

Multi-omics Approaches Identify Uremic Toxins and Immune Regulators as Circulating Biomarkers of Metastatic RCC

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ABSTRACT

BACKGROUND: The diagnosis and treatment of metastatic renal cell carcinoma (mRCC) is challenging. Drug combinations targeting the immune checkpoint and angiogenesis have improved clinical outcomes. However, non-invasive diagnostic and prognostic biomarkers of mRCC are lacking.

RESULTS: Using plasma samples from 56 RCC patients (n=22 localized versus n=34 metastatic disease), we leveraged two recent methods: (1) high-throughput proteomics and (2) gut microbiome-focused targeted metabolomics, for non-invasive biomarker identification of localized vs mRCC (Two-sample t-tests: FDR<20% and p<0.027). Elevated biomarkers in mRCC included soluble PD-L1 (sPD-L1, p<0.0001) and CLMP (p=0.002), as determined by proteomics; and indole (p<0.0001), 5-hydroxy-indole acetic acid (5-HIAA, p=0.002), and phenyl-lacetylglutamine (PAGln, p=0.005), as determined by microbial metabolomics.

CONCLUSIONS: sPD-L1, indole derivatives, and PAGln, which have links to immune regulation and nephric integrity, were identified as biomarkers of mRCC. Follow-up studies with larger cohorts will address the oncogenic mechanisms and relevance of these biomarkers in therapy response.

KEYWORDS:

Multi-omics, Uremic Toxins, Circulating Biomarkers, Metastatic RCC

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INTRODUCTION

Clinical outcomes for patients diagnosed with renal cell carcinoma (RCC) have dramatically improved over the last few decades and the identification of the best management strategies are now being actively debated¹. In particular, the approval of immunotherapy (IO) for adjuvant RCC and IO-based combinations in patients with metastatic RCC (mRCC) have improved overall survival (OS)²⁻⁵. However, despite these improvements, biomarkers for localized versus mRCC as well as biomarkers of OS are severely lacking. Currently available algorithms estimating the risk of RCC recurrence after surgery for localized RCC, as well as prognostic scoring models for patients with mRCC, are primarily comprised of clinical features and do not account for the heterogeneity of underlying disease biology⁶⁻⁹. The need for novel biologically-driven biomarkers is thus critical.

Metastatic kidney cancer presents unique biological challenges in the development of circulating biomarkers. Although there has been some success in using cell-free diagnostic tools, kidney cancers do not shed biological material efficiently, which has limited the utility of these approaches in biomarker discovery¹⁰. The development of tumors in the kidney disrupts glomerular architecture and filtration and thereby offers an opportunity to leverage modern technologies to diagnose this filtration defect in mRCC patients. Plasma Kidney Injury Molecule 1 (KIM-1) represents one such recent example of a circulating biomarker of nephric damage that is being extensively exploited for annotation of disease stage and treatment outcomes^{11,12}.

The development of the multiplexed Olink® proteomics platform approach has offered new technology for biomarker discovery in many human disease contexts¹³. The discovery of KIM-1 as a biomarker of therapy response in RCC was made using such multiplexed proteomics tools¹². Olink® uses a ‘proximity extension assay’ where the same target protein is recognized using two independent antibodies, each with a conjugated complemen-

tary DNA tail¹⁴. The proximal association of the two antibodies can be quantified using sequencing-based strategies. The technology has now matured, and commercial Olink® panels now cater to specific functionally annotated protein target panels (e.g., metabolic panels, immunological panels, etc.). These focused approaches have reduced biological noise and improved biomarker discovery.

Gut microbial dysbiosis and the use of microbial agents as adjuvants are both relevant determinants of immunotherapy response in kidney cancer¹⁵⁻¹⁷. The nephric damage induced by RCC lesions, especially in the proximal tubules, compromises estimated Glomerular Filtration Rates (eGFR). One unexpected consequence of this disruption is the re-entry of gut microbiome derived metabolites into circulation. Improved understanding of gut microbial composition and their metabolic products, in concert with the development of highly sensitive, targeted mass spectrometry approaches has led to exponential leaps in our ability to utilize microbial metabolites as biomarkers in human disease. However, these technologies have thus far not been leveraged for biomarker discovery in mRCC.

In this study we objectively evaluate our ability to use these recent technological advances for biomarker discovery in mRCC. Remarkably, our studies have revalidated soluble PD-L1 (sPD-L1) as a biomarker of disease progression and led to the identification of several novel protein and microbial metabolite biomarkers that are elevated in mRCC patients. Finally, we have also identified circulating protein candidates associated with overall survival in these patients.

METHODS

IRBs and Patient consent details

All plasma samples were collected upon consent from kidney cancer patients at the Cleveland Clinic under the institutionally approved protocols (IRB4639, CASE5816, and CASE12815), which were combined into a single standalone IRB 22-676 to support this DOD funded study.

STATISTICAL METHODS

Baseline biomarker levels were summarized using mean and SD by disease group. Two-sample t-test was used to compare each biomarker's circulating levels between disease groups. False discovery rate (FDR) control was performed using beta-uniform method with 20% FDR rate. Spearman's correlation test was used to correlate metabolite biomarkers with Olink® biomarkers. Due to a large number of significant correlation p-values, FDR was set more stringently at 10% to identify significantly correlated biomarker pairs. Overall survival (OS) was estimated by the Kaplan-Meier method. Log rank test was used to compare OS between disease groups and biomarkers, where biomarkers were dichotomized at median and the FDR of the log rank test p-values was set at 20%. All tests were two-sided and p-values passing FDR thresholds were considered statistically significant. Statistical analysis was carried out using SAS Studio 3.7 (SAS Institute, Cary, NC) and R version 4.4 (R Foundation, Vienna, Austria).

Olink® Analysis

The Olink® analysis was performed on three panels (described in Supplementary Table 1). The patient plasma samples were first randomized and the users were blinded to the sample identity. The localized and mRCC samples were spread uniformly (and randomly) on the analysis plates. All assays were done using 1 µl of the plasma, which was aliquoted by thawing frozen plasma samples. All prepared sample plates were held overnight at -80°C until the day of experiment. The analysis was done by incubating samples with paired antibodies linked to DNA oligonucleotides for 16-22 hours. Enzymatic extension was performed and the new (hybridized) DNA sequences were amplified and quantified using the Q100 instrument. Each plate underwent quality control analysis by the immune monitoring laboratory's technical staff. Only plates passing quality control were

analyzed and compiled into final assay results.

LC-MS/MS based targeted metabolomics

Stable isotope dilution liquid chromatography with quadrupole mass spectrometer (LC-MS/MS) was used for quantification of gut microbiome-associated metabolites in plasma. 20 µl of plasma was used for extraction. In brief, 80 µl of isotope labeled internal standard mix was added to 20 µl of plasma, vortexed for 1 min, followed by centrifuging at 20000 g at 4 °C for 15 min. 30 µl of supernatant was transferred to a MS vial with insert, then 30 µl of LCMS water was added to the MS vial for the final analysis. Plasma samples were analyzed on a Thermo Vanquish liquid chromatograph coupled with a Thermo TSQ Quantiva. 0.2% of formic acid in LC-MS water was used as mobile phase A

	Localized RCC [N=22] N (% or range)	Metastatic RCC [N=34] N (% or range)
Gender	17 (77)	24 (71)
Male	5 (23)	10 (29)
Female		
Age at Diagnosis	61 (47-84)	60 (44-81)
Histology		
Clear Cell	18 (82)	25 (74)
Non clear cell	4 (18) ¹	9 (26) ²
Sarcomatoid/Rhabdoid	4 (18)	7 (21)
Features		
Nephrectomy		
Grade ≥ 3	22 (100)	26 (76)
pT ≥ 3	21 (95)	20 (77)
Node positive	16 (73)	19 (73)
	3 (14)	2 (8)
IMDC risk	N/A	
Favorable		7 (21)
Intermediate		23 (67)
Poor		4 (12)
Sites of metastatic disease	N/A	
Lymph Node		23 (67)
Lung		18 (53)
Bone		9 (26)
Liver		7 (21)

TABLE 1. Patient characteristics. ¹ One each: FH-deficient, papillary, unclassified, chromophobe. ² Papillary (n=4), Unclassified (n=3), mucinous/tubular/spindle cell (n=1), unknown (n=1).

and 0.2% formic acid in acetonitrile was used as mobile phase B. The separation was conducted using the following gradient: 0 min, 5% B; 0-2 min, 5% B; 2-8 min, 5%-100%B; 8-16 mins, 100%B; 16-16.5 mins, 5%B; 16.5-25 mins, 5%B. The flow rate was set at 0.2 ml/min. Samples were injected at 2 μ l onto a Phenomenex Gemini C18 analytical column (2.0 $\times$ 150 mm, 3 μ m). Column temperature was set at 25 $^{\circ}$ C. Multiple reaction monitoring (MRM) ion transitions are shown in Table 2. The acquired data was processed by Thermo Xcalibur 4.3 software to calculate the concentrations.

RESULTS

Cohort Characteristics

A total of 56 patients with RCC who had at least one blood sample available for analysis were included (Table 1). Of these patients, 22 had localized RCC and 34 had mRCC. Of the patients with localized disease, most were male (77%) with a median age of 61 (range, 47-84). All patients with localized RCC underwent a nephrectomy as primary treatment for their disease with clear cell RCC (82%) as the most common histology. Patients with metastatic disease were mostly male (71%), median age of 60 (range, 44-81), with the majority having undergone a prior nephrectomy (76%). Other clinical features including histology and metastatic sites of disease were reflective of a typical mRCC cohort (Table 1). The median OS for patients with localized disease was not reached (NA-NA) and for patients with mRCC was 44.32 months (35.84-NA; $p=0.005$).

Circulating protein biomarkers in mRCC

We analyzed banked plasma samples from the RCC cohort using three Olink® T96 panels: immune response, inflammation, and metabolism (Figure 1A and Supplementary Table 1), altogether representing 276 unique proteins (92 unique protein targets in each panel, with no overlap among the three panels). The circulating levels of these proteins were measured using the standard manufacturer recommended protocol; however, to minimize bias, localized and mRCC samples were randomized and then added to the same plate. Proteins were quan-

tified and the difference in circulating levels was compared using a two-sample t-test (adjusted $p \leq 0.027$). The differences in circulating levels of the proteins in the two cohorts were visualized using a volcano plot and showed 13 upregulated and 3 downregulated proteins in the mRCC cohort (Figure 1B).

We noted significant biological value in these results. Most notably, we observed that circulating levels of SPD-L1 – a regulator of immune checkpoint response – were elevated in mRCC patients (Figure 1C). This is consistent with a previous report showing SPD-L1 to be a biomarker of progressive disease in RCC¹⁸. Other notable proteins that were elevated in mRCC included the cell state regulatory proteins [e.g., Meteorin like glial cell differentiation regulator (METRNL), Nectin Cell Adhesion Molecule 2 (NECTIN2), and Neuronal Proliferation and Differentiation Control 1 (NPDC1)] and signaling receptors [e.g., LDL Receptor related Protein 11 (LRP11), Receptor tyrosine kinase like orphan receptor 1 (ROR1), and FAM3 Metabolism Regulating Signaling Molecule C (FAM3C)] (Figures 1B to 1F and Supplementary Table 2). The elevated circulating levels of these proteins are consistent with the epithelial-mesenchymal transition in mRCC and the potential immune checkpoint activation in these cancers. In contrast, we found that the circulating levels of Dopamine Decarboxylase (DDC) and Tripartite Motif-Containing Protein 21 (TRIM21) were reduced in mRCC patients. Altogether, this analysis offered many candidate biomarkers of metastatic disease.

Prognostic markers in mRCC

As expected, we noted that patients with mRCC had worse overall survival (OS) than patients with localized disease (Supplementary Figure 1). We then re-analyzed the metastatic cohort and probed for prognostic markers in the Olink® panel. We stratified the mRCC patients into high- and low-level groups based on the median circulating protein level and compared the association between circulating protein levels and OS. The results of this analysis were surprising. For example, none of the proteins with elevated circulating levels in the

mRCC patients (relative to localized RCC) showed any statistically significant associations with OS within the mRCC patient cohort. Instead, we found ~12 proteins whose higher circulating levels correlated with worse OS in mRCC (Supplementary

Table 3). Besides the well-known oncogenic transcription factor, JUN, we identified transcriptional regulatory proteins [e.g., HEXIM P-TEFb complex subunit 1 (HEXIM1) and Zinc Finger and BTB domain containing 16 (ZBTB16)] and signaling pro-

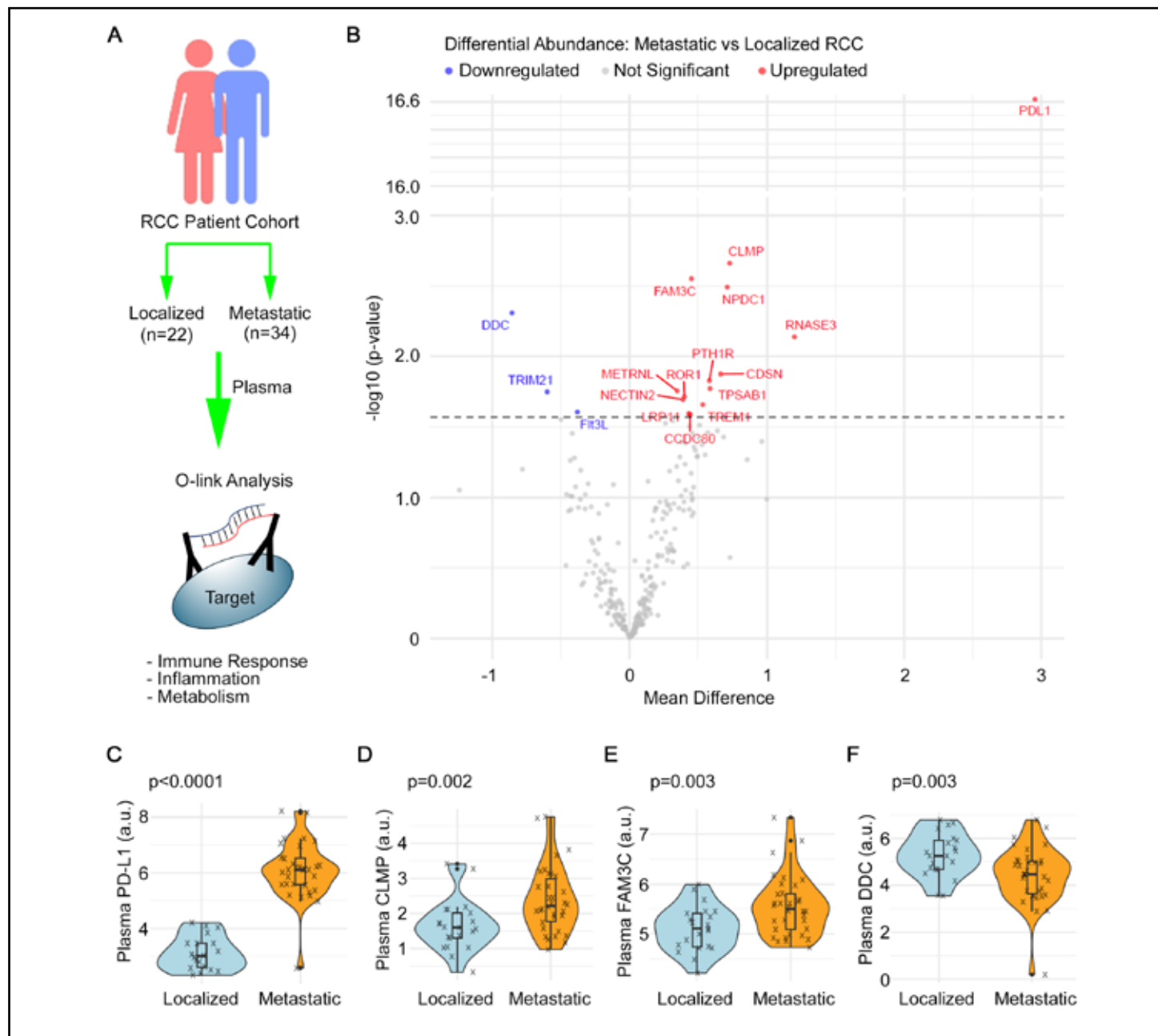


FIGURE 1. Biomarker Discovery in mRCC using Olink® Analysis. (A) Schema of experimental design and the biochemical principle of the Olink® based 'proximity extension assay'. (B) Volcano plot of fold-change in protein abundance in metastatic (n=34) versus localized RCC (n=22) patients. Statistically significant differences are highlighted above the dotted line with the elevated proteins labeled in red and downregulated proteins shown in blue. (C-F) Violin plots of certain representative proteins, as indicated, that were either up- or down-regulated in metastatic disease. All statistical comparisons were done using the two-sample t-test with correction for multiple testing and a $p \leq 0.027$ was considered significant.

teins [e.g., Hematopoietic cell-specific Lyn substrate 1 (HCLS1) and SRSF protein kinase 2 (SRPK2)] among the proteins associated with worse outcomes in mRCC (Figure 2 and Supplementary Table 3). Note that circulating levels of these proteins were not increased in mRCC relative to localized disease, thus highlighting the need to distinguish circulating protein levels from potential functional consequences of these proteins. Altogether, our Olink® analysis was able to identify new biomarkers associated with mRCC disease.

Discovery of differentially abundant microbial metabolites

One concern with the Olink® biomarkers was that, except for sPD-L1, the other markers showed relatively small fold-change differences in metastatic versus localized disease. We therefore sought to leverage our expertise to interrogate other novel biological processes, such as the gut microbial metabolites, for biomarker discovery.

The gut microbial community synthesizes and catabolizes many key nutrients and in fact can be considered an endocrine organ producing unique metabolites that systemically enter circulation^{19, 20}. Moreover, gut dysbiosis is a clinically relevant feature of kidney cancer¹⁵⁻¹⁷. We hypothesized that the disruption of the kidney architecture in RCC patients affects the circulating levels of many of these metabolites and interrogated the relevance of these metabolites as mRCC biomarkers. To this end, we

No.	Metabolite	Polarity	Precursor (m/z)	Product (m/z)
1	TMAO	Positive	76.1	58.1
2	Choline	Positive	104.1	60
3	N-Acetyl-5-hydroxytryptamine	Positive	219.1	160.1
4	5-Hydroxy-Indole-acetic acid	Positive	192.1	146.0
5	Phenyl-acetyl-glutamine	Positive	265.1	130
6	Phenyl-acetyl-glycine	Positive	194.1	91.0
7	Lactoyl-phenylalanine	Positive	238.1	120.1
8	3-Methyl-2-oxindole	Positive	148.1	130
9	Indole	Positive	118.1	91.1
10	Indole-3-acetate	Positive	176.1	130.0
11	Indole-3-propionic acid	Positive	190.1	130.0
12	Indole-3-butyric acid	Positive	204.1	186.0
13	3-Methyl-Indole	Positive	132.1	117.0
14	TMAO-d9	Positive	85.3	66.1
15	N-Acetyl-5-hydroxytryptamine-d3	Positive	222.4	160
16	5-Hydroxy-Indole-acetic acid-d6	Positive	198.3	151
17	Phenyl-acetyl-glutamine-d5	Positive	270.3	130
18	Phenyl-acetyl-glycine-d5	Positive	199.3	96.1
19	Indole-3-acetate-d2	Positive	178.3	132.1
20	Indole-3-propionic acid-d2	Positive	192.3	130
21	Indole-3-butyric acid-d2	Positive	206.3	188
22	3-Methyl-Indole-d3	Positive	135.3	117
23	N-Butylglycine	Negative	144.1	74.1
24	DL-p-hydroxyphenyllactic acid	Negative	181.1	162.9
25	3-hydroxyhippuric acid	Negative	194.1	150.0
26	4-Hydroxybenzoic acid	Negative	137.1	93.0
27	Hippuric acid	Negative	178.1	134.0
28	p-Cresol-glucuronide	Negative	283.2	175
29	3-Phenyllactic acid	Negative	165.1	147
30	4-Hydroxybenzoic acid-d4	Negative	141.3	97
31	Hippuric acid-d2	Negative	180.3	136.0
32	p-Cresol-glucuronide-d7	Negative	290.4	113

TABLE 2. Multiple reaction monitoring (MRM) transition for each metabolite quantified by LC-MS/MS.

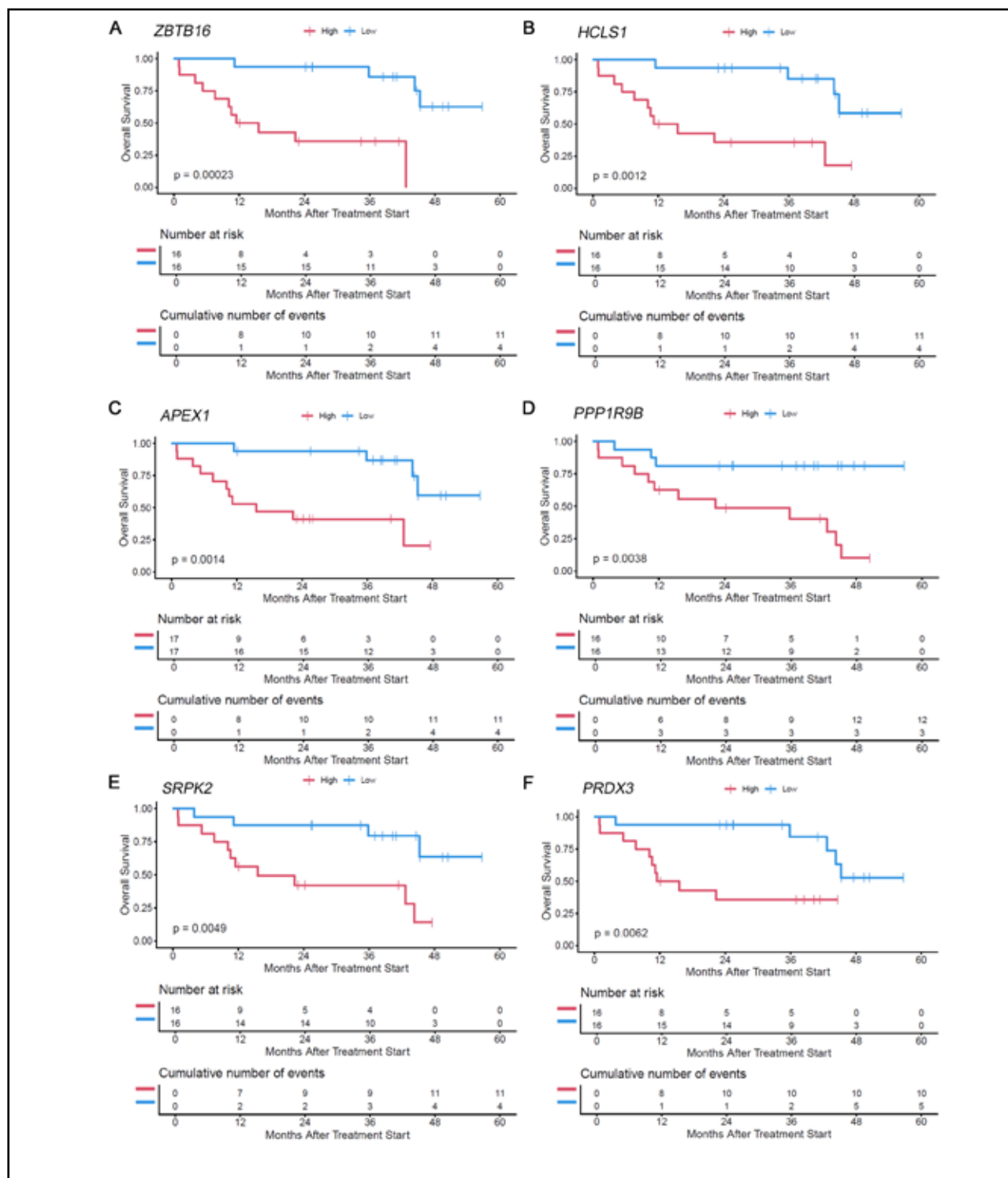


FIGURE 2. Overall Survival (OS) Analysis in Metastatic RCC Patients. (A to F) Kaplan-Meier curves and corresponding survival data tables of metastatic RCC patients showing significantly worse outcomes (lower OS) in patients with elevated expression of ZBTB16 (A), HCLS1 (B), APEX1 (C), PPP1R9B (D), SRPK2 (E), and PRDX3 (F), as indicated.

deployed a targeted stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) method to measure the abundance of a panel of circulating metabolites that are known to originate from gut bacteria (Figure 3A and Table 2). We then compared the circulating levels of metabolites derived from indole and choline in the plasma of mRCC (versus localized RCC) patients, using the same statistical parameters described for the proteomics analysis (two-sample t-test, adjusted p-value ≤ 0.027). These comparisons identified three metabolites whose circulating levels were elevated in mRCC patients, including bacterially-derived metabolites of tryptophan, such as indole (fold-change: 4.1; p-value: $1.51\text{E-}06$) and 5-hydroxy-indole acetic acid (5-HIAA, fold-change: 1.7; p-value: $2.42\text{E-}03$) (Figure 3B and 3C, and Supplementary Table 2). We also found that levels of the bacterial phenylalanine metabo-

lite phenylacetylglutamine (PAGln) were elevated in metastatic patients (fold-change: 2.3; p-value: $4.90\text{E-}03$) (Figure 3D and Supplementary Table 2).

The discovery of indole derivatives and PAGln as biomarkers of mRCC deserves further investigation, given the emerging literature supporting a role for the gut microbiome in ccRCC²¹⁻²³. The indole metabolites are derived from tryptophan; however, their effects on tumor growth have been controversial^{24,25}. Plant-derived indole metabolites (e.g., the indole alkaloids Vinblastine and Vincristine and indole-3-carbinol), are known for their anti-tumor properties²⁶. Interestingly, indole is metabolized by gut microbes into indoxyl sulfate, a uremic toxin associated with chronic renal damage²⁷. Moreover, studies in mRCC patients indicate that indole (and its derivatives) are associated with immunosuppression and driving

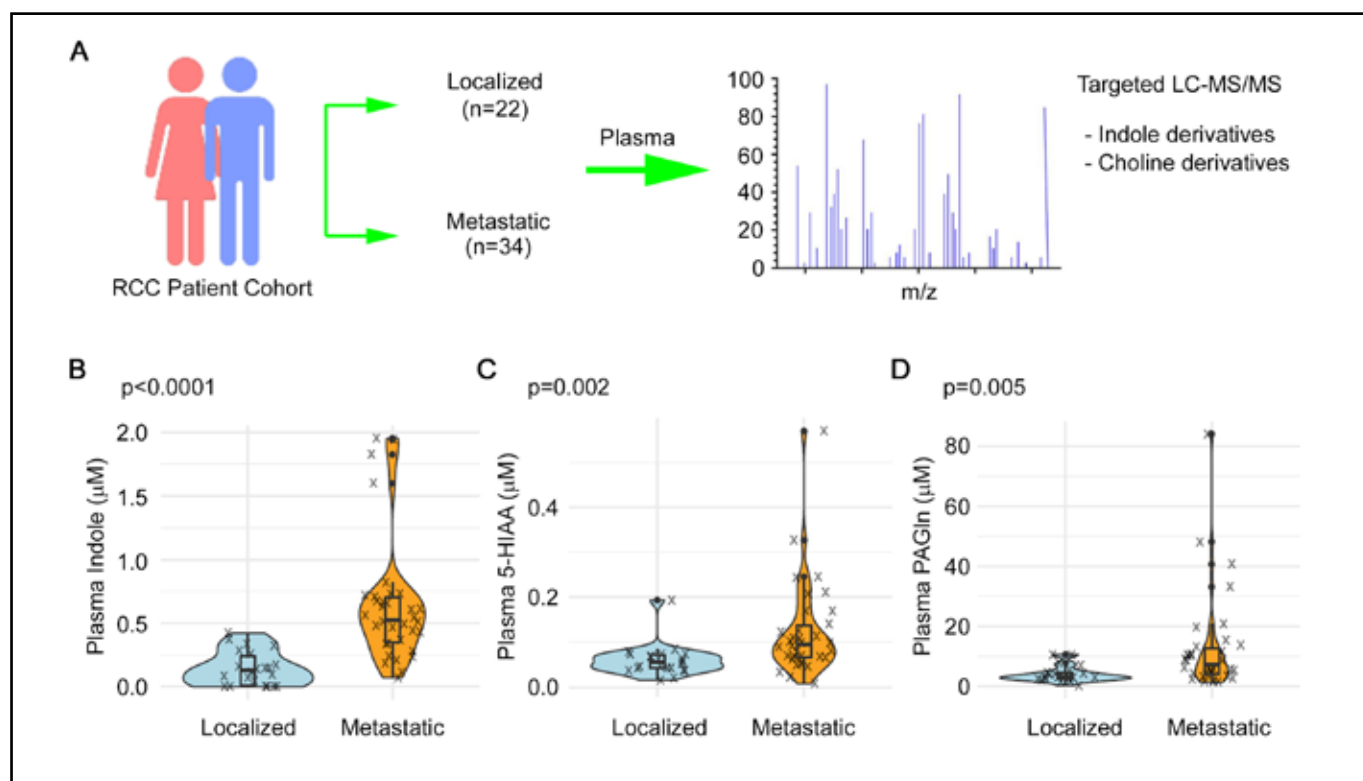


FIGURE 3. Microbial Metabolomics Analysis for Biomarker Discovery in mRCC. (A) Schema of experimental design for LC-MS/MS based metabolomics analysis of gut-derived metabolites in the indole and choline pathways. (B-D) Violin plots of certain representative metabolites that were upregulated in metastatic disease (n=34) versus localized RCC (n=22) patients. All statistical comparisons were done using the two-sample t-test with correction for multiple testing (FDR $\leq 20\%$; $p \leq 0.027$ was considered significant).

metastatic disease²⁸. These findings are consistent with the idea that elevated circulating levels of indole in patients with mRCC might be associated with dysfunctional tumor-immune interactions and ultimately promote tumor growth in kidney cancer.

In contrast to the indole derivatives, the relevance of PAGln is relatively understudied in cancer biology; however, this molecule is associated with worse cardiovascular risk and with renal dysfunction, which has led to its previous characterization as a uremic toxin²⁹⁻³³. Therefore, elevated PAGln (like indoxyl sulfate) could potentially be a marker of renal damage in mRCC patients.

Correlation analysis of the Olink® and microbial metabolite panels

Our initial studies analyzed the Olink® and microbial metabolite panels independently to identify novel biomarkers in mRCC patients. Next, we performed correlation analysis between these two datasets to identify biological features that co-oc-

cur with high propensity. To perform this analysis, we did pairwise comparisons between the microbial metabolites with each of the proteins in the Olink® panel. We calculated statistical significance using the Spearman's correlation test (FDR: 10%, adjusted p-value ≤ 0.0482). Our analysis identified ~735 protein-metabolite pairs that showed statistically significant correlations (Supplementary Figure 2 and Supplementary Table 4).

We then focused on the three microbial metabolites (Indole, 5-HIAA, and PAGln) that showed significant elevation in mRCC patient samples and shortlisted the statistically significant correlations for each of these metabolites (Figure 4A and Supplementary Table 4). All three metabolites correlated with a panel of ~20 proteins (Figure 4A). To infer the biological implications of these associations, we performed enrichment analysis on the proteins that showed statistically significant correlations with these metabolites, using the g:Profiler³⁴. These studies demonstrated that the

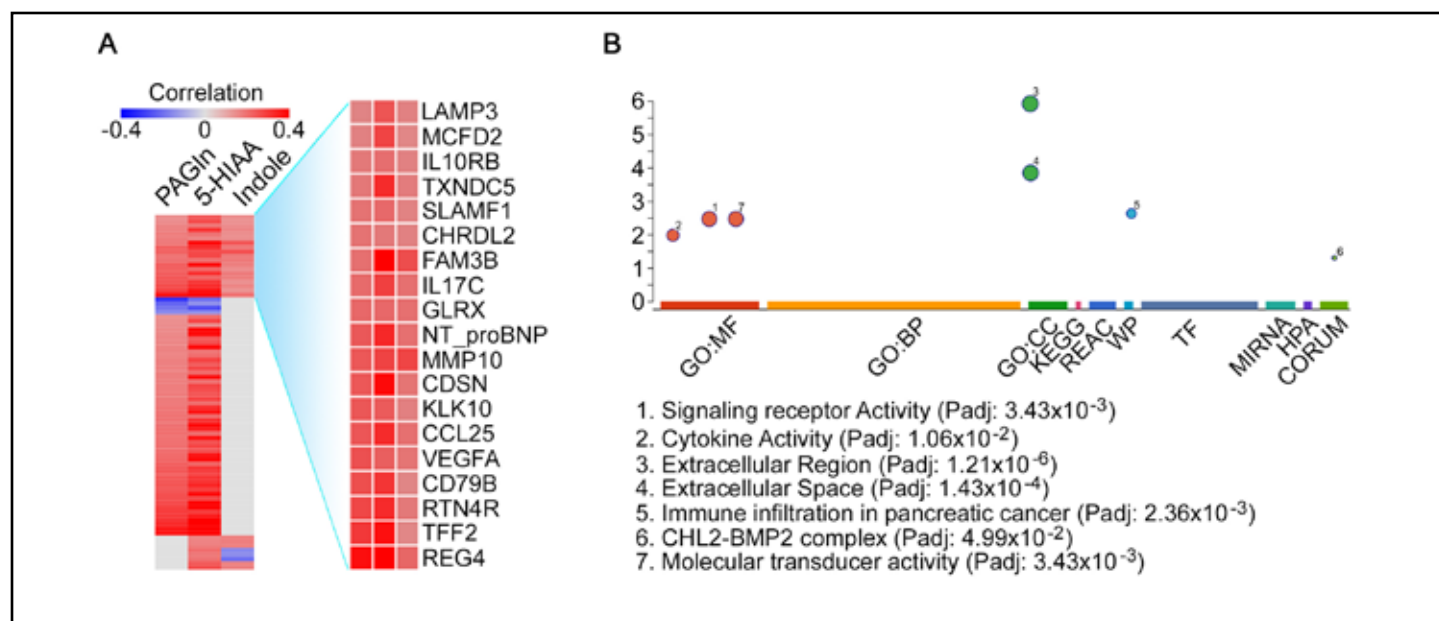


FIGURE 4. Correlation Analysis between Olink® and Metabolomics Datasets. (A) Heatmap representing the statistically significant positive correlations between the proteins detected by Olink® analysis and the microbial metabolites phenylacetylglutamine (PAGln), 5-hydroxy indole acetic acid (5-HIAA), and Indole, as indicated. Significant correlations were identified using the Spearman correlation analysis (FDR $\leq 10\%$, $p \leq 0.0482$ considered significant). (B) Functional enrichment analysis, using the g:profiler algorithm to identify biological features that are overrepresented in the proteins identified via correlations studies in (A).

three metabolites correlated with elevated circulating levels of proteins involved in chemokine signaling, receptor-ligand signaling modules, and chemotaxis/migration (Figure 4B), consistent with the immunological dysregulation and inflammatory state that is typical of metastatic tumors.

DISCUSSION

Renal cell carcinoma (RCC) is one of the most common cancers in the United States with approximately 80,000 new diagnoses per year and 14,000 deaths each year³⁵. Despite the high prevalence and lethality of RCC, there are no established blood-based biomarkers for early detection or treatment selection. The International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) is the most commonly used prognostic criteria currently used in clinical practice^{7,36}, but it relies solely on clinical characteristics. It does not account for biological heterogeneity, such as underlying genomic, metabolomic, or microbial differences between RCC patients. While it functions well as a prognostic marker for mRCC patients undergoing systemic therapy, it does not guide treatment selection or serve as a predictor of response or resistance to therapy. Other algorithms in RCC are also predominantly limited to clinical features.

Recently, investigations into the genomic heterogeneity of RCC and an attempt to incorporate their use in prognostication and treatment selection has gained momentum. Efforts to incorporate genomic components (e.g., PBRM1, BAP1, etc.) into the IMDC prognostic criteria are ongoing. Studies such as OPTIC (NCT05361720), BIONI-KK (NCT02960906), and CARE1 (NCT06364631) are using genomic biomarkers to guide treatment selection. Moreover, studies investigating the role of blood-based biomarkers such as KIM-1 to detect RCC before clinical diagnosis or as a means to monitor effective therapeutic response are also underway¹¹⁻¹². Despite this progress, no validated biomarkers are currently available, highlighting the need for novel approaches in biomarker development for RCC. Our study provides a template to complement these ongoing trials with

biomarker discovery by leveraging some recent technological developments.

Although large-scale biomarker discovery was initially limited to nucleic acids, recent technological developments have allowed us to directly measure small molecules and proteins. The development of Olink® has been one such example. This multiplexed assay relies on proximity ligation. Two monoclonal antibodies targeting different epitopes on the same protein, each with a complementary DNA tail, are bound to biological samples. The juxtaposition of the antibodies results in DNA hybridization, which can be efficiently quantified using real-time-qPCR analysis. The abundance of the hybridized product thus offers a quantitative proxy for binding affinity. This technological approach has now matured and begun offering the opportunity for large-scale biomarker discovery. Our work has leveraged this relatively novel tool to address the clinically urgent need for biomarker discovery in mRCC.

Within the last decade, there have been numerous reports linking the gut microbiome and metaorganismal metabolic pathways to diverse cancers³⁷⁻³⁹. However, the vast majority of cancer-related microbiome studies are correlative, simply cataloguing stool microbiome or tumor-associated microbiome in cancer patients using metagenomic sequencing approaches. Undoubtedly, high throughput sequencing approaches have uncovered a number of compelling microbiome-cancer links. However, just because a bacterial sequence is detectable and is associated with disease does not mean that there is a functional alteration in the gut microbiome. In parallel to the rapid growth in sequencing-based approaches, advances in the fields of mass spectrometry and computational biology have allowed for the identification of natural products and metabolites that originate solely from the gut microbial endocrine organ^{19,20}. Here, we have leveraged access to LC-MS/MS methods to quantify several metabolites that are known to be exclusively derived from bacterial sources. Although, here, we have only established associations between the metabolites and mRCC, these

findings could be mechanistically developed to address the importance of microbe-host crosstalk.

There is now a wealth of evidence supporting a role for metaorganismal tryptophan metabolism in diverse cancers. In general, the host derived tryptophan metabolite kynurenine has been linked to cancer promotion⁴⁰⁻⁴², whereas gut microbe-derived indole metabolites of tryptophan such as indole-3-propionic acid and indole-3-acetic acid have been shown to enhance anti-tumor immunity⁴⁰⁻⁴². The current thought is that bacterially-derived indole metabolites are sensed by the host nuclear hormone receptor aryl hydrocarbon receptor (AhR) to elicit some of their biological activity in the host^{43,44}. However, we still incompletely understand potential mechanisms by which microbial and host-derived tryptophan metabolites impact ccRCC metastasis. Interestingly, both AhR and the Hypoxia Inducible Factor (HIF) transcription factor compete for binding with the Aryl Hydrocarbon receptor Nuclear Translocator (ARNT, also called HIF1 β). HIF is a well-known oncogenic driver in ccRCC and we speculate that indole-derived metabolites could thus functionally impact HIF-dependent oncogenic programs by regulating the available intracellular pools of ARNT. Altogether, additional studies are warranted to explore the potential role for bacterially-derived indole metabolites in ccRCC progression.

Our data also indicates elevated levels of the gut microbe-derived phenylalanine metabolite PAGln in mRCC patients. PAGln has recently gained attention as a cardiovascular disease-associated metabolite, where it is known to signal through host adrenergic receptors to promote pro-thrombotic effects²⁹⁻³³. We thus speculate that gut microbe-derived PAGln may similarly activate adrenergic pathways in the kidney or metastatic niches to promote ccRCC. Collectively, the current study identifies several potential metaorganismal nutrient metabolism pathways (tryptophan > indole; phenylalanine > PAGln) that deserve further mechanistic investigation in the setting of ccRCC metastasis. Although both these metabolites have associations

with nephric integrity, we did not have clinical measures of renal function from our patients and thus could not statistically correlate circulating levels of these metabolites with renal damage. In addition, the potential influence of nephrectomy status and residual renal function on circulating metabolite levels could not be evaluated in this cohort.

There are several inherent limitations in this study. Given the relative expense associated with these analyses, we have presently limited our studies to a pilot 'risk-assessment' scale with 56 patient samples, which has unfortunately diminished our ability to make broader associations with disease outcomes. Moreover, clinically, the study contains a relatively small and heterogeneous population of patients with mRCC and does not define for which specific histology, stage, or systemic treatment these findings are likely to be most relevant. Another important limitation is that there were no standardized collection time points for these samples. As such, timing of plasma collection relative to nephrectomy, metastatic diagnosis, etc. varied between patients and may have impacted results. Finally, the study is also conducted at a single institution and thus requires external validation in larger cohorts. Importantly, sPD-L1 was the most notable biomarker identified in our Olink® analysis and circulating levels of this protein (previously called soluble PD-L1) have been reported as a biomarker of RCC disease progression¹⁸. Thus, while additional studies are indeed necessary, our preliminary findings do have some external validation and thus bear potential for future clinical translation.

Finally, it is critical to highlight that the metastatic biomarkers (i.e., those elevated in mRCC vs localized RCC) did not translate into an association with OS within mRCC patients. In other words, biomarkers of a disease state are not necessarily prognostic biomarkers within a specific disease setting. We acknowledge the caveat that the abundance of a given protein and its association with OS neither establishes causation nor provides evidence of a mechanistic link between the protein (and/or metabolite) and mRCC development. Therefore, we cautiously

consider these differentially regulated proteins and metabolites as biomarkers associated with metastatic disease rather than as evidence of causality or therapeutic feasibility. Future studies will address the functional relevance of these proteins/metabolites as (potential) drivers of metastatic disease by leveraging cell-based assays, animal models of kidney cancer, and human patient-derived organoids/xenografts. Altogether, the biomarkers identified in this study have provided us with important results that serve as a foundation for future clinical and laboratory-based validation studies.

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The authors declare no conflicts of interest with the findings presented in this work. All data is provided in the manuscript or as supplementary data. The following author contributions are credited. Conceptualization: MCO, JMB, AAC; Performed Experiments: XY, SD, VV, MP; Experimental Data Analysis: XY, SD, VV, ML, MP, CMDM; Statistical Analysis: WW; Wrote Manuscript: MCO, JMB, AAC.

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DATA AVAILABILITY STATEMENT

All the data necessary to evaluate the conclusions of the manuscript are provided in the paper and/or the Supplementary Materials.

SUPPLEMENTAL INFORMATION

Supplemental information is available online: <https://kidney-cancer-journal.com/>

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