

# Prognostic Role of Circulating Tumor Cells in Metastatic Renal Cell Carcinoma: A Large, Multicenter, Prospective Trial

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Disclosures of potential conflicts of interest may be found at the end of this article.

**Key Words.** Circulating tumor cells • Renal cell carcinoma • Metastases • Sunitinib • Pazopanib • Biomarker

## ABSTRACT

**Background.** Circulating tumor cells (CTCs) correlate with adverse prognosis in patients with breast, colorectal, lung, and prostate cancer. Little data are available for renal cell carcinoma (RCC).

**Materials and Methods.** We designed a multicenter prospective observational study to assess the correlation between CTC counts and progression-free survival (PFS) in patients with metastatic RCC treated with an antiangiogenic tyrosine kinase inhibitor as a first-line regimen; overall survival (OS) and response were secondary objectives. CTC counts were enumerated by the CellSearch system at four time points: day 0 of treatment, day 28, day 56 and then at progression, or at 12 months in the absence of progression.

**Results.** One hundred ninety-five eligible patients with a median age of 69 years were treated with sunitinib (77.5%) or pazopanib (21%). At baseline, 46.7% of patients had one

or more CTCs per milliliter (range, 1 to 263). Thirty patients had at least three CTCs, with a median PFS of 5.8 versus 15 months in the remaining patients ( $p = .002$ ; hazard ratio [HR], 1.99), independently of the International Metastatic RCC Database Consortium score at multivariate analysis (HR, 1.91; 95% confidence interval [CI], 1.16–3.14). Patients with at least three CTCs had a shorter estimated OS of 13.8 months versus 52.8 months in those with fewer than three CTCs ( $p = .003$ ; HR, 1.99; multivariate analysis HR, 1.67; 95% CI, 0.95–2.93). Baseline CTC counts did not correlate with response; neither did having CTC sequencing counts greater than or equal to one, two, three, four, or five.

**Conclusion.** We provide prospective evidence that the presence of three or more CTCs at baseline is associated with a significantly shorter PFS and OS in patients with metastatic RCC. *The Oncologist* 2021;26:740–750

**Implications for Practice:** This prospective study evaluated whether the presence of circulating tumor cells (CTCs) in the peripheral blood correlates with activity of first-line tyrosine kinase inhibitors in metastatic renal cell carcinoma (RCC). This

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study demonstrated that almost half of patients with metastatic RCC have at least one CTC in their blood and that those patients with at least three CTCs are at increased risk of early progressive disease and early death due to RCC. Studies incorporating CTC counts in the prognostic algorithms of metastatic RCC are warranted.

## INTRODUCTION

The treatment of metastatic renal cell carcinoma (mRCC) was revolutionized in the early 2000s by the advent of oral tyrosine kinase inhibitors (TKIs) targeting the vascular endothelial growth factor receptor and platelet-derived growth factor receptor, with a significant improvement in patients' progression-free survival (PFS) and overall survival (OS) [1]. The activity of TKIs can today be significantly increased by the adjunct of anti-programmed cell death-1 (PD-1) monoclonal antibodies [2].

However, patients with mRCC are extremely heterogeneous in terms of response to treatment. Prognostic stratification according to 6 independent clinical parameters was validated using the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) in the era of targeted therapies [3], but the IMDC score does not predict radiological response to TKIs. Moreover, the evaluation of radiological response to antiangiogenic agents may be troublesome if we resort to the classic RECIST criteria [4, 5].

Several studies have investigated a wide range of circulating biomarkers in patients with mRCC [6], but so far none of them has been approved for use in clinical practice.

According to the Foundation for the National Institutes of Health Biomarkers Consortium, it is currently accepted that circulating tumor cells (CTCs) may be identified by the expression of markers of epithelial origin (cytokeratins and epithelial cell adhesion molecule [EpCAM]) and the absence of the hematopoietic marker cluster differentiation 45 (CD45) antigen [7], as applied in the CellSearch semiautomated enrichment system. Yet, many alternative tools may be used, such as the more recent biophysical label-free enrichment strategies [8–10].

A pivotal study in breast cancer clearly demonstrated that CTC counts above five cells per 7.5 mL predict a dismal prognosis in patients with metastatic disease [11]. Likewise, the prognostic role of CTCs was also confirmed in metastatic colon cancer [12], prostate cancer [13, 14], lung [15] and other solid tumors [16]. Conversely, very little data are available concerning the role of CTCs in mRCC. Using the CellSearch system, 25% of specimens from 11 patients with mRCC showed at least two CTCs in a pilot study [17]; subsequently, 16% of 25 patients with mRCC reported the presence of at least one CTC in another small study [18].

TKIs were developed as antiangiogenic agents, but they also have intrinsic antitumoral and immunomodulatory activity [19, 20]. It may thus be speculated that these agents might modify the release and spread of CTCs in the blood and interfere with their metastatic capability [20, 21], although no clinical evidence is available so far.

We first designed a pilot study to determine the feasibility of CellSearch assay in mRCC [22] and found that CTCs were present in 58.5% of 53 treatment-naïve patients. Once we were able to identify apoptotic circulating tumor cells

with the expression of the M30 neopeptide [23, 24], we found that a higher balance of apoptotic to live CTCs correlated with improved outcome in terms of disease control [22].

We therefore planned a prospective noninterventional multicenter trial designed to evaluate the distribution and prognostic/predictive role of CTCs in a wider, but homogeneous, cohort of patients with mRCC receiving a TKI as their first-line regimen for metastatic disease.

## MATERIALS AND METHODS

### Patients

Eligibility criteria were as follows: histological diagnosis of renal cell carcinoma (RCC; clear cell or non-clear cell subtype) performed on either a resected kidney tumor or on a biopsy of a metastatic site; any age and Fuhrman grade; the presence of metastatic disease candidate to receive a TKI as a first-line regimen.

As previously reported, the first 53 patients were enrolled at the Istituto Oncologico Veneto (IOV) and three Italian centers from June 2008 to September 2010 [22]. The remaining patients were enrolled in 15 Italian centers from May 2013 to December 2015 as part of a large multicenter study approved first by the IOV Ethics Committee on April 15, 2013, and then by the Ethics Committee of participating Centers. All patients gave their written consent for the collection of serial blood samples and their clinical data for this observational study. The choice of TKI and dose reductions were left to the treating physician's discretion.

As an observational trial, the study did not involve a predefined schedule of assessments, since each Investigator followed international guidelines for baseline staging and for the monitoring of systemic therapies in mRCC, mainly with contrast-enhanced CT scan of the chest and abdomen. Blood tests (hematology with renal and liver function, and thyroid function tests) were repeated approximately every month. The status of the disease was mainly reevaluated by means of contrast-enhanced CT scan performed every 3 to 4 months. Further radiological exams (such as magnetic resonance, ultrasound, or bone scintigraphy) were performed whenever needed. Tumor response and progression were assessed locally according to RECIST, version 1.1 [4].

### CTC Count Schedule and Analysis

Blood samples had to be taken at baseline on the day of the first TKI dose, and then on day 28, day 56 and at the time of progression, or after 12 months in the absence of progression.

Whole blood samples were collected into CellSave tubes (Code # 790005, Menarini, Italy). Samples were maintained at room temperature and dispatched to IOV for the

centralized evaluation of CTCs and Circulating Endothelial Cells (CECs), to be completed within 96 hours. CTCs were enumerated in whole blood through the CellSearch System (Menarini, Italy), according to the manufacturer's instructions for the Circulating Tumor Cell (CTC) Kit (Code # 7900001, Menarini, Italy) and the user guidelines for in vitro diagnostic use [11, 25].

In order to identify nucleated epithelial cells, CTCs were enriched by the automated platform via anti-EpCAM antibodies and then stained for DAPI and cytokeratin 8, 18, and 19, whereas CD45 staining, which identifies leukocytes, was used as specificity control [7].

An event was classified as CTC when its morphological features were consistent with that of a nucleated cell with round or oval morphology, with a visible nucleus (DAPI-positive) of at least 4 microns in diameter, and uniform cytoplasmic staining for cytokeratins 8, 18, 19. Moreover, at least 50% of the nucleus and cytoplasm areas had to overlap. CTC numbers were expressed as the number of cells per 7.5 mL of blood.

## OBJECTIVES

This study's primary objective was to analyze the prognostic role of CTC counts on the PFS of patients with mRCC starting a first-line regimen with a TKI. Basal values of total CTCs and change of CTC counts over time were assessed. Secondary objectives included correlation between CTC counts and OS, radiological tumor response, clinical parameters and IMDC group stratification.

## STATISTICAL ANALYSIS

As an observational prospective trial, this study did not have a target sample of accrual. However, based on the results of our pilot study on 53 patients [21] and given the risk of patient dropout, we planned to enroll at least 150 new evaluable patients in the study's second multicenter cohort.

Analyses of correlation between baseline CTC counts and clinical parameters (gender, age (<70 vs. ≥70 years), Fuhrman grade (1–2 vs. 3–4), previous surgery on primary kidney tumor versus none, time to metastases (less than or more than 1 year), neutrophil-to-lymphocyte ratio (<3 or ≥3), site of metastases (bone, liver and brain), histology (clear cell vs. other) and IMDC prognostic group (good, intermediate or poor) were performed using a chi-squared test and a Fisher's exact test. Several cutoff values for basal CTCs were tested: zero versus at least one CTC, fewer than two versus at least two CTCs, fewer than three versus at least three CTCs, fewer than four versus at least four CTCs, and fewer than five versus at least five CTCs.

PFS was measured from the day of the baseline CTC count (i.e., the first day of treatment) until radiological or clinical progression. Since patients with mRCC may experience long survival after first-line regimen treatment failure, we decided to censor the PFS of patients lost to follow-up on the last date they were known to be alive without progression. OS was calculated from the day of the first CTC count to death, whatever the cause.

Cox proportional hazards analysis was used to estimate univariate and multivariate hazard ratios for the whole cohort's PFS and OS. Subgroups were compared according to the two-tailed log-rank test. Since the IMDC classification incorporates the most relevant and independent prognostic factors for patients with mRCC [3], we performed the multivariate analysis with the IMDC group and dichotomized CTC levels according to several cutoff values (one, two, three, four, and five).

Subsequently, we stratified patients into four groups according to the values of CTC sequencing obtained in each patient: patients with negative counts at baseline and during treatment (Group 1a), patients with a baseline count of at least one CTC then switching to negative (Group 1b), patients with a baseline negative count switching to at least one CTC (Group 1c) or patients showing CTC count at least one at baseline and during treatment (Group 1d). The same classification could be applied with different cutoff values of two (Groups 2a, 2b, 2c, and 2d), three (Groups 3a, 3b, 3c, and 3d), four (Groups 4a, 4b, 4c, and 4d), and up to five CTCs (Groups 5a, 5b, 5c, and 5d).

Statistical analyses were carried out using SPSS statistical software.

## RESULTS

Out of a total of 246 enrolled patients, 51 were excluded due to technical problems during the despatch or analysis of the first blood sample, which was considered to be a mandatory baseline evaluation, or due to early loss to follow-up. For the present analysis 195 patients were eligible. These patients' detailed clinical characteristics are reported in Table 1. All patients started at least one cycle of TKI, which was sunitinib (77.5%), pazopanib (21%) or sorafenib (1.5%).

## CTC Counts

At baseline, 91 of 195 patients (46.7%) had one or more CTCs, median two, range 1 to 263 CTCs. The mean number of CTCs in positive patients was 8.6. Some representative images of CTCs isolated from four patients with mRCC at baseline, before starting therapy (T0) and on day 28 (T1), are shown in supplemental online Figure 1.

The probability of having at least one CTC at baseline did not correlate with gender ( $p = .67$ ), age ≥70 years ( $p = .12$ ), Fuhrman grade (1–2 vs. 3–4,  $p = .70$ ), previous removal of primitive tumor ( $p = .78$ ), time to metastases (less than or more than 1 year,  $p = .36$ ) or neutrophil-to-lymphocyte ratio (<3 or ≥3,  $p = .27$ ). Interestingly, 56.3% of patients with synchronous metastases (detected within 0 to 3 months from diagnosis) were CTC positive compared with 38.8% of patients with metachronous metastases ( $p = .015$ ). All the above clinical parameters were not significant with a cutoff value of two, three, four, or five CTCs.

Site of disease did not appear to correlate with the presence of CTCs: 52% positivity in patients with liver, 43.5% in patients with bone and 33% in patients with brain metastases (chi-squared test,  $p = .12$ ), although in the bone group the percentage of patients with at least three CTCs almost

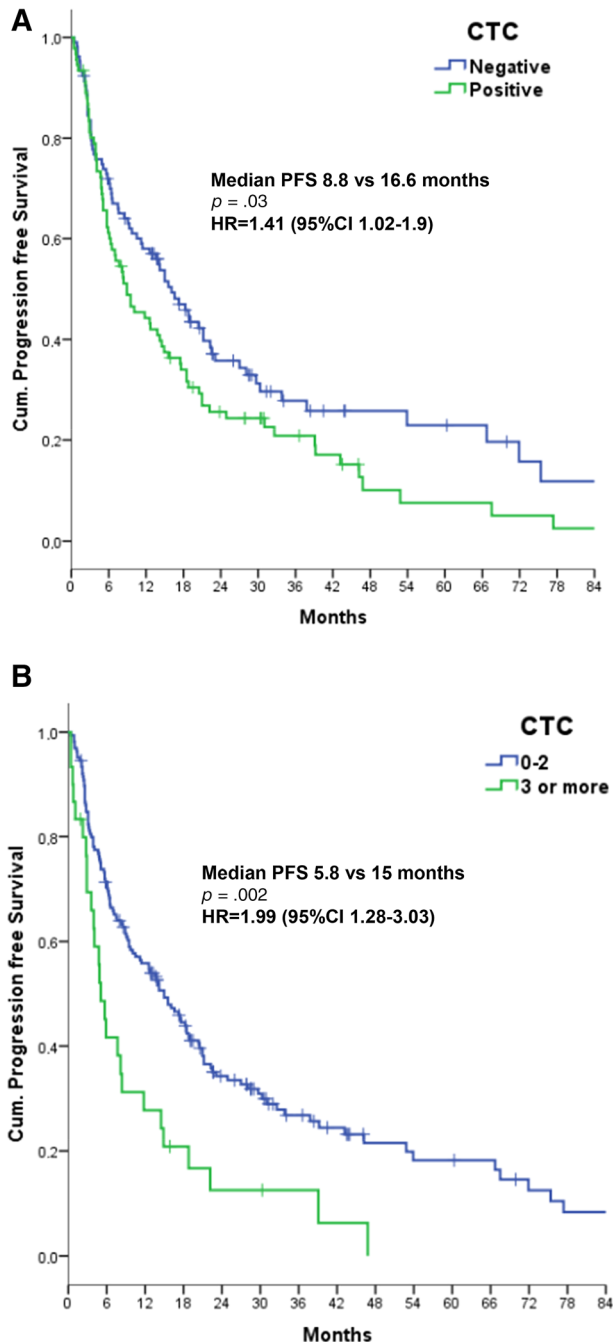
**Table 1.** Characteristics of 195 eligible patients

Characteristics	<i>n</i> (%)
Age, median (range), yr	69 (27–91)
Gender	
Male	140 (71.8)
Female	55 (28.2)
Karnofsky performance status at enrollment	
100	63 (32.3)
90	52 (26.7)
80	45 (23.0)
70	30 (15.4)
60	5 (2.6)
Prior surgery	
Partial nephrectomy	10 (5)
Radical nephrectomy	148 (76)
Biopsy/not specified	37 (19)
Histology	
Clear cell	160 (82.0)
Papillary	12 (6.2)
Chromophobe	4 (2.0)
Undifferentiated/unspecified	19 (9.8)
Fuhrman grade (available in 143 patients)	
G1	4 (2.7)
G2	50 (35.0)
G3	53 (37.1)
G4	36 (25.2)
Site of disease	
Lung	100 (51.3)
Lymph nodes	43 (22)
Bone	39 (20)
Kidney	25 (12.8)
Liver	25 (12.8)
Adrenal	9 (4.6)
Pancreas	7 (3.6)
Cerebral	6 (3%)
Soft tissue	5 (2.6%)
IMDC score (available in 151 patients)	
Good	48 (32.0)
Intermediate	77 (51.3)
Poor	25 (16.7)
Type of first-line tyrosine-kinase inhibitor	
Sunitinib	151 (77.5)
Pazopanib	41 (21)
Sorafenib	3 (1.5)

**Distribution of baseline CTC counts according to type of TKI, *n* (%)**

TKI	CTC negative	CTCs $\geq 1$	CTCs $\geq 2$	CTCs $\geq 3$	CTCs $\geq 4$	CTCs $\geq 5$
Sunitinib	75	76	41	24	15	9
Pazopanib	28	13	8	6	3	2
Sorafenib	1	2	0	0	0	0
Total	104 (53.3)	91 (46.7)	49 (25.1)	30 (15.4)	18 (9.2)	11 (5.6)

Abbreviations: CTC, circulating tumor cell; IMDC, International Metastatic Renal Cell Carcinoma Database Consortium; TKI, tyrosine kinase inhibitor.



**Figure 1.** PFS according to negative versus positive baseline CTC counts (two-tailed log-rank test,  $p = .03$ ) (A) and fewer than three versus at least three CTCs ( $p = .002$ ) (B). Abbreviations: CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio; PFS, progression-free survival.

doubled compared with patients with uninvolved bone (25.6% vs. 12.6%,  $p = .045$ ).

Interestingly, 15 out of 26 (57.6%) patients with non-clear cell histology were CTC positive at baseline compared with 43 out of 160 (26.9%) in the clear cell group ( $p = .002$ ). The difference was also maintained by applying a cutoff of three CTCs (35.4% vs. 16.5%,  $p = .045$ ). However, when only taking the 160 patients with clear cell histology into consideration, the presence of sarcomatoid features did not appear to correlate with the presence of

**Table 2.** Response rates according to cutoff values of baseline CTC counts (185 evaluable patients)

CTC cutoff value	Responses in patients below cutoff, n (%)	Responses in patients above cutoff, n (%)	<i>p</i> value
One CTC	38/98 (38.7)	38/87 (43.6)	.49
Two CTCs	52/136 (38.2)	24/49 (48.9)	.19
Three CTCs	66/156 (42.3)	10/29 (34.4)	.34
Four CTCs	72/168 (42.9)	4/17 (23.5)	.12
Five CTCs	74/174 (42.5)	2/11 (18.2)	.16

Abbreviation: CTC, circulating tumor cell.

CTCs according to the different cutoffs of one, two, three, four, and five CTCs tested.

At baseline, the IMDC risk score percentage of patients with at least one CTC increased from 33.3% favorable (16/48 patients), to 45.5% intermediate (35/77 patients) and 64% poor (16/25 patients) ( $p = .043$ ). Considering the cutoff of at least three CTCs, the IMDC risk score percentage increased from 6.3% favorable (3/48 patients), to 15.6% intermediate (12/77 patients) and 28% poor (7/25 patients) ( $p = .042$ ).

### Response

After a 10.1-month median duration of treatment, the investigator-assessed best response in 185 evaluable patients was 3.8% complete response (7 patients), 37.3% partial response (69 patients), 33% stable disease (61 patients), and 25.9% progression (48 patients).

Responses were reported in 38.7% of patients who were CTC-negative at baseline versus 43.6% of patients with at least one CTC ( $p = .49$ ) and in 42.3% of patients with fewer than three CTCs versus 34.4% with at least three CTCs ( $p = .34$ ) (Table 2). Considering only 48 IMDC favorable risk patients, 25 of them achieved a response (52%), but no significant difference was found in terms of the response rate between patients below or above the cutoff values of one CTC (56.2% vs. 43.7%,  $p = .41$ , respectively), two CTCs (50.0% vs. 66.7%,  $p = .44$ ), and three CTCs (53.3% vs. 33.3%,  $p = .50$ ). The number of favorable risk patients above the cutoff value of four and five CTCs was very low.

Only the baseline CTC count was available in 43 patients. Table 3 summarizes the distribution of the remaining 152 patients according to the serial variations of CTCs using the different cutoff values of one, two, three, four, and five CTCs, which did not correlate with response. Furthermore, when considering the subgroup of favorable risk patients, responses did not appear to correlate with the pattern distribution of dynamic changes in CTCs.

### Progression-Free Survival

After a median follow-up of 31.5 months, the median PFS was calculated as 13.6 months (23% censored; 95% confidence interval [CI], 9.9–17.3 months). The IMDC score confirmed its statistically prognostic role, with a median PFS of 32.6 months in favorable, 11.2 months in intermediate, and 3.1 months in poor risk patients ( $p < .001$ ).

**Table 3.** Best radiological response rate according to distribution of dynamic CTC counts and different CTC cutoff values

Best response	Group 1a CTCs always negative	Group 1b CTC $\geq 1$ at baseline and then negative	Group 1c CTC negative at baseline and then $\geq 1$	Group 1d CTC always $\geq 1$	$p = .75$
Response	15	16	22	13	66 (43.4)
No response	14	20	33	19	86 (56.6)
Total	29 (19.1)	36 (23.7)	55 (36.2)	32 (21.0)	Total: 152 (100)
	Group 2a All CTC values $< 2$	Group 2b $\geq 2$ CTCs at baseline to $< 2$	Group 2c $< 2$ CTCs at baseline to $\geq 2$	Group 2d All CTC values $\geq 2$	$p = .71$
Response	26	11	21	8	66 (43.4)
No response	34	14	32	6	86 (56.6)
Total	60 (39.5)	25 (16.4)	53 (34.9)	14 (9.2)	Total: 152 (100)
	Group 3a All CTC values $< 3$	Group 3b $\geq 3$ CTCs at baseline to $< 3$	Group 3c $< 3$ CTCs at baseline to $\geq 3$	Group 3d All CTC values $\geq 3$	$p = .74$
Response	41	6	17	2	66 (43.4)
No response	51	12	19	4	86 (56.6)
Total	92 (60.6)	18 (11.8)	36 (23.7)	6 (3.9)	Total: 152 (100)
	Group 4a All CTC values $< 4$	Group 4b $\geq 4$ CTCs at baseline to $< 4$	Group 4c $< 4$ CTCs at baseline to $\geq 4$	Group 4d All CTC values $\geq 4$	$p = .19$
Response	53	3	8	2	66 (43.4)
No response	58	8	19	1	86 (56.6)
Total	111 (73.0)	11 (7.3)	27 (17.8)	3 (1.9)	Total: 152 (100)
	Group 5a All CTC values $< 5$	Group 5b $\geq 5$ CTCs at baseline to $< 5$	Group 5c $< 5$ CTCs at baseline to $\geq 5$	Group 5d All CTC values $\geq 5$	$p = .12$
Response	59	1	5	1	66 (43.4)
No response	64	6	13	3	86 (56.6)
Total	123 (80.9)	7 (4.6)	18 (11.9)	4 (2.6)	Total: 152 (100)

All values are *n* or *n* (%).

Abbreviation: CTC, circulating tumor cell.

Patients with at least one CTC had a significantly shorter PFS compared with CTC-negative patients (8.8 vs. 16.6 months,  $p = .03$ ; Cox univariate hazard ratio [HR], 1.41; 95% CI, 1.02–1.9) (Fig. 1A).

The difference was lost with the cutoff of at least two CTCs (log-rank test  $p = .16$ ; HR, 1.29; 95% CI, 0.90–1.84), but became significant when the cutoff of at least three CTCs was considered: these patients had a median PFS of 5.8 months compared with 15 months for the remaining patients (log-rank test  $p = .002$ ; Cox univariate HR, 1.99; 95% CI, 1.28–3.03) (Fig. 1B).

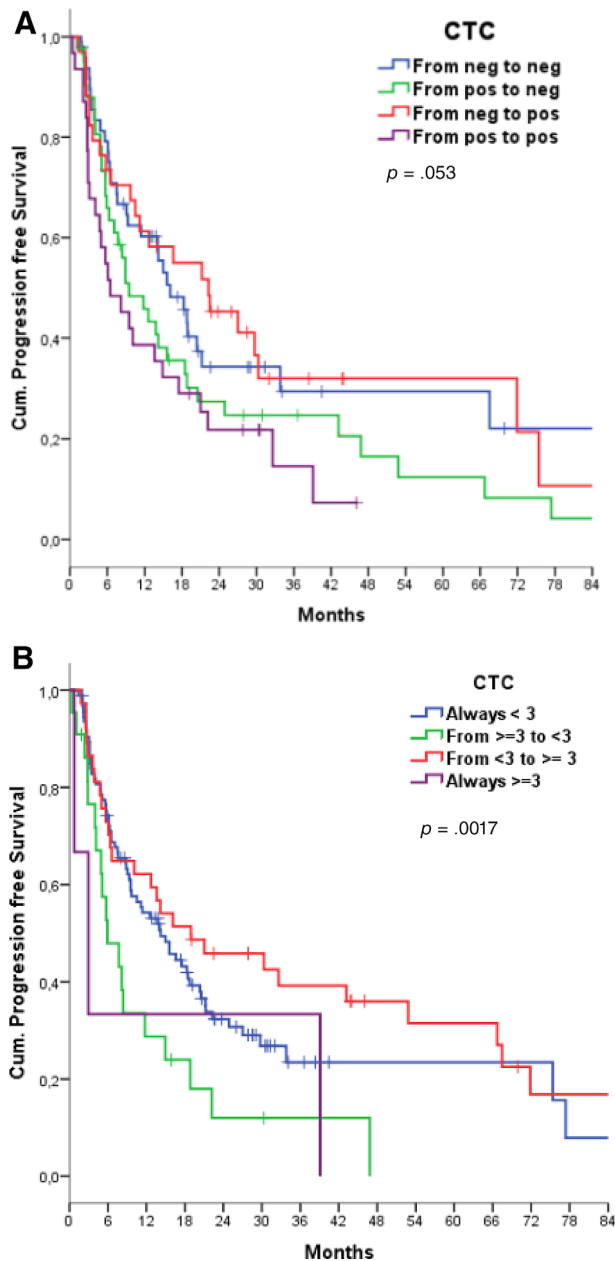
Differences in PFS remained significant with upper cutoff values of at least four CTCs (log-rank test  $p = .005$ ; HR, 2.02; 95% CI, 1.21–3.36) and at least five CTCs ( $p = .003$ ; HR, 2.59; 95% CI, 1.34–4.95). However, since the number of positive patients decreased significantly (Table 1), three CTCs were chosen as the cutoff for multivariate analysis.

With a Cox multivariate analysis taking the IMDC score and CTCs into consideration, having three or more CTCs

retained its statistical significance, HR 1.91 (95% CI, 1.16–3.14;  $p = .011$ ) (supplemental online Table 1).

The median PFS in 32 favorable risk patients with no CTCs was 53.9 months compared with 21.0 months in 16 patients with at least one CTC (33.3%) ( $p = .06$ ; HR, 1.21; 95% CI, 0.89–2.32) (supplemental online Fig. 2). The median PFS in 45 favorable risk patients with fewer than three CTCs was 32.6 months compared with 8.3 months in three patients with at least three CTCs ( $p = .17$ ).

PFS analysis, according to the dynamic change in CTCs over time (using Group 1a as a reference), showed an unexpected trend in favor of patients categorized as Group 1c (CTC counts negative at baseline and subsequently at least one), with a median PFS of 19.2 months ( $p = .053$ ) (Fig. 2A). We then repeated the same analysis on serial counts using the cutoff of at least two cells and no difference was found ( $p = .50$ ). With the cutoff value of three cells (using Group 3a as a reference), we found that Group 3c (CTC counts fewer than three at baseline and



**Figure 2.** Progression-free survival according to dynamic changes in CTC counts using the cutoff value of one or more CTCs (up, two-tailed log-rank test,  $p = .053$ ) (A) or three or more CTCs ( $p = .017$ ) (B).

Abbreviation: CTC, circulating tumor cell.

subsequently at least three) paradoxically had the longer PFS of 18.9 months ( $p = .017$ ) (Fig. 2B).

Thereafter, we also tested the correlation of PFS according to the same four different patterns of dynamic changes with cutoff values of four and five CTCs, but no differences were detected ( $p = .084$  and  $p = .79$ , respectively).

We repeated the same analysis for the 48 favorable risk patients and again, no differences were found in all the CTC cutoff values tested.

### Overall Survival

The estimated OS rate at 24 months was 59.9%. As expected, the IMDC score strongly impacted on OS, with a

median OS not reached in favorable patients, 24.9 months reached in intermediate patients and 5.7 months reached in poor risk patients ( $p < .001$ ).

We only observed a nonsignificant trend in increased OS for CTC-negative patients when compared with those with one or more CTCs (log-rank test  $p = .207$ ; Cox univariate HR, 1.28; 95% CI, 0.86–1.91) (Fig. 3A). Similarly, no difference was found with the cutoff value of at least two CTCs (log-rank test  $p = .18$ ; HR, 1.34; 95% CI, 0.87–2.07). However, patients with at least three CTCs had a shorter estimated OS of 13.8 versus 52.8 months in the remaining patients ( $p = .003$ ; Cox univariate HR, 1.99; CI, 1.17–3.2) (Fig. 3B).

Differences in OS remained significant with upper cutoff values of at least four CTCs ( $p = .005$ ; HR, 2.25; 95% CI, 1.25–4.05) and at least five CTCs ( $p < .001$ ; HR, 3.89; 95% CI, 1.68–6.82); we consequently also confirmed three CTCs as the cutoff value for multivariate analysis for OS.

Taking the IMDC score into consideration, a CTC count of at least three CTCs at baseline had an HR of 1.67 (95% CI, 0.95–2.93;  $p = .076$ ) (supplemental online Table 1).

Very few deaths (8 out of 48 patients) were observed in favorable risk patients. At 24 months, 87.5% of patients without CTCs were still alive compared with 87.5% of patients among those with at least one CTC ( $p = .38$ ), whereas 86.7% of patients with zero to two CTCs were alive compared with 100% of those with at least three CTCs ( $p = .46$ ).

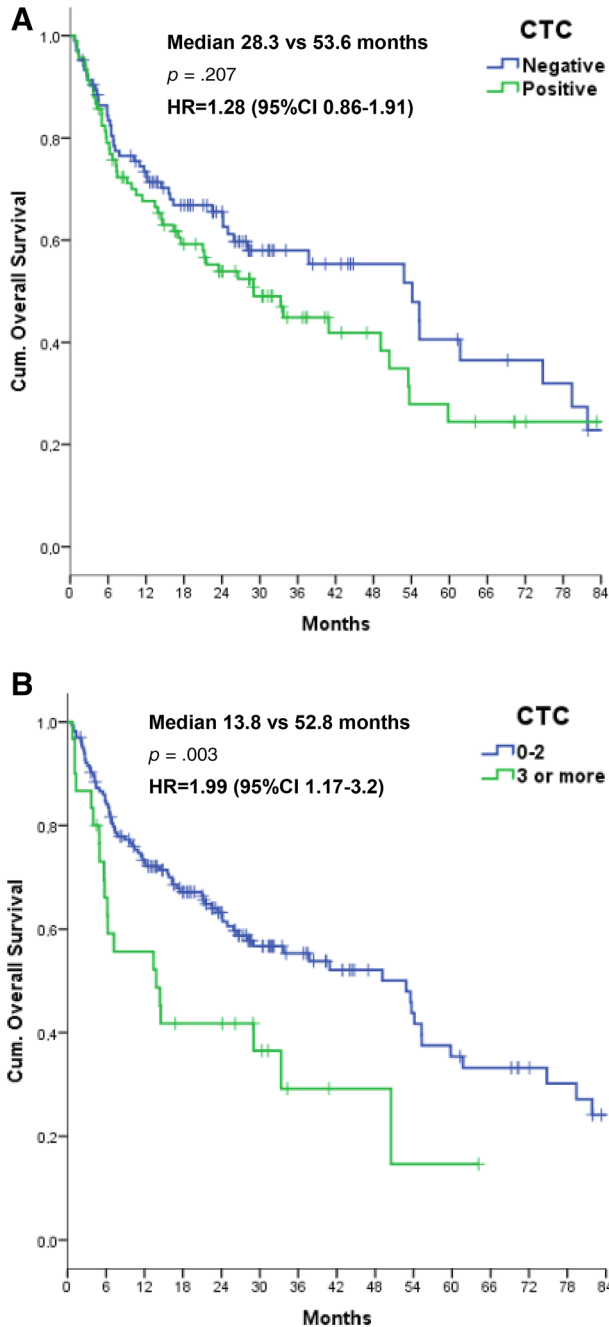
OS did not differ according to the dynamic change in CTC counts over time during treatment (Group 1a as a reference, log-rank test  $p = .433$ ) with a cutoff of one CTC (Fig. 4A). No differences were found in terms of OS when repeating the same analysis on serial CTC counts using the cutoff value of at least three cells, although a small trend for Group 3c appeared (log-rank test  $p = .076$ ) (Fig. 4B).

We then went on to test the correlation of OS according to the same four different patterns of dynamic changes with cutoff values of four and five cells, but no differences were detected (two-tailed log-rank test  $p = .810$  and  $p = .68$ , respectively).

We repeated the same analyses for the 48 favorable risk patients, and again no differences were found across all the five cutoff values that were assessed.

### DISCUSSION

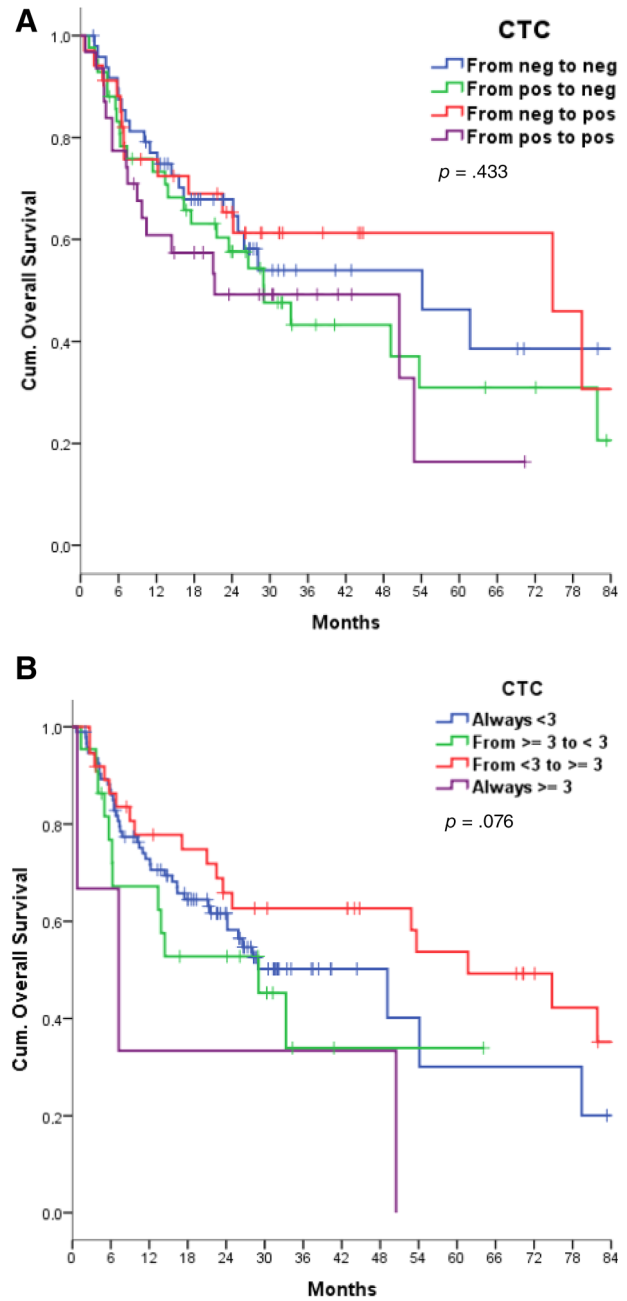
RCC is a highly vascularized neoplasia resistant to standard chemotherapy, but sensitive to antiangiogenic drugs and immunotherapy. The IMDC algorithm is a mandatory stratification factor for clinical trials and identifies intermediate and poor risk patient candidates who today can receive the most recent immunotherapy combination of Nivolumab plus Ipilimumab [26]. TKIs are synergic with immunotherapy, and combination regimens can be given to patients in all risk groups [2]. There is wide debate on whether monotherapy with TKIs could still be a reasonable and cost-effective choice for patients with a favorable IMDC risk and a low metastatic burden in the lung and/or lymph nodes [27].



**Figure 3.** Overall survival according to negative versus positive baseline CTC counts (two-tailed log-rank test,  $p = .207$ ) (A) and fewer than three or at least three CTCs ( $p = .003$ ) (B). Abbreviations: CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio.

In any case, IMDC criteria do not discriminate between patients responsive to TKIs, and no validated blood biomarker is available to guide the choice between the current first-line options for patients with mRCC (combined regimens vs. TKI monotherapy) and to predict response during treatment [6].

CTCs appear to be promising for monitoring response to treatment in patients with colorectal cancer [28] and castration-resistant prostate cancer [29], but limited data are available for mRCC. Allard and colleagues [17] were the first to demonstrate the feasibility of detecting CTCs using



**Figure 4.** Overall survival according to dynamic changes in CTC counts using the cutoff value of one or more CTCs (two-tailed log-rank test,  $p = .053$ ) (A) or three or more CTCs ( $p = .076$ ) (B). Abbreviation: CTC, circulating tumor cell.

the CellSearch platform in 11 patients with RCC: 25% of specimens had at least two CTCs, whereas none of them had at least five CTCs. In 25 patients with mRCC, Gradilone and colleagues [18] reported four CellSearch CTC-positive patients out of 25 (16%). Other authors reported that 41% of patients with clear cell RCC were EpCAM-positive ( $\geq 5\%$  of staining) [30]. CTCs are a heterogeneous population in patients with mRCC, with different metastatic capabilities, similar to what is reported for other malignancies [31]. Therefore, intensive efforts were focused on non-EpCAM-based technologies, but few papers addressed this matter



in patients with mRCC and even then, with inconclusive results [8–10].

Broncy and colleagues tried to identify CTCs in the peripheral blood of patients with RCC according to a cytomorphological method termed “isolation by size of epithelial tumor cell technique” (ISET). Twenty out of 30 patients, with mainly localized RCC, were CTC positive with this method (66.6%) [9].

Similarly, Klezl and colleagues [10] used an EpCAM-independent size-based enrichment method and reported 86.6% prevalence of CTC-positive samples in patients with RCC undergoing surgery. Another group recently reported that the positivity of CTCs detected by ISET in 13 out of 36 patients (36.1%) correlated with clinical tumor-node-metastasis (TNM) staging, whereas the presence of CTCs detected by CellSearch in the same patients (19.4%) did not. Eventually, CTC counts measured through both methods did not correlate with patients’ PFS and OS [32].

Taking into account the need to exploit standardized, automated assays, suitable for multicenter clinical studies [33], we decided to apply the U.S. Food and Drug Administration–cleared CellSearch platform to patients with RCC.

We demonstrated that CTCs could be isolated in almost 60% of the 53 patients with mRCC before treatment with sunitinib [22], a prevalence that surely exceeds that reported by other small studies conducted in mRCC with CellSearch [17, 18, 32]. This difference could possibly be related to the different sample size as well as to the selection bias, since all our patients with metastatic disease were deemed eligible to receive a systemic treatment with a TKI in view of burden of disease and/or the presence of symptoms.

Most importantly, we obtained the proof of principle that EpCAM expression could be acquired during disease evolution (e.g., negative in the primary tumor and positive in the metastases), supporting the rationale of employing EpCAM-based technology even if the primary tumor tissue did not express this antigen [22]. Moreover, we know that EpCAM is highly expressed in cancer stem cells and may play a role in cancer migration. We can, therefore, infer that assessment of EpCAM in CTCs may better reflect the dynamic biological changes of invasive and metastatic cell populations, as with other dynamic tumor markers in general. It also has to be remembered that EpCAM expression is higher in papillary and chromophobe histological subtypes of RCC (80%) [34].

We therefore designed a second large multicenter observational trial in order to enroll at least 150 additional evaluable patients with RCC (with either clear or non-clear histology) treated homogeneously with a first-line TKI, with the aim of assessing the prognostic as well as the predictive role of CTCs in terms of radiological response.

We confirmed the presence of at least one CTC/7.5 mL of peripheral blood in almost half of the patients (46.7%), and three or more CTCs in 15.4% of cases. Globally, CTC counts in our study appear inferior when compared with other solid tumors. Up to 93.3% of patients with metastatic prostate cancer may be CTC positive [14]. Nevertheless, the number of previous lines of hormonal and radiotherapy

treatments in either an adjuvant or metastatic setting may represent an important bias in such comparisons between patients with prostate cancer and our cohort of treatment-naïve patients with mRCC.

CTC counts at baseline did not correlate with any of the clinical parameters tested, and only the presence of synchronous metastases showed a mild but statistically significant correlation with the presence of at least one CTC. This can be interpreted as a correlation with a more aggressive behavior, although significance was lost with the cutoff value of two or three CTCs, possibly due to lower patient numbers.

Interestingly, non-clear cell histology correlated with a higher probability of detecting at least one CTC (57.6%) compared with the clear cell phenotype (26.9%,  $p = .002$ ). The difference was maintained with the cutoff value of two or three CTCs, thus confirming the different biology of the two histological groups. As for the site of metastases, we found a correlation between bone metastases and a higher chance of having a CTC count of at least three, but not with the presence of liver or brain metastases. The correlation between bone metastases and circulating CTC counts has already been reported in breast cancer [35] and other tumors, and several studies are currently focusing on the complex balance of quiescence or proliferation of disseminated tumor cells in the bone metastatic niche.

In our cohort, baseline counts of CTCs became consistently prognostic for PFS and OS, starting from the cutoff value of three CTCs (HR for progression 1.99 and death 1.99). The number of patients with values of at least four CTCs or at least five CTCs was very low (Table 1), and these cutoff values did not achieve predictive significance for radiological response (see below). We thus propose three CTCs as the reference prognostic cutoff value for mRCC when using the CellSearch platform. In a multivariate analysis incorporating the IMDC score and CTC counts, the presence of three or more CTCs maintained an adverse impact on progression (HR, 1.91;  $p = .011$ ) and a trend towards significance for OS (HR, 1.67;  $p = .076$ ) (supplemental online Table 1).

Since TKI monotherapy is currently considered to be an option mainly for IMDC favorable risk patients [27], the same analyses for PFS and OS were performed in this subgroup. We obtained comparable results in terms of correlation of baseline CTC counts of at least one with PFS (supplemental online Fig. 2), albeit not statistically significant due to lower patient numbers and fewer events. The number of patients with CTCs above three, four, or five was too small to draw any further comparisons. Very few overall survival events were registered in this subgroup.

The availability of data concerning the correlation between CTC counts and radiological response is limited. Indeed, an early CTC change following therapy did not correlate with the RECIST response in a pooled analysis conducted on patients with various types of advanced cancer treated with chemotherapy and other agents within phase I trials [36]. This underlines the fact that shrinkage of tumor masses is not unequivocally correlated with a reduction in the population of blood CTCs.

Our study failed to demonstrate a correlation between baseline CTC counts and radiological response (as judged by the treating physician) (Table 2). Contrary to what is reported on other solid tumors with the same CellSearch system [11, 12, 14], the pattern of dynamic change of CTC counts over time with all the different cutoff values of CTCs could not even be correlated with response to TKIs (Table 3), PFS (Fig. 2) and OS (Fig. 4). We carried out the same analyses in the subgroup of 48 IMDC favorable risk patients. However, here again, no significant correlation between baseline and dynamic CTC counts and radiological response was registered.

The different mechanisms of action of TKIs, which are mainly antiangiogenic rather than direct cytotoxic drugs, could partly explain such findings. This is corroborated by the fact that roughly one-third of our patients showed a fluctuation in CTC values over time, for example, increasing and reducing over time in the same patient (or vice versa). Moreover, there have been reports that prolonged inhibition of angiogenesis might paradoxically increase metastasis of tumor cells in animal models [21]. These experimental findings raise the question as to whether CTC spread might even increase *in vivo* due to an efficacious antiangiogenic pressure induced by TKIs, thus biasing the analysis of correlation between dynamic changes of CTC counts and tumor shrinkage. It would be very interesting to conduct a comparable prospective study on CTCs in patients with mRCC receiving modern PD-1–based immunotherapy [2, 26], in order to see if PD-1 blockade can influence the shedding and circulation of CTCs and their correlation with radiological response, PFS and OS. Indeed, a small study conducted on patients with lung cancer recently demonstrated that CTC counts are correlated with the survival of patients with lung cancer treated with nivolumab [37].

This study's limitations are related to the use of an EpCAM-based automated assay CellSearch system, and the distinctive schedule of blood draws that we applied to patients compared with other studies. The loss of certain time points and the heterogeneity in the timing of the radiological reevaluation of patients might have biased such results, potentially resulting in delayed recognition of progression.

Further analyses are ongoing on apoptotic markers in CTCs and concomitant counts of circulating endothelial cells collected in the same cohort of patients. The aim is to confirm our preliminary observation that the ratio of apoptotic to live CTCs could better reflect the response to treatment and also the duration of disease control [22].

Nevertheless, the results of our study do not provide a rationale for changing treatment on the basis of an unfavorable dynamic change in CellSearch-detected CTC counts during treatment with first-line TKIs in mRCC. Moreover,

large prospective trials with alternative cytomorphological enrichment strategies are still warranted, especially in patients receiving new immunotherapy regimens.

## CONCLUSION

In this large, homogeneous prospective cohort of patients with metastatic RCC treated in the first line with TKIs, we provide evidence that the presence of three or more CTCs at baseline detected by the EpCAM-based CellSearch system is associated with a significantly shorter PFS and OS. This impact is independent of the IMDC classification in multivariate analysis for PFS. Baseline and dynamic CTC counts were not predictive of radiological response.

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## DISCLOSURES

**Umberto Basso:** Pfizer, Novartis, Bristol-Myers Squibb, Astellas, Janssen, Ipsen, Sanofi-Aventis (H), Pfizer, Novartis, Merck Sharp and Dohme, Incyte, Sanofi-Aventis (C/A); **Vincenza Conteduca:** Astellas, Janssen, Sanofi-Aventis (H), Bayer (C/A); **Alessandra Bearz:** Takeda, Boehringer Ingelheim, Novartis, Pfizer, Merck Sharp and Dohme (C/A, H), Jansen, Roche (other—travel); **Ugo De Giorgi:** Astellas, Janssen, Bayer, Sanofi Aventis, Pfizer, Novartis, Merck Sharp and Dohme, Ipsen (C/A). The other authors indicated no financial relationships.

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