISUP-The International Society of Urological Pathology (ISUP) nuclear grade.
[b] PADUA-Preoperative Aspects and Dimensions Used for an Anatomical (PADUA) Classification of Renal Tumours.
[c] Leibovich score-Prognostic score that is based on T stage, size, lymph node status, nuclear grade and presence of tumor necrosis (Higher score gives worse prognosis (0–11)).

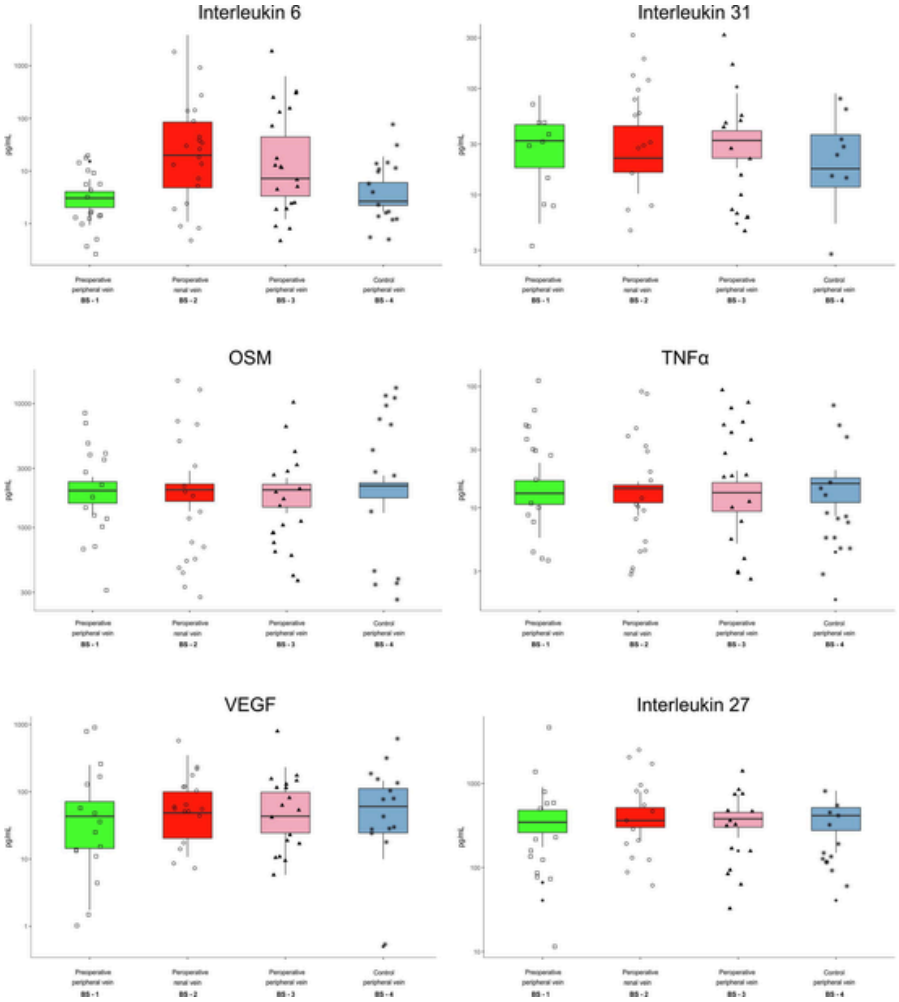

Fig. 2. The figure shows the all the values with boxplots for six cytokines measured in blood samples (BS) preoperatively (BS-1), intraoperatively and simultaneously from the renal vein (BS-2) and peripherally (BS-3) and at postoperative control after 4–6 weeks (BS-4). The intraoperative measurement from the renal vein (BS-2) of IL-6 is significantly higher than in the preoperative (BS-1) and postoperative (BS-4) samples ($p = 0.005$ and $p = 0.032$, respectively). For the other cytokines there are no significant differences.

For the CCRCC patients, during surgery, the mean ratio between RV and peripheral IL-33R and gp130 levels (BS-2/BS-3) with confidence intervals, were significantly different and higher than the expected ratio of 1 (Fig. 3b).

PRCC patients demonstrated significantly higher levels of IL-6Rα in both BS-1 and BS-2 (Supplementary Fig. 1). Otherwise, there were no significant differences in receptor levels between CCRCC and PRCC/CHRCC.

3.2.3. Correlation between cytokines/receptors across measurements

By correlating all cytokines and receptors, the best correlation was found for the individual cytokine / receptor (intraclass). IL-6 showed the least overall intraclass correlation, while IL-27, OSM, IL-33R and VEGF demonstrated the highest. Between cytokines / receptors, the highest overall correlation was seen between different measurements of IL-33R and VEGF, IL-6Rα and OSM, IL-1Ra and IL-27 and IL-6Rα and IL-27. Supplementary Table 2 demonstrates the correlations for both intraclass and between cytokines/receptors among CCRCC patients.

3.3. Immunohistochemistry for CCRCC

We calculated the levels of CD3, CD68, FoxP3, IL-6 and IL-6R positive cells in the patients' tumors and the surrounding tissue (n = 24). The density and number of positive cells were scored semi-quantitatively and subjectively. The following number of patients had 10% or more expression by immunohistochemistry: CD3 positive tumor lymphocytes 24/24; CD3 positive lymphocytes in interphase zone 19/23: CD68 positive cells in tumor 20/24: CD68 positive interphase zone cell 6/23: FoxP3 in tumor infiltrating lymphocytes 4/24: FoxP3 in interphase zone lymphocytes 3/24. FoxP3 in tumor cells 0/24 (Fig. 5).

Regarding IL-6, none of the patients showed expression in tumor lymphocytes and only one in interphase zone lymphocytes. On the

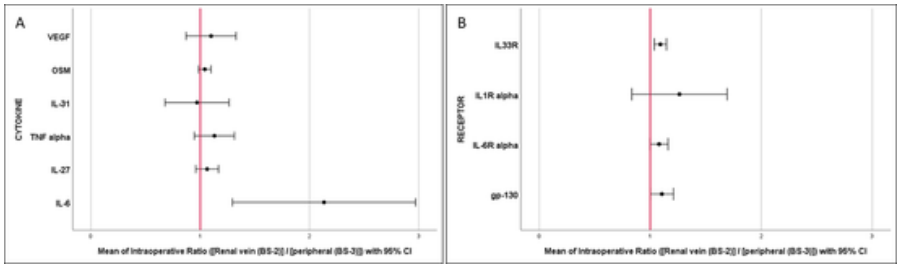

Fig. 3. The figures shows the mean of the intraoperative ratios (renal vein (BS-2)/peripherally (BS-3) with confidence intervals for A) cytokines and B) receptors. The red line represent the expected BS-2 /BS-3 ratio given an even distribution in the body. IL-6, IL-33R and gp130 have confidence intervals that does not include 1.

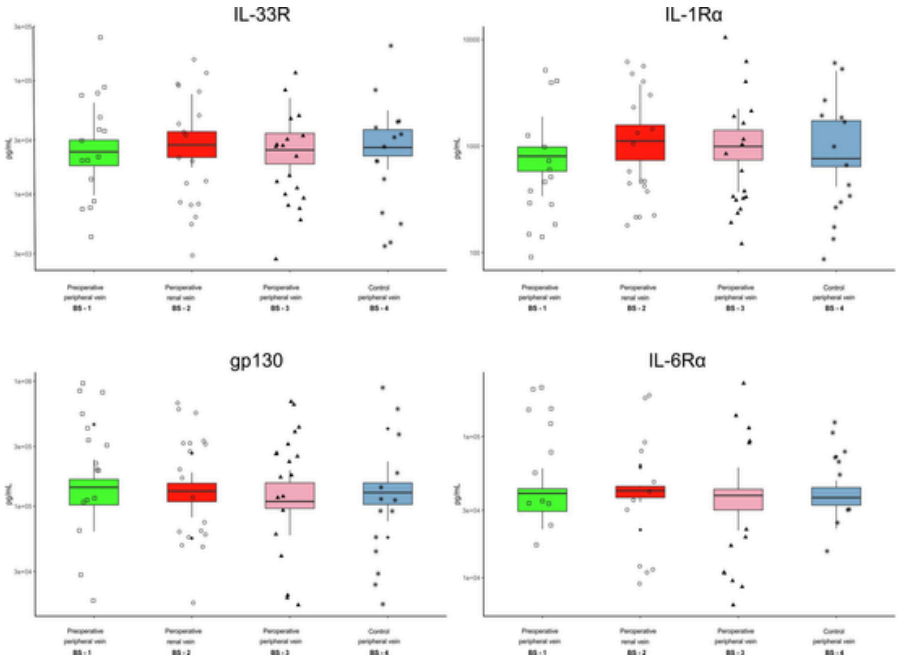

Fig. 4. The figure shows the all the values with boxplots for four receptors measured in blood samples (BS) preoperatively (BS-1), intraoperatively and simultaneously from the renal vein (BS-2) and peripherally (BS-3) and at postoperative control after 4–6 weeks (BS-4). The intraoperative measurement from the renal vein (BS-2) of gp130 is significantly lower than in the preoperative (BS-1) and postoperative (BS-4) samples ($p = 0.023$ and $p = 0.037$, respectively). IL-1Rα showed increased values in the renal vein compared to preoperatively (BS-2 vs. BS-1, $p = 0.008$). Otherwise, no significant differences were observed.

other hand, 23/24 were IL-6 positive in tumor cells and 20/23 in the vasculature (Fig. 5). Expression of IL-6R in tumor cells was seen in 23/24 of the studied patients (Table 3).

Comparing CCRCC patients with low IL-6 and those with high, there was a difference between them concerning expression of IL-6 in tumor cells ($p < 0.001$). Furthermore, there is a much higher expression of IL-6R in tumor cells ($p < 0.001$) and FoxP3 in tumor lymphocytes in those with higher pre-operative IL-6 ($p = 0.039$). There was no difference in expression of CD3 nor CD68 in lymphocytes between those two groups (data not shown).

FoxP3 in the interphase zone lymphocytes correlated to s-IL-6 intraoperatively (BS-2 and BS-3, $p = 0.01$ and $p = 0.042$, respectively). s-IL-6 preoperatively (BS-1) and at control (BS-4) correlated with IL-6 tumor lymphocytes, $p = 0.011$, and $p = 0.034$, respectively. Preoperatively. IL-6 (BS-1) correlates with IL-6 in tumor cells ($p = 0.018$) and IL-6R in tumor cells ($p = 0.013$). Stage and size correlate to IL-6R in tumor cells ($p = 0.032$ and $p = 0.028$, respectively). There was no other correlation between IHC and known histopathological risk factors.

3.4. Interactions between contrast enhancement, IL-6 measurements, and immunohistochemistry within CCRCC

The interrater reliability for CE on CT-scans was high ($k = 0.61$). For the following analyses, we used the result from one reader (GG). Data from the other reader (KMH) showed similar results (data not shown). Comparing CE and the IL-6 values, there was a significant correlation with both the IL-6 samples taken during surgery (BS-2 and BS-3 with a p-value < 0.01 and $p < 0.05$, respectively). No significant

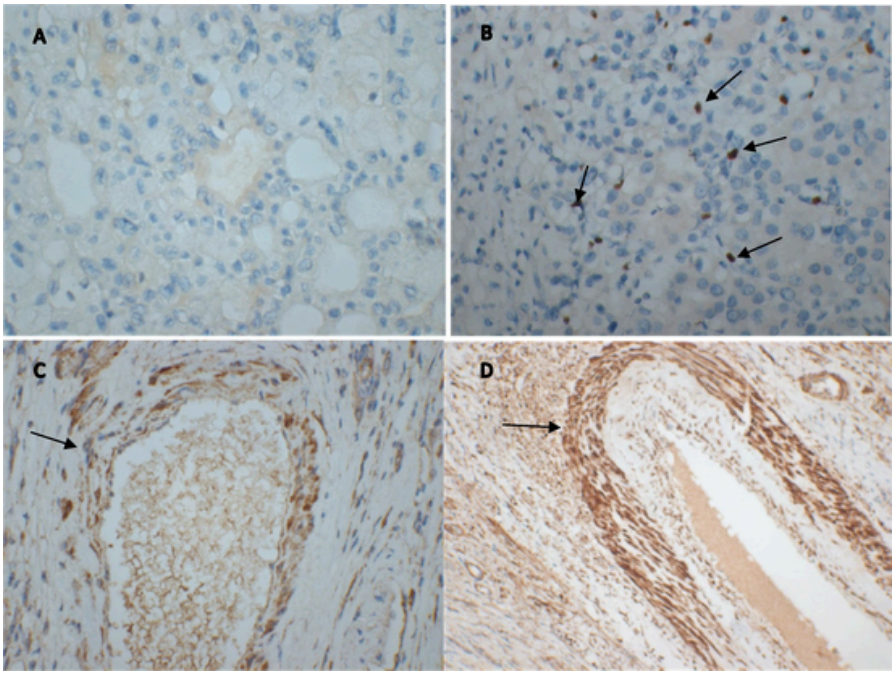

Fig. 5. Immunohistochemical staining for FOXP3 and IL-6 in clear cell renal cell carcinoma. A) Tumor tissue negative for FOXP3, score 0. B) Arrows pointing at FOXP3 positive intramural lymphocytes, score 1 (slight and scattered infiltration). C) Arrow pointing at IL-6 positive medial smooth muscle in an intrarenal artery, score 1 (10% positive cells). D) Arrow pointing at IL-6 positive medial smooth muscle cells in intrarenal artery, score 2 (10–50% positive cells).

correlation was found between IL and 6 changes and IHC, nor between IHC and CE. In a linear regression model, only higher CE remained an independent predictor of increased levels of IL-6 in the RV ($p < 0.001$) with an explained variance (r^2) of 0.595.

4. Discussion

There are two main findings in this this small pilot study investigating serum IL-1 - and IL-6 family cytokines and related receptors in CCRCC patients before, during and after surgery. Firstly, the stability of the majority of cytokines and receptors and secondly, the observed increase in IL-6 intraoperatively.

The remarkably constant level of the measured cytokines and cytokine receptors from the pre-treatment samples to the six week post-treatment samples was unexpected, but adds substantial validity to one-sample studies regarding (RCC) cancer. Scientific understanding of the half-life of human cytokines in blood is lacking. The elimination half-life for IL-6 is approximately 15 h and 12 h for rats and mice, respectively [26]. In humans, the elimination half-life is approximately 13 h [27]. To our knowledge, there are no previous studies investigating the elimination half-life of IL-6 in RCC patients. This study supports a relatively long elimination half-life (5–15 h) in humans because of the measured stability of the cytokine concentrations. Furthermore, the stability of many of the different cytokine concentrations throughout treatment suggests a "thermostat" that regulates cytokine concentrations and the liver is a possible candidate for this [28].

Our results have demonstrated that serum concentration of IL-6 increased during surgery. IL-6 is a cytokine, which is produced by many cells as a response to stimuli [12]. Physical exercise, such as long-distance walking, has been shown to increase IL-6 up to 10 times over 24 h [29]. Thus, it is likely that a physical trauma like open surgery may increase the general level of IL-6 both during surgery and immediately afterwards. We found a 3:1 ratio between IL-6 samples collected from the RV compared to preoperatively for all patients and 2:1 for CCRCC. This is lower than the 10:1 ratio that Blay et al. previously published in a series of three patients [30]. However, based on our intraoperative measurements, which show a significant difference between the samples from the renal vein and peripherally, extrarenal production of IL-6 is probably not the whole explanation for this increase.

The concentrations of s-IL-Rα and s-gp130 measured in this study changed minimally. This supports that the hypothesis that measured IL-6 concentrations are functionally relevant given both IL-6 concentrations acting on the membrane bound IL-6 receptor and the complex of IL-6/sIL-6Rα stimulated the relevant cell more. This is further supported by minimal change in s-gp130 concentrations. The changed IL-6 levels appear therefore to be physiologically relevant.

Based on the supporting results in this study, we hypothesize that a substantial part of the increase in IL-6 is due to production within the tumor cells and/or from the tumor vasculature. The present IHC data demonstrates the general expression of IL-6/s-IL-6R in tumor cells and IL-6 in vasculature as evidence of tumor IL-6 synthesis which confirms earlier results [11]. When comparing patients with high versus low preoperative serum levels of IL-6, the former were shown to have both higher density of IL-6 and higher expression of IL-6R in tumor cells, which supports the theory that the tumor as a source for circulating serum IL-6. Moreover, the CE is an indicator of vascularization and blood flow through the tumor. The larger increase in IL-6 values in the RV among those with higher tumor CE, also indicates that RCC tumors are associated with IL-6 production. Overall, our results are compliant with a hypothesis that RCC tumor cells secrete IL-6 and likely stimulate the vascular cells to do the same.

Table 3
Description of immuno-histochemical analyses patients with CCRCC, staining assessment and numbers of patients in each group. Each selected slide contained both tumor tissue corresponding to the tumor nuclear grade and an area bordering on and comprising kidney parenchyma (interphase zone). The samples (n = 38) were scored in a semi-quantitative fashion, reviewed by an expert in pathology (LB) and further transformed into numeric values for statistical analyses according to the following: +++ = 3, ++(+) = 2.5, ++ = 2, +(+) = 1.5, + = 1, ± = 0.5 and – = 0.0.

	– = 0.0	± = 0.5	+ = 1.0	+(+) = 1.5	++ = 2.0	++(+) = 2.5	+++ = 3.0
CD3-positive tumor lymphocytes	0	0	8	4	6	5	1
CD3-positive lymphocytes in interphase zone[1]	2	2	8	6	4	1	0
CD68-positive cells in tumor	0	4	4	9	5	1	1
CD68-positive interphase zone cells[1]	7	10	4	1	1	0	0
FoxP3 in tumor lymphocytes	8	12	3	1	0	0	0
FoxP3 in interphase zone lymphocytes[1]	7	13	0	3	0	0	0
FoxP3 in tumor cells	24	0	0	0	0	0	0
IL6 in tumor lymphocytes	20	4	0	0	0	0	0
IL6 in interphase zone lymphocytes	18	5	1	0	0	0	0
IL6 in tumor cells	1	0	4	0	4	0	15
IL6 in vasculature	1	3	8	0	10	1	1
IL6 receptor in tumor lymphocytes[2]	0	7	0	0	0	0	0
IL6R in interphase zone lymphocytes[2]	0	7	0	0	0	0	0
IL6R in tumor cells	1	0	8	0	3	0	12

1) n = 23, 2) n = 7.
The proportion of IL-6 and IL6R-positive tumor cells were scored as "no positive tumor cells" (0), "less than 10% positive tumor cells "(± 0.5)", "10% positive tumor cells" (1 +), "10–50% positive tumor cells" (2 +), or "more than 50% positive tumor cells" (3 +). For CD3, CD68 and FOXP3, 1 + means slight and scattered infiltration, 2 + moderate infiltration and 3 + the dense infiltration of positive cells in more than 50% of the area.

Previously, we have shown that both IL-6 and IL-27, when measured at diagnosis, predicted recurrence and DSS to a similar extent [11]. These cytokines share the gp130 receptor, i.e. the β-part of the receptor. Regarding these two cytokines, the present study suggests it is not the membrane bound gp130 receptor, which is the sole mechanism for the survival predictions. Further studies on this are warranted. In the case of IL-6, we have studied the soluble receptors IL-6Rα and soluble gp130 levels. The decoy receptor gp130 had decreased concentration versus no significant change regarding the *trans*-activating IL-6Rα. Thus, it is supported that both the IL-6 classical- and *trans*-activation will be strengthened through these soluble receptors with increased serum IL-6 as part of RCC pathophysiology. Regarding the IL-1 family cytokines and receptors, we have shown that s-IL-33R concentration were increased in the RV. IL-33R is considered a decoy receptor [20]. However, most published studies on soluble (decoy) receptors indicate worse cancer prognosis with increased such concentrations [20]. This could be explained by the cellular turnover of tumors but this needs to be studied in more detail. We have shown a considerable presence of T lymphocytes, both within the tumor and the interphase. On the other hand, fewer lymphocytes were FoxP3 positive, suggesting few T regulatory cells. Interestingly, the presence of IL-6R on lymphocytes was more abundant with higher IL-6 serum levels suggesting that IL-6 may also inhibit T lymphocyte function though classical activation.

To our knowledge, this is the first study to investigate the levels of IL-6- and IL-1-family cytokines in consecutive samples from the same cancer patients before, during and after surgery. However, published data for comparison and benchmarking is limited. Further limitations are that it is a small pilot study with few patients, and there is presence of selection bias because only patients undergoing open surgery were included. This approach was chosen because it is technically only feasible to attain blood from the RV during open surgery. However, the surgical trauma by itself might be a confounder that complicates the understanding of the changes in IL-6 measurements. Furthermore, in this study there is a gender imbalance with more men (4.7:1) than the usual 1.5–2:1 ratio known from other cohorts [31]. A strength of this study is that each patient serves as their own control. We were able to study individual sample values, and therefore examine trends on an individual basis at multiple points in time. The intraoperative RV samples add considerable value to these findings.

5. Conclusions

Serum levels of IL-6 increased during surgery. Intraoperative IL-6 and s-IL-33R values were higher in the RV compared to the periphery, which suggests secretion from the tumor or tumor microenvironment itself. Supportive of this is an almost general expression of IL-6/s-IL-6R in tumor cells and IL-6 in vasculature in the RCC tumor microenvironment. Other studied cytokines were remarkably stable across all the measurements.

Declarations

Funding - The study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit-sectors.

Ethics approval and consent to participate - All patients were given oral and written information about the study, and they gave written informed consent. The study was approved by the Regional Committee for Medical Research Ethics in Western Norway (Approval No. 2017/1757), and the database was approved by the Norwegian Social Science Data Services.

Consent for publication - Not applicable.

Availability of data and material - The approval from the ethical committee and informed consent do not cover a full open publication of the dataset. The raw data may be made available in unidentified form on request, and if needed contact the corresponding author.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2021.155586.

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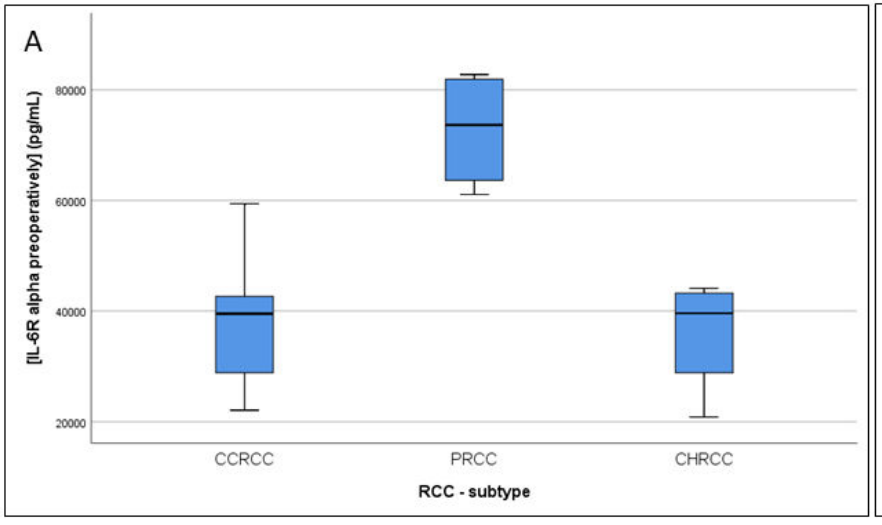
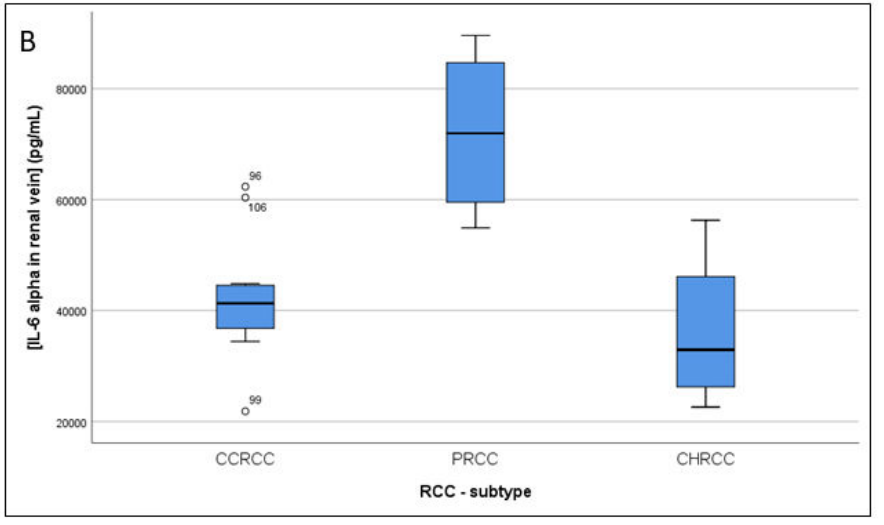

A
[IL-6R alpha preoperatively] (pg/mL)
80000
60000
40000
20000
CCRCC
PRCC
CHRCC
RCC - subtype


B
[IL-6 alpha in renal vein] (pg/mL)
80000
60000
40000
20000
96
106
99
CCRCC
PRCC
CHRCC
RCC - subtype

Supplementary Table 1: Immunohistochemistry

Antibody	Source	Epitope retrieved	Dilution	Incubation time (min)	Detection kit
CD3 (A0452)	DAKO	CC1, 36 min	1:100	32	UV
CD68 (KP1,M0814)	DAKO	CC1, 64 min	1:5000	32	UV
FOXP3 (560044, clone:259D/C7)	BD Biosciences	CC1, 64 min	1:20	32	UV
IL6 (ab 9324)	Abcam	CC1, 48 min	1:200	120	OV
IL6R (ab 128008)	Abcam	CC1, 48 min	1:800	32	OV

Supplementary Table 2. Pearson correlation matrix for correlations across measurements (BS-1-BS-4) for each cytokine/receptor (intraclass (blue squares)) and between cytokines/receptors. Significant correlations are highligthed with orange for intraclass correlations, and yellow ($p<0.05$) and green ($p<0.01$) for correlations between different cytokines/receptors. The latter with overall highest correlation are marked with red squares.

[1]) Preoperative sample (BS-1), [2]) Intraoperative peripheral sample (BS-3), [3]) Intraoperative sample renal vein sample (BS-2), [4]) Control sample 4-6 weeks after surgery (BS-4), *p<0.05, **p<0.01

	IL-6[1]	IL-6[2]	IL-6[3]	IL-6[4]	gp130[1]	gp130[2]	gp130[3]	gp130[4]	IL-27[1]	IL-27[2]	IL-27[3]	IL-27[4]	IL-6Ra[1]	IL-6Ra[2]	IL-6Ra[3]	IL-6Ra[4]	TNFa[1]	TNFa[2]	TNFa[3]	TNFa[4]	IL-1RA[1]	IL-1RA[2]	IL-1RA[3]	IL-1RA[4]	IL-1b[1]	IL-31[1]	IL-31[3]	IL-31[4]	OSM[1]	OSM[2]	OSM[3]	OSM[4]	ST2[1]	ST2[2]	ST2[3]	ST2[4]	VEGF[1]	VEGF[2]	VEGF[3]	VEGF[4]
IL-6[1]	1	-0.091	-0.077	0.441																																				
IL-6[2]	-0.091	1	0.706**	-0.179																																				
IL-6[3]	-0.077	0.706**	1	-0.168																																				
IL-6[4]	0.441	-0.179	-0.168	1																																				
gp130[1]	0.013	-0.168	-0.084	-0.060	1	0.670**	0.910**	-0.12																																
gp130[2]	0.327	0.108	0.201	0.459	0.670**	1	0.790**	0.464																																
gp130[3]	0.096	-0.143	-0.025	0.180	0.910**	0.790**	1	0.221																																
gp130[4]	0.216	0.740**	0.669**	-0.083	-0.012	0.464	0.221	1																																
IL-27[1]	0.314	-0.082	-0.081	0.330	0.343	0.471	0.307	0.253	1	0.835**	0.706**	0.743**																												
IL-27[2]	0.408	0.000	-0.119	-0.329	0.538	0.127	0.224	0.265	0.835**	1	0.930**	0.920**																												
IL-27[3]	0.419	-0.025	-0.063	-0.224	0.618*	0.326	0.418	0.398	0.706**	0.930**	1	0.853**																												
IL-27[4]	0.269	0.171	0.252	-0.193	0.663*	0.203	0.430	0.187	0.743**	0.920**	0.853**	1																												
IL-6Ra[1]	-0.372	-0.381	-0.344	0.196	0.717*	0.221	0.512*	-0.498	0.338	0.057	0.490	0.460	1	0.888**	0.709**	0.332																								
IL-6Ra[2]	0.037	0.097	0.154	0.017	0.550	0.324	0.400	-0.120	0.401	0.933**	0.817**	0.71.2*	0.777**	1	0.912**	0.588																								
IL-6Ra[3]	-0.134	0.019	0.083	-0.026	0.525	0.285	0.516	-0.196	0.609	0.887**	0.804**	0.882**	0.708*	0.912**	1	0.480																								
IL-6Ra[4]	0.521	0.059	0.112	-0.331	0.605	0.115	0.359	0.234	0.766*	0.964**	0.852*	0.810*	0.332	0.588	0.480	1																								
TNFa[1]	0.064	-0.257	-0.185	0.400	0.426	0.603*	0.422	-0.129	0.461	0.331	0.269	0.244	0.492	0.192	0.090	0.434	1	0.563*	0.455	0.421																		
TNFa[2]	0.183	0.043	-0.043	0.219	0.115	0.411	0.171	0.198	0.488	0.272	0.203	0.418	0.046	0.205	0.149	0.052	0.563*	1	0.636**	0.526*																		
TNFa[3]	0.333	0.044	0.218	0.125	0.454	0.704**	0.604**	0.168	0.298	0.218	0.357	0.340	0.011	0.137	0.193	0.111	0.455	0.636**	1	0.301																		
TNFa[4]	0.431	0.246	0.295	0.003	0.025	0.278	0.065	0.485	0.602*	0.433	0.257	0.619*	-0.340	-0.268	-0.246	0.492	0.421	0.526*	0.301	1																		
IL-1RA[1]	0.149	-0.150	-0.078	0.090	0.479	0.462	0.377	0.208	-0.018**	0.762**	0.749**	0.845**	0.337	0.520	0.664*	0.699**	0.408	0.479	0.402	0.321	1	0.778**	0.670**	0.268														
IL-1RA[2]	0.218	-0.016	-0.123	0.592	0.374	0.532*	0.359	0.377	0.789**	0.364	0.470	0.323	0.041	0.351	0.271	0.270	0.298	0.529*	0.360	0.370	0.778**	1	0.482*	0.410														
IL-1RA[3]	0.125	0.454	0.753**	0.579*	0.207	0.512	0.190	0.363	0.709**	0.045	0.185	0.243	-0.320	0.268	0.217	0.160	0.436	0.361	0.493*	0.294	0.670**	0.482*	1	0.410														
IL-1RA[4]	-0.066	-0.165	-0.188	0.336	-0.040	-0.183	-0.167	-0.155	0.625*	0.202	-0.003	0.273	0.103	0.194	0.112	0.386	0.188	0.280	0.183	0.263	0.268	0.410	0.410	1														
IL-31[1]	-0.047	-0.068	-0.160	-0.023	0.161	0.372	0.323	0.616	0.234	0.054	0.018	0.214	0.560	0.257	0.083	0.444	0.551	0.328	0.053	0.204	-0.218	0.076	0.197	0.297	1	-0.772*	0.604	0.538										
IL-31[3]	-0.199	0.356	0.092	-0.304	-0.170	0.165	-.0092	0.770	-0.107	0.080	-0.011	-0.098	-0.277	0.084	0.152	-0.083	0.140	0.258	-0.043	0.251	-0.235	-0.006	0.158	-0.125	0.772*	1	0.778**	0.537										
IL-31[4]	-0.218	0.095	0.018	-0.418	-0.192	0.039	-0.109	0.431	-0.250	0.214	-0.204	0.284	-0.222	-0.011	0.348	0.226	0.008	-0.070	-0.043	0.156	-0.446	0.378	-0.136	-0.084	0.604	0.778**	1	0.892**										
IL-31[4]	0.025	-0.288	-0.311	-0.388	0.449	0.148	0.143	0.182	-0.239	0.218	-0.096	0.194	-0.790	-0.675	0.952*	0.481	0.207	-0.112	0.074	0.429	-0.377	0.293	-0.295	0.141	0.538	0.537	0.892**	1										
OSM[1]	-0.393	0.127	0.110	0.243	-0.108	-0.283	-0.273	-0.202	-0.020	0.064	-0.227	0.146	0.528	0.390	0.249	-0.080	0.316	-0.016	-0.522*	-0.243	-0.122	-0.149	-0.065	0.308	0.510	0.232	0.081	-0.521	1	0.713**	0.443	0.696**						
OSM[2]	-0.375	0.083	-0.202	-0.315	0.036	-0.495	-0.333	-0.080	-0.046	0.250	0.133	0.367	0.481	0.729**	0.651*	0.508	-0.019	0.142	-0.401	-0.138	-0.014	-0.007	-0.192	0.360	0.256	0.312	0.089	-0.260	0.713**	1	0.853**	0.873**						
OSM[3]	-0.259	-0.001	-0.133	-0.284	-0.069	-0.470	-0.255	-0.138	0.007	0.281	0.262	0.413	0.251	0.595*	0.605*	0.635*	0.091	0.033	-0.276	-0.022	0.119	0.054	-0.059	0.353	0.077	0.171	0.098	-0.245	0.443	0.859**	1	0.802**						
OSM[4]	-0.179	0.201	0.182	-0.343	0.015	-0.476	-0.328	-0.051	0.315	0.692*	0.477	0.343	0.336	0.606*	0.425	0.717*	0.219	-0.035	-0.387	0.099	0.334	0.006	-0.013	0.406	0.188	0.148	0.039	-0.092	0.696**	0.853**	0.802**	1						
ST2[1]	0.042	0.266	0.324	0.148	-0.225	0.221	-0.299	-0.154	0.350	0.128	-0.171	0.287	0.216	0.393	0.512	-0.079	0.128	0.150	-0.267	-0.064	0.204	0.075	-0.144	0.127	-0.226	-0.213	0.120	-0.594	0.403	0.351	0.139	0.195	1	0.964**	0.954**	0.817**		
ST2[2]	0.103	0.118	0.059	0.418	-0.220	0.001	-0.125	-0.132	0.427	0.175	-0.219	0.071	0.157	0.361	0.519	-0.270	0.239	0.139	-0.147	-0.191	0.217	0.207	.098	0.083	-0.237	-0.264	0.369	-0.632	0.322	0.167	0.049	-0.082	0.964**	1	0.988**	0.959**		
ST2[3]	00.43	0.121	0.057	0.343	-0.216	-0.042	-0.134	-0.155	0.269	0.262	-0.306	0.080	0.145	0.375	0.524	-0.220	0.283	0.087	-0.171	-0.206	0.163	0.110	0.043	0.490	-0.274	-0.260	0.291	-0.581	0.308	0.197	0.087	-0.066	0.954**	0.988**	1	0.849**		
ST2[4]	0.429	0.085	0.159	0.572*	-0.069	0.339	0.110	-0.080	0.245	0.092	-0.074	0.010	0.365	0.432	0.624	-0.322	0.031	0.233	0.142	-0.065	0.288	0.247	0.179	-0.108	-0.347	-0.419	0.435	-0.680	0.205	-0.136	-0.188	-0.240	0.817**	0.969**	0.849**	1		
VEGF[1]	0.760**	-0.081	0.098	0.413	0.093	-0.025	-0.084	-0.004	0.374	0.293	0.236	0.265	0.368	0.464	0.592	0.092	0.052	0.017	-0.116	0.125	0.100	0.050	-0.050	0.094	0.109	-0.232	0.226	-0.491	0.169	0.099	0.109	0.132	0.927*	0.602*	0.546*	0.665**	1	0.841**	0.678**	0.814**
VEGF[2]	0.260	-0.155	0.203	-0.037	0.099	-0.314	-0.202	-0.224	0.411	0.335	0.240	0.442	0.540	0.624*	0.708*	0.067	0.130	0.019	-0.362	-0.266	0.096	0.066	-0.219	0.208	0.217	-0.037	0.062	-0.401	0.452	0.544*	0.434	0.355	0.682**	0.619**	0.612**	0.445	0.841**	1	0.940**	0.829**
VEGF[3]	0.016	-0.051	0.061	-0.104	0.149	-0.375	-0.254	-0.296	0.412	0.257	0.152	0.442	0.414	0.567	0.683*	-0.007	0.268	-0.107	-0.431	-0.463	0.100	-0.147	-0.155	0.138	0.075	-0.058	0.021	-0.368	0.468	0.543	0.455	0.340	0.763**	0.712**	0.730**	0.437	0.678**	0.940**	1	0.682**
VEGF[4]	0.455	0.098	0.094	0.014	0.048	-0.262	-0.215	-0.005	0.733**	0.614*	0.404	0.515	0.308	0.367	0.481	0.480	0.079	-0.026	-0.391	0.301	0.386	0.046	-0.054	0.400	0.172	-0.235	0.297	-0.544	0.439	0.390	0.389	0.531*	0.558*	0.392	0.356	0.301	0.814**	0.829**	0.682**	1

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