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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.

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.

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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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 of 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 may seed tumor cells into the blood or lymphatic circulation 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 form 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.

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

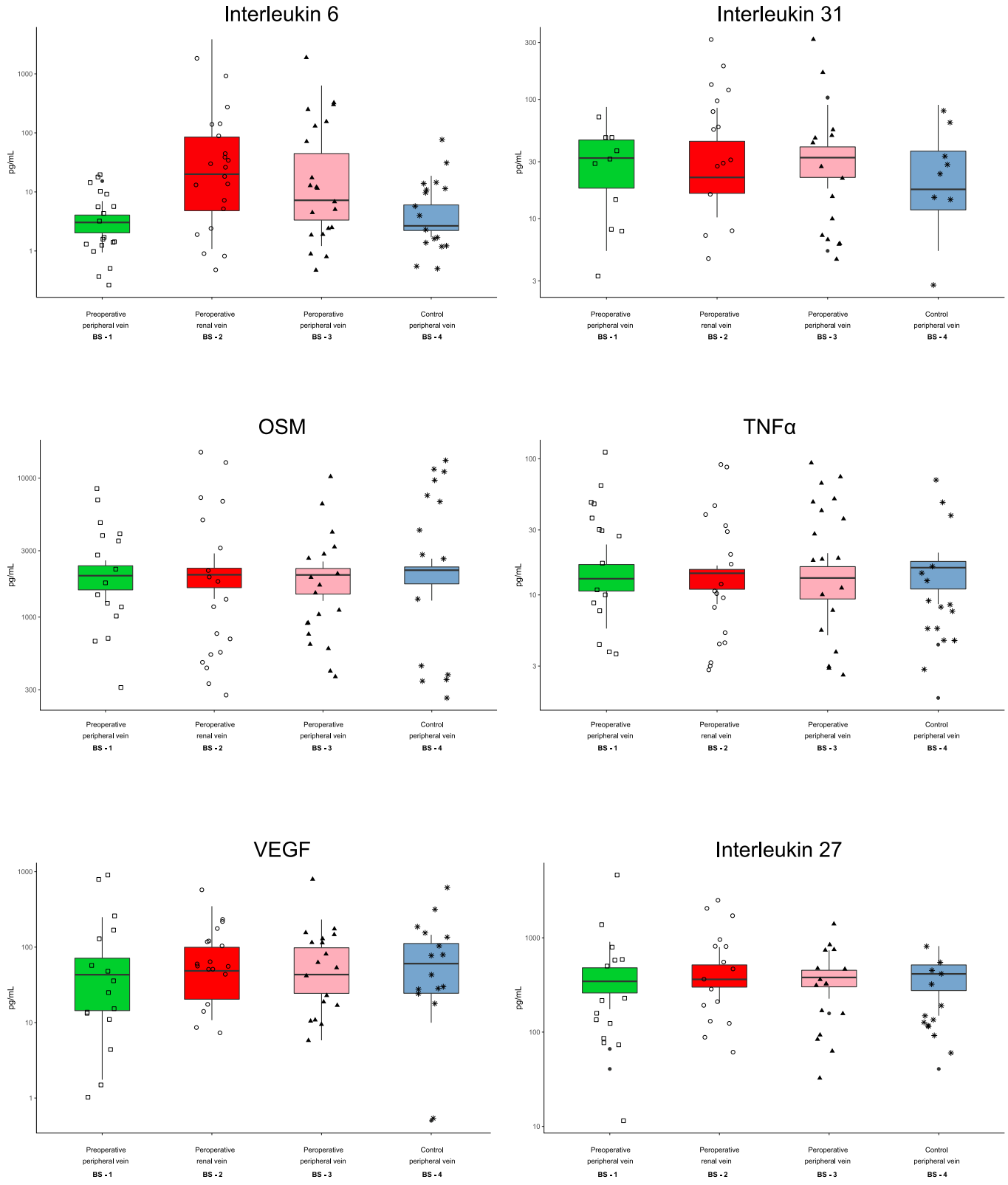

Fig. 1. CT-scan on the left and macroscopic presentation on the right of a patient treated with nephrectomy for a 12 cm (pT2b) tumor in the lower pole of right kidney, PADUA-score 13. Histopathological examination confirmed clear-cell RCC with ISUP nuclear grade 2. The arrows indicate the characteristic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

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 analyzed 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).

3. Results

3.1. Tumor and patients 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).

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 Collabarotive 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)).

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.

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. 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 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).

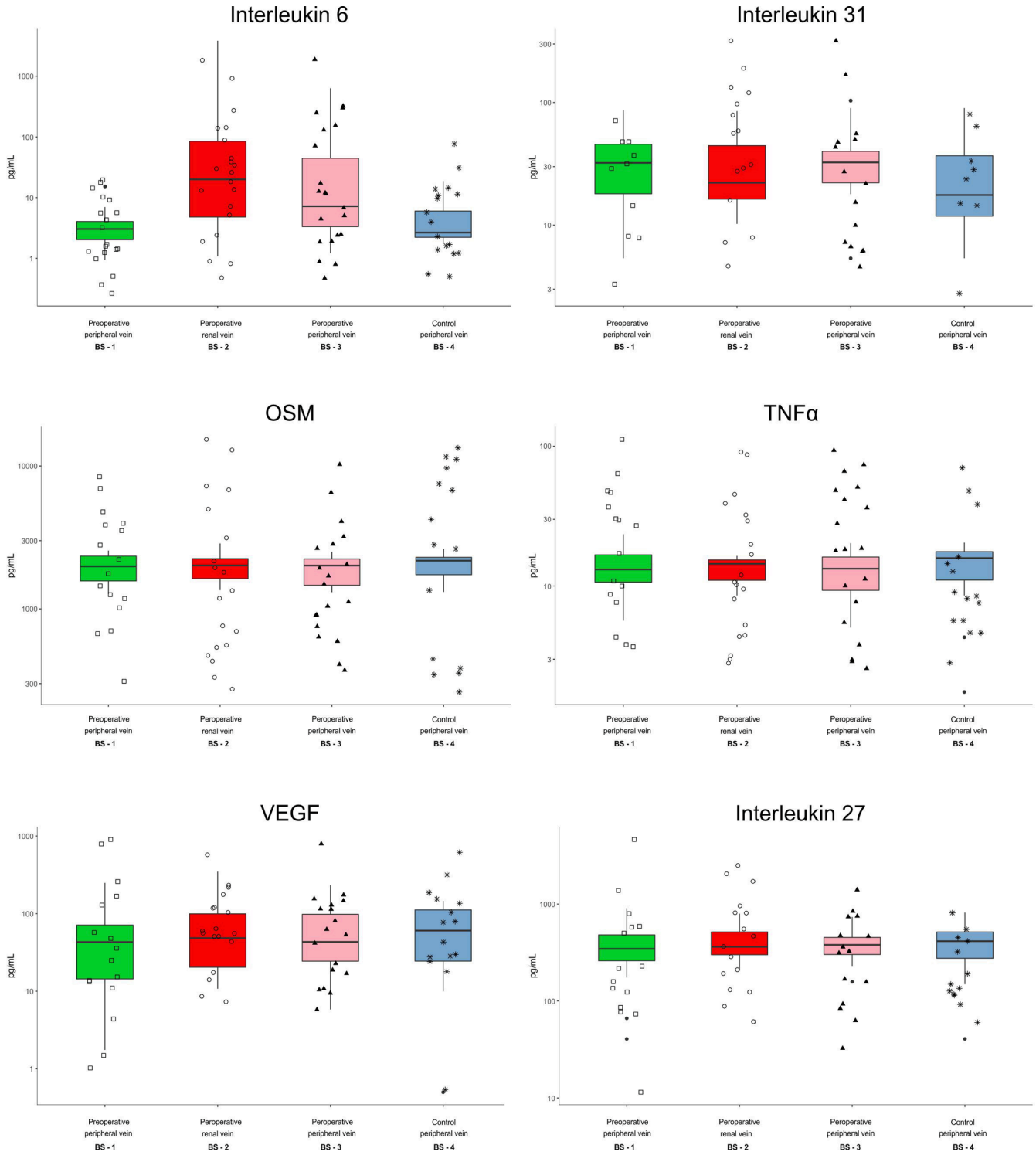

Fig. 2. The figure shows the all the values with boxplots for six cytokines measured in blood samples (BS) preoperatively (BS-1), intraoperatively and simultaneously from the renal vein (BS-2) and peripherally (BS-3) and at postoperative control after 4–6 weeks (BS-4). The intraoperative measurement 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.

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

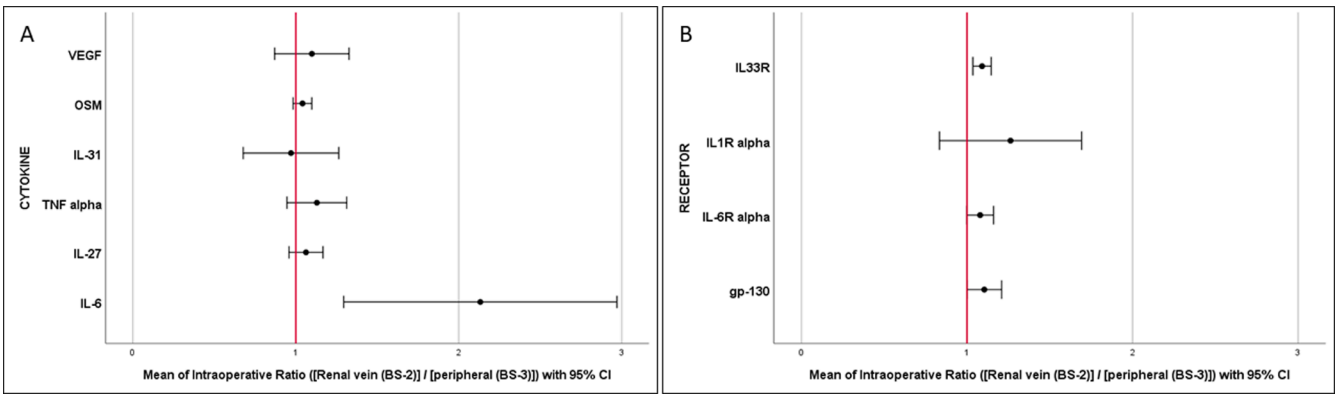

Fig. 3. The figures shows the mean of the intraoperative ratios (renal vein (BS-2)/peripherally (BS-3) with confidence intervals for A) cytokines and B) receptors. The red line represent the expected BS-2 /BS-3 ratio given an even distribution in the body. IL-6, IL-33R and gp130 have confidence intervals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

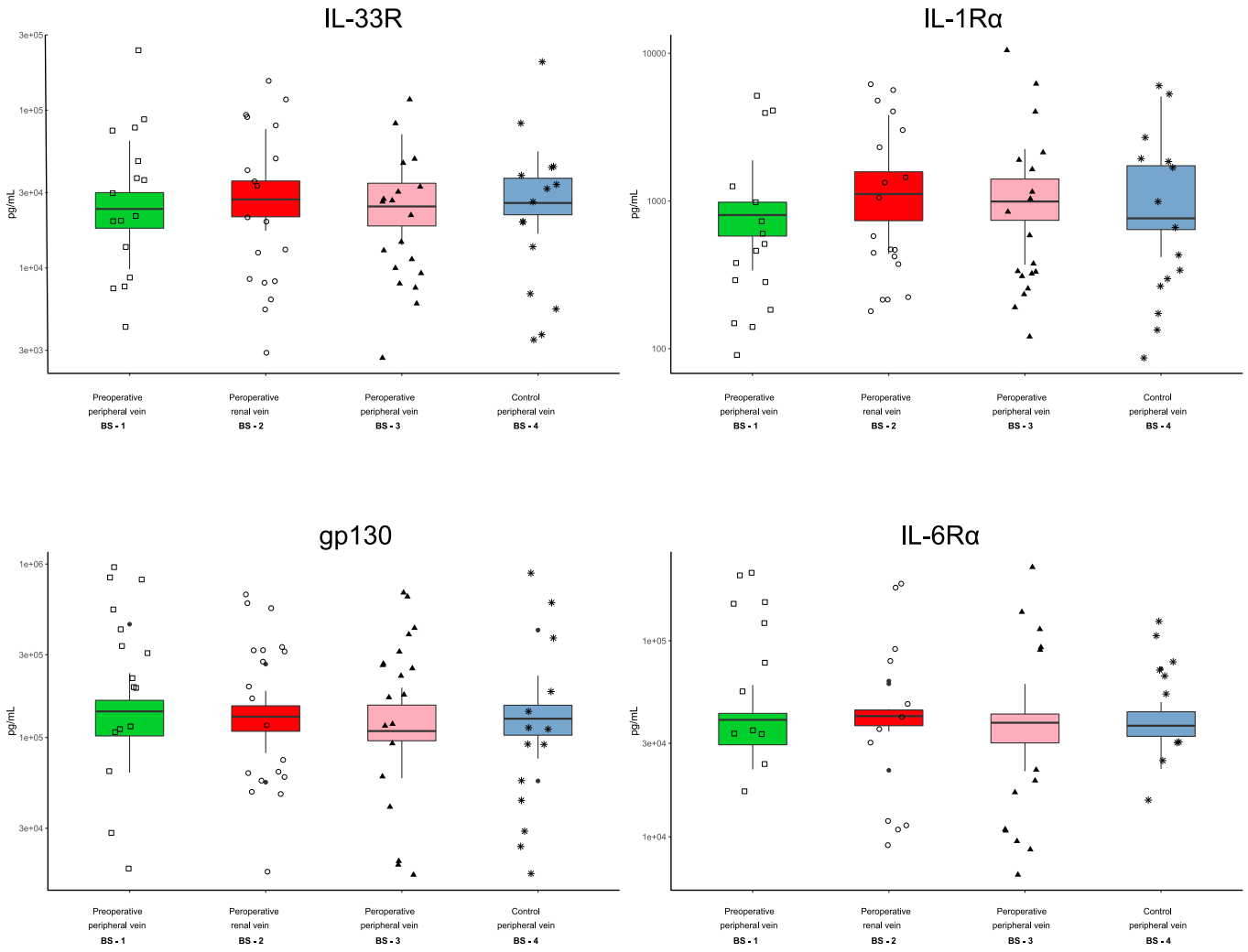

Fig. 4. The figure shows the all the values with boxplots for four receptors measured in blood samples (BS) preoperatively (BS-1), intraoperatively and simultaneously from the renal vein (BS-2) and peripherally (BS-3) and at postoperative control after 4–6 weeks (BS-4). The intraoperative measurement 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.

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 correlation was found between IL-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.

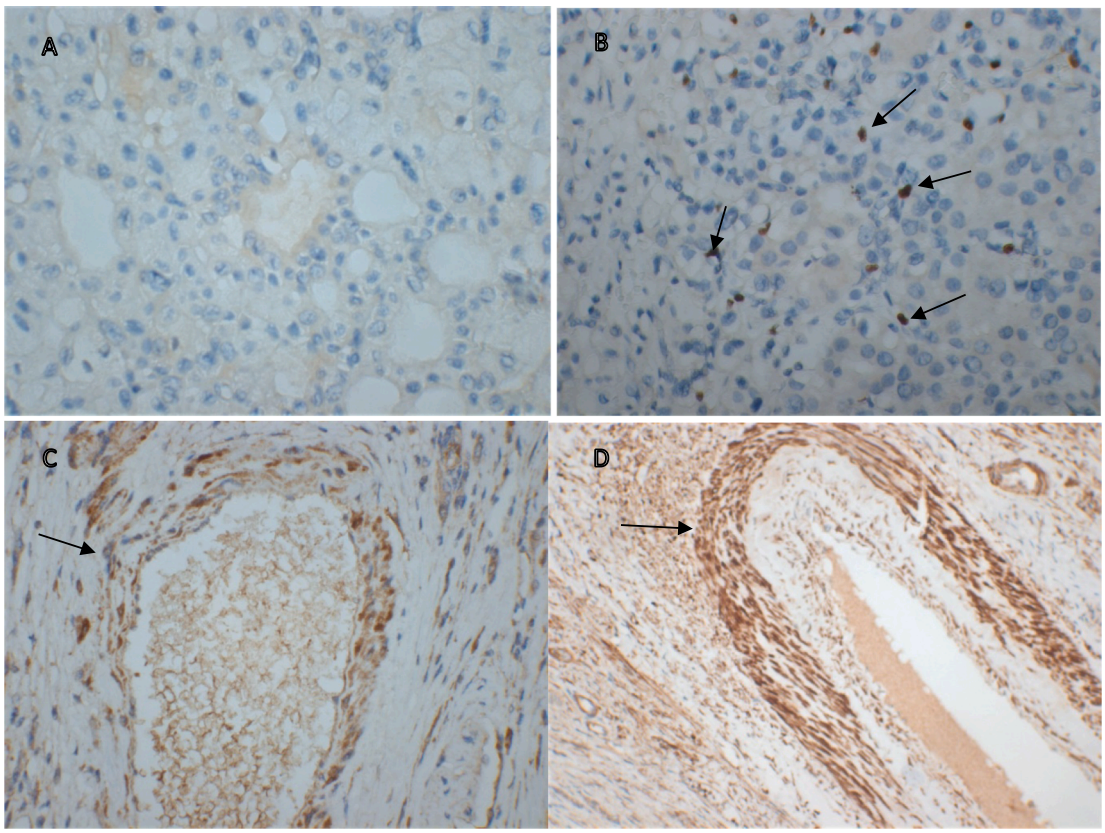

Fig. 5. Immunohistochemical staining for FOXP3 and IL-6 in clear cell renal cell carcinoma. A) Tumor tissue negative for FOXP3, score 0. B) Arrows pointing at FOXP3 positive intramural lymphocytes, score 1 (slight and scattered infiltration). C) Arrow pointing at IL-6 positive medial smooth muscle in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

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-6Rα 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.

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

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.

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.

Author's contribution

Conceptualization, visualization and methodology: Gudbrandsdottir, Gigja, Aarstad, Hans Jørgen, Beisland, Christian.

Data curation and investigation: Gudbrandsdottir, Gigja; Bostad, Leif; Reisæter, Lars; Hjelle, Karin M, Aarstad, Helene and Førde, Kristina.

Formal analysis and validation: Gudbrandsdottir, Gigja; Beisland, Christian; Reisæter, Lars.

Software: Beisland, Christian, Gudbrandsdottir, Gigja and Reisæter, Lars. IBM® SPSS® Statistics software (Release 26.0). Boxplots were made using R version 4.0.4 (www.r-project.org), utilizing the packages {foreign}, {plyr}, and {ggplot2}.

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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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