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Research Article

# Contrasting Circulating Tumor Cells and Free Circulating DNA Responses in Men Treated for Prostate Cancer after Primary Versus Salvage Radiotherapy

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ABSTRACT

**Purpose:** To investigate the relationships between circulating tumor cells (CTCs), free circulating DNA (fcDNA) and biochemical response in prostate cancer patients treated primarily versus salvage radiotherapy (RT).

**Methods and Materials:** Blood was collected prospectively from patients, enrolled in two institutional Phase II trials for primary and salvage RT. Three blood samples were collected at: (i) prior to treatment [RT or androgen deprivation therapy (ADT)], (ii) last week of RT, and (iii) three months post-RT. CTCs were quantified in 31 samples from 12 primary patients and 30 samples from 12 salvage patients; fcDNA were analyzed in 11 primary (28 samples) and 5 (9 samples) salvage patients. CTCs were visualized by immunofluorescence after microfilter capture and fcDNA was quantified using real-time Polymerase chain reaction (PCR). CTCs and fcDNA were correlated with early biochemical response by subdividing patients into early favorable and unfavorable response at 3 months after RT.

**Results:** For those treated primarily, there was a direct correlation with CTC counts and prostate specific antigen (PSA) pre-RT that changed to a reciprocal relationship 3 months post-RT. CTCs increased significantly (p=0.03) at 3 months after primary RT in the biochemical favorable patients, while no significant association was observed for fcDNA. Correspondingly, post-RT fcDNA levels were inversely related to CTC counts. In salvage patients, the number of CTCs was related to pre-RT PSA, but it was not correlated to RT response. In post-RT series, a significant direct correlation was observed between CTCs and PSA.

**Conclusion:** Our preliminary studies suggest that RT affects CTC counts, which are thus associated with prostate cancer biochemical response. A larger cohort with longer follow-up will be needed to establish the association with more recognized treatment endpoints.



## Introduction

Radiotherapy (RT) for prostate cancer is the most common primary treatment alternative to prostatectomy, resulting in similar long-term tumor control rates with preservation of anatomy and, consequently, improved functional outcome. However, about 25-50% of men with intermediate to high risk disease ultimately develop biochemical failure (BF) after RT; BF is a harbinger of distant metastasis (DM) [1, 2]. The

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risk of DM after radiotherapy is biphasic, with an early wave peaking at 0-4 years post-RT and a second that has been related to risk group and local vs distant failure patterns [3]. The data indicate that early DM is related to risk group (high risk greater than intermediate risk) that is in turn related to the components of unrecognized micrometastases present prior to RT and local persistence after RT [3]. Late DM (8+ years after completion of treatment) is seen more in intermediate risk and is most consistent with local persistence after RT because RT dose escalation reduces the risk [3-7]. Radiation dose escalation for prostate cancer has been shown to reduce biochemical and distant failure overall, presumably through the main mechanism of reduction in local persistence of disease. Likewise, there are reports that dose escalation improves outcome when salvage RT is used after prostatectomy, suggesting a local component to biochemical failure in this setting as well [8]. However, even with salvage RT dose escalation, failure rates can reach over 50% [7].

Micrometastatic disease is a significant risk in patients treated with RT primarily and for salvage. Markers of this risk are needed to better stratify patients who require more aggressive treatment, such as the concomitant use of androgen deprivation therapy (ADT), the length of ADT treatment, chemotherapy and/or abiraterone [9, 10]. The blood biomarkers of circulating tumor cells (CTCs) and free circulating DNA (fcDNA) have the potential to better define the effectiveness of RT treatment with and without ADT [11]. In prostate cancer, CTCs and fcDNA have been used to follow response [12, 13]. CTCs are an attractive tumor surrogate that may represent the genetic and phenotypic composition of the determinate clone(s) of the primary tumor [14]. On the other hand, fcDNA may serve as a biomarker for malignant tumor detection and follow up in patients with a variety of solid tumors. There are no published reports on fcDNA in serum and/or CTCs in prostate cancer patients assessed immediately prior to and after external beam RT. In this paper we describe the changes in CTC and fcDNA levels pre- and post-RT in men enrolled in two prospective clinical trials for primary and salvage radiation treatment. We also compare CTC and fcDNA changes to early biochemical response and contrast these associations in patients treated primarily to those treated for salvage after prostatectomy.

## Materials and Methods

### I Study Population

CTCs and fcDNA were analyzed in patients, participating in two institutional phase III randomized trials: a primary radiotherapy treatment trial (IRB #20100635; NCT01411332) and a salvage treatment trial (IRB# 20101056; NCT01411345). Both trials were approved by the Institutional Review Board at University of Miami and all patients signed appropriate informed consent for treatment and the analysis of blood components for research purposes.

The main objective of the primary treatment trial is to compare the efficacy of administering a multiparametric magnetic resonance imaging (mpMRI)-targeted external beam boost to standard dose and standard fraction definitive external beam radiation for intermediate to high risk prostate cancer patients. In this randomized trial the experimental arm includes daily dose-painted escalation to the MRI-defined tumor volume at 2.35 Gy per fraction, while the rest of the prostate and proximal seminal vesicles receive 2.0 Gy per fraction to 76 Gy [15]. The boost region receives an absolute dose of 89.3 Gy. Assuming an $\alpha/\beta$ ratio of 3.0, this would be equivalent to 95.5 Gy in 2.0 Gy fractions. The patients randomized to the standard intensity-modulated RT (IMRT) arm receive 80 Gy at 2.0 Gy per fraction to the prostate and proximal seminal vesicles. The primary endpoint is a positive prostate biopsy and/or clinical/biochemical failure at 2 years after completion of all treatment. Short term ADT is permitted; in all patients ADT and fiducial marker placement took place after the pretreatment research blood sample was taken.

The salvage treatment trial is a phase III randomized trial of MRI-mapped dose-escalated salvage RT post-prostatectomy. This trial evaluates 68 Gy in 34 fractions to the prostate bed, versus the same dose and fractionation to the bed plus a daily simultaneous integrated boost of 2.25 Gy per fraction (76.5 Gy; biologically equivalent to 80 in 2.0 Gy fractions, assuming an $\alpha/\beta=3$) to the mpMRI identified suspicious lesion (required for eligibility). The primary endpoint of the trial is an undetectable PSA 24 months post salvage radiation treatment. All treatments were delivered using IMRT with daily image guidance. ADT was not permitted on the study.

### II Sample Collection

Blood samples for CTCs and fcDNA analysis were collected in accordance with the primary and salvage treatment protocols. Blood samples were collected in one 7.5 ml CellSave$^{TM}$ Preservative Tube (Immunicon, PA) and one 10 ml serum BD Vacutainer tubes (Becton, Dickinson and Co, NJ) at three time points during the clinical trials: *(i)* Prior to treatment (RT or ADT), *(ii)* the last week of treatment; and *(iii)* three months after treatment.

### III CTC Enumeration

Blood samples were processed for enumeration of CTCs as described before [16]. Briefly, the sample was fixed in 1% formalin for 10 minutes and processed through a microfiltration platform for CTC capture [17]. After filtration the filter was washed with 1×DPBS (Life technologies Corp, CA) and subjected to pan-Cytokeratin and CD45 (Dako North America Corp, CA) followed by Alexa 488 and Alexa 594 secondary antibody labeling (Life technologies Corp, CA) and mounted with ProLong Gold antifade mounting media with DAPI (Life Technologies). CTCs were then identified as Cytokeratin positive/DAPI positive/CD45 negative cells with additional morphology criteria. PC-3 cells (ATCC, VA) and peripheral blood mononuclear cells were used as positive controls for staining after cytospinning onto slides.

### IV fcDNA Quantification

Serum was separated by centrifugation at 3,500 rpm for 15 minutes at room temperature, after which DNA was extracted immediately. DNA was extracted from 1 ml serum using QIAamp UltraSens virus kit (Qiagen, Germany) following the manufacturer's protocol and stored at −20°C until further analysis. Samples were processed for fcDNA quantification as described before [18]. Briefly, serum DNA was quantitated by real-time Polymerase chain reaction (PCR) for

*glutathione S-transferase, pi* (*GSTP1*), based on the previous study by Bastian *et al* [19]. Amplification primers (forward, 5′ AGG CCT TCG CTG GAG TTT C 3′; reverse, 5′ CCA TGC TGG GAG CTC TGA G 3′) and an amplicon-specific fluorogenic hybridization probe (6FAMCGC CGC AGT CTT CGC CAC CTAMRA) were used. PCR was carried out in an iCycler (Biorad Laborotories, CA). The PCR mixture consisted of 12.5 µl of Taqman Universal master mix (Applied Biosystems, CA), 5 pmoles of probe, and 5 pmoles each of the forward and reverse primer in a 25 µl reaction volume. Each sample was analyzed in triplicate. All PCR runs included a negative control using water blanks. A standard curve was generated for each PCR run using serial dilutions of human placental DNA (Sigma Aldrich LLC, MO) at concentrations ranging between 160 ng/µl to 180 pg/µl. For calculation of DNA concentrations, the standard curve was interpolated with the threshold cycle of unknown target samples.

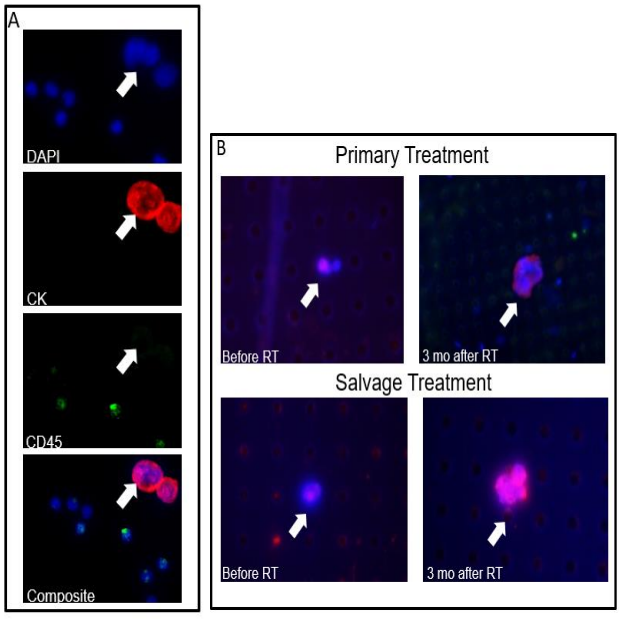


**Figure 1: (A)** Microscopy of CTCs in patient's blood. Three (DAPI, Cytokeratin (CK) and CD45) fluorescent channels of a microscopy field with representative cells (arrowed) defined as CTCs. The composite image from the three markers is represented below; **(B)** Composite images of CTCs in primary treatment and salvage treatment radiotherapy (RT) trials, visualized before and three months after RT, illustrating clustering of the cells.

### V Statistical Analysis

The distribution of CTCs and fcDNA in both pre- and post-treatment samples was summarized with descriptive statistics. Patients were divided into biochemical favorable (PSA ≤ 1 ng/ml for primary treatment and PSA ≤ 0.1 ng/ml for salvage treatment) and unfavorable (PSA > 1 ng/ml for primary treatment and PSA > 0.1 ng/ml) for salvage at 3 months post-RT [20]. The changes in CTCs and fcDNA pre- and post-RT were investigated using Student's *t*-test. The relationship between CTCs/fcDNA and PSA was assessed at different experimental time points by Pearson correlation coefficient. Significance was determined using probability values of $p < 0.05$ from two-tailed tests. Similarly, the relationship between CTCs and fcDNA data at different experimental time points was also investigated.

## Results

### I Patients

Blood for analysis was collected from 12 patients treated primarily and 12 patients treated for salvage. The clinical characteristics of the patients are summarized in (Table 1).

**Table 1:** Clinical characteristics of patients.

| | **Primary Treatment Trial** | **Salvage Treatment Trial** |
|---|---|---|
| **Patients (N)** | **12** | **12** |
| **Age** | | |
| **Median (yrs):** | **73** | **55** |
| **Range (yrs):** | **64 - 86** | **44 - 65** |
| **Gleason Score*** | | |
| **6** | | **1 (8%)** |
| **3+4** | **9 (75%)** | **4 (33%)** |
| **4+3** | **1 (8%)** | **4 (33%)** |
| **8** | **2 (17%)** | **1 (8%)** |
| **9** | | **2 (17%)** |
| **Tumor Category†** | | |
| **T1** | **4 (33%)** | |
| **T2** | **8 (67%)** | **9 (75%)** |
| **T3** | | **3 (25%)** |
| **Initial PSA‡** | | |
| **Median (ng/ml)** | **5.9** | **0.6** |
| **Range (ng/ml)** | **1.4 - 15.5** | **0.2 – 1.8** |
| **ADT** | **2 (17%)** | **N/A**** |
| **Treatment Arm** | | |
| **Standard** | **8 (67%)** | **6 (50%)** |
| **Experimental** | **4 (33%)** | **6 (50%)** |

***Abbreviations*:** PSA – Prostate Specific Antigen; ADT - androgen deprivation therapy

* Post surgical Gleason score is reported for salvage radiation treatment patients.

† Clinical stage is reported for primary treatment; pathologic stage – for salvage.

‡ PSA before any treatment (radiotherapy or androgen deprivation therapy).

** ADT was not allowed on the salvage treatment trial.

### II Visualization of Microfilter Captured CTCs by Fluorescence Microscopy

CTCs were visualized by fluorescence microscopy after microfilter capture and characterized as positive for the common epithelial cytokeratins (CK), negative for the leukocyte marker CD45, containing intact nuclei, having no evidence of apoptosis, and being morphologically distinct from leukocytes**.** PC-3 cells admixed with peripheral blood mononuclear cells (PBMCs) were used a positive control (Figure 1A). CTCs are generally larger than leucocytes, but cell size alone was insufficient for accurate tumor cell identification. An example of a composite image of CTCs before and after radiation treatment is shown in Figure 1B. A clustering of the cells post-RT, which was previously reported, can be seen in the samples post-RT [21, 22].

### III CTC and fcDNA Enumeration in Primary and Salvage Treatment Trials

CTCs were quantified in 12 primary and 12 salvage patients and fcDNA - in 11 primary and 5 salvage patients. In the primary treatment trial, 31 samples (11 pre-RT, 11 last week of RT and 9 at 3 months post-RT) were analyzed for CTCs and 28 (11 pre-RT, 10 last week of RT and 7 at 3 months post-RT) for fcDNA. In the salvage treatment trial, 30 (12 pre-RT, 11 last week of RT and 7 at 3 months post-RT) and 9 (4 pre-RT, 3 last week of RT and 2 at 3 months post-RT) samples were analyzed for CTCs and fcDNA, respectively. CTC and fcDNA measurements are presented in Figure 2 at each experimental time point**.** In the primary treatment trial, CTCs increased from (mean±SD) 33±43 (4/11 [36%] patients had undetectable CTCs) pre-RT to 51±42 during the last week of RT and 57±55 3 mo post-RT. In contrast, fcDNA levels decreased from 46 ± 28 (ng/ml) to 30 ± 32 during the last week of RT and increased to 55±97 at 3 months post-RT. In the salvage patients, the CTCs counts changed from 34±22 to 21±16 during the last week of RT and were 37 ± 23 post-RT. Similarly, the fcDNA level fluctuated from 209 ± 340 to 16 ± 19 and 321 ± 326 post-RT. None of these changes were significantly significant relative to pre-RT measurement. Similarly, there was no significant difference in CTC/fcDNA between the experimental and standard arms of the two trials (Supplemental Figure 1).

While there was no significant difference ($p<0.05$) between any CTC/fcDNA measurements, a gradual increase in the CTC levels can be observed in the primary treatment group. Note that the pre-treatment CTC levels in both primary and salvage treatment groups of patients were very similar. If we combine the CTC counts from the two post-RT time points, CTCs increased post-RT markedly relative for the salvage patients (mean±SD: 54 ± 47 vs 27 ± 20, p = 0.031). The changes in fcDNA are harder to characterize because of the low number of samples.

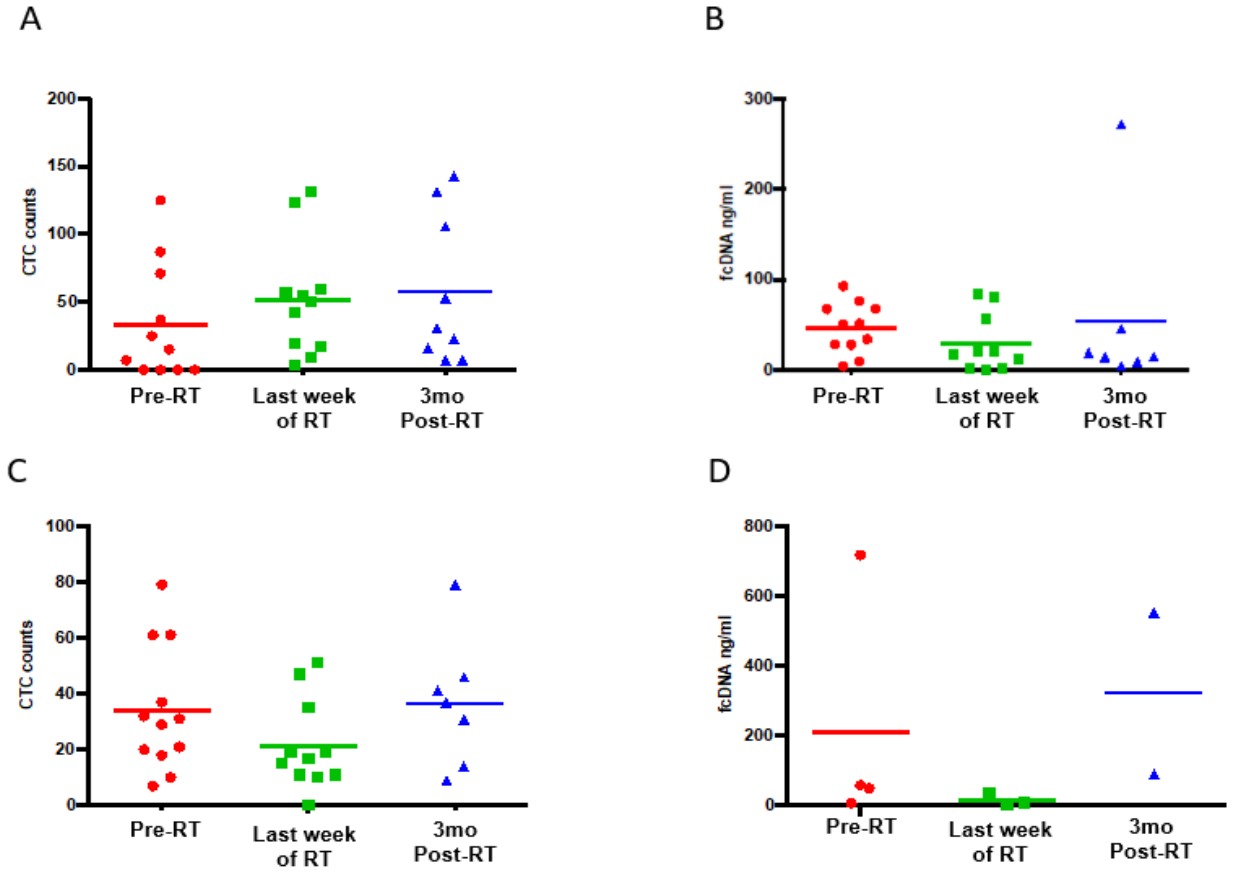


**Figure 2:** CTC and fcDNA distribution at time points: pre-RT, last week of RT and 3 months post-RT in patients from: **(A, B)** Primary treatment clinical trial; **(C, D)** Salvage treatment clinical trial. Horizontal line represents the mean.

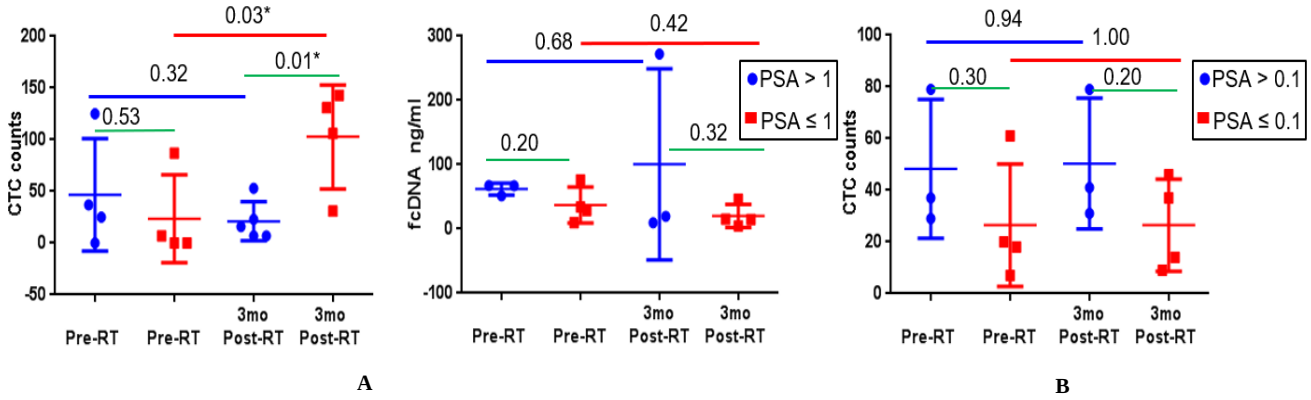


**Figure 3: (A)** Change in CTCs and fcDNA following RT in primary treatment clinical trial patients with biochemical favorable response (PSA ⩽ 1 ng/mL at 3 months post-RT) and biochemical unfavorable response (PSA > 1 ng/mL at 3 months post-treatment). CTC counts increased significantly relative to pre-treatment counts for the responder's group (p = 0.03) (*denotes statistical significance). **(B)** Changes in CTC following RT in salvage treatment clinical trial patients with biochemical favorable response (PSA ⩽ 0.1 ng/mL at 3-month post-treatment) and biochemical unfavorable response (PSA > 0. 1 ng/mL at 3 months post-treatment).

### IV Relationship Between Early CTC/fcDNA Measures and RT Treatment Response

The median follow-up of the analyzed primary and salvage treatment patients was 2.5 months (range: 5.76 to 25.2) and 4.44 months (range: 1.32 to 23.64), respectively. Figure 3A shows changes in CTC and fcDNA levels in the two response groups of primary treatment patients. CTCs increased significantly after RT for the "favorable response" group (p=0.03), while the counts did not change for the "unfavorable response" group (p = 0.32). Note that the CTC values at 3 months were significantly different between the two groups (p = 0.01). No significant differences were observed for fcDNA, possibly because of the small number of measurements. Figure 3B shows the changes in CTCs following RT in salvage treatment patients with a favorable biochemical response and those with unfavorable response biochemical failure There was no difference between the pre-RT and post-RT points. It appears, though, that non-responders showed consistently higher CTC counts than responders at the two time points.

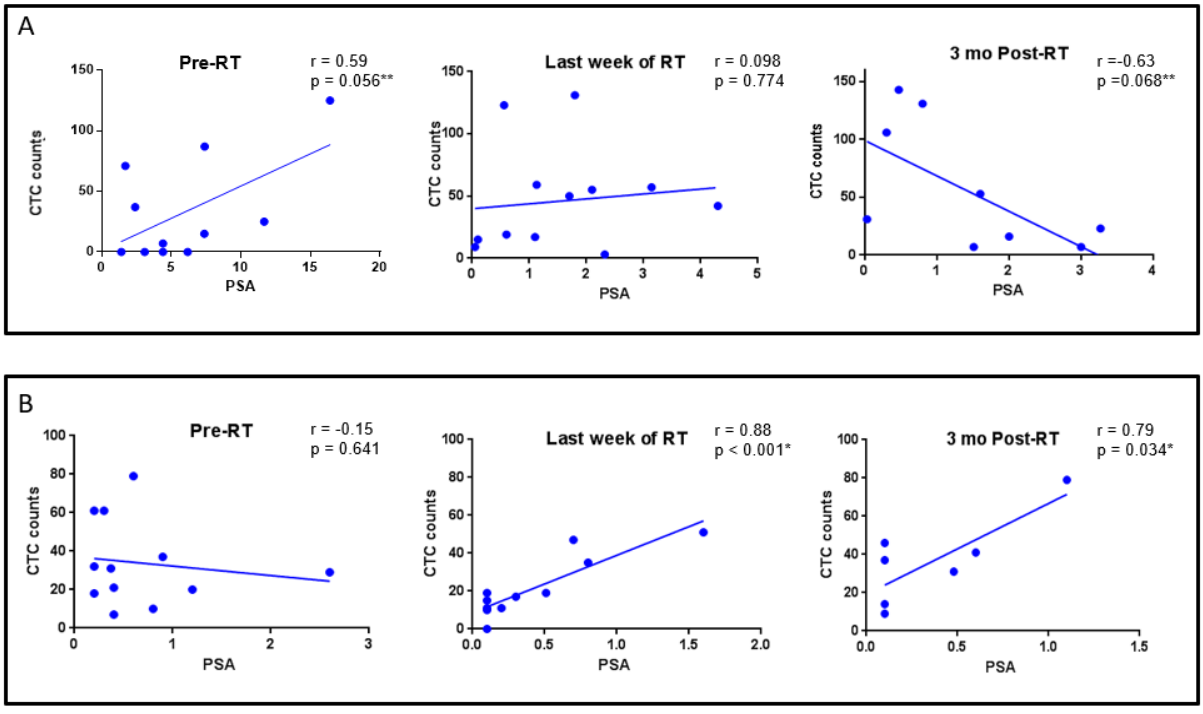


**Figure 4:** Correlation between CTC and PSA at 3 different time points (pre-RT, last week of RT and 3 months post-RT) **(A)** Primary treatment clinical trial, and **(B)** Salvage treatment clinical trials. Undetectable PSA values (< 0.01) are set to 0.01. (*denotes statistical significance; ** denotes marginal statistical significance).

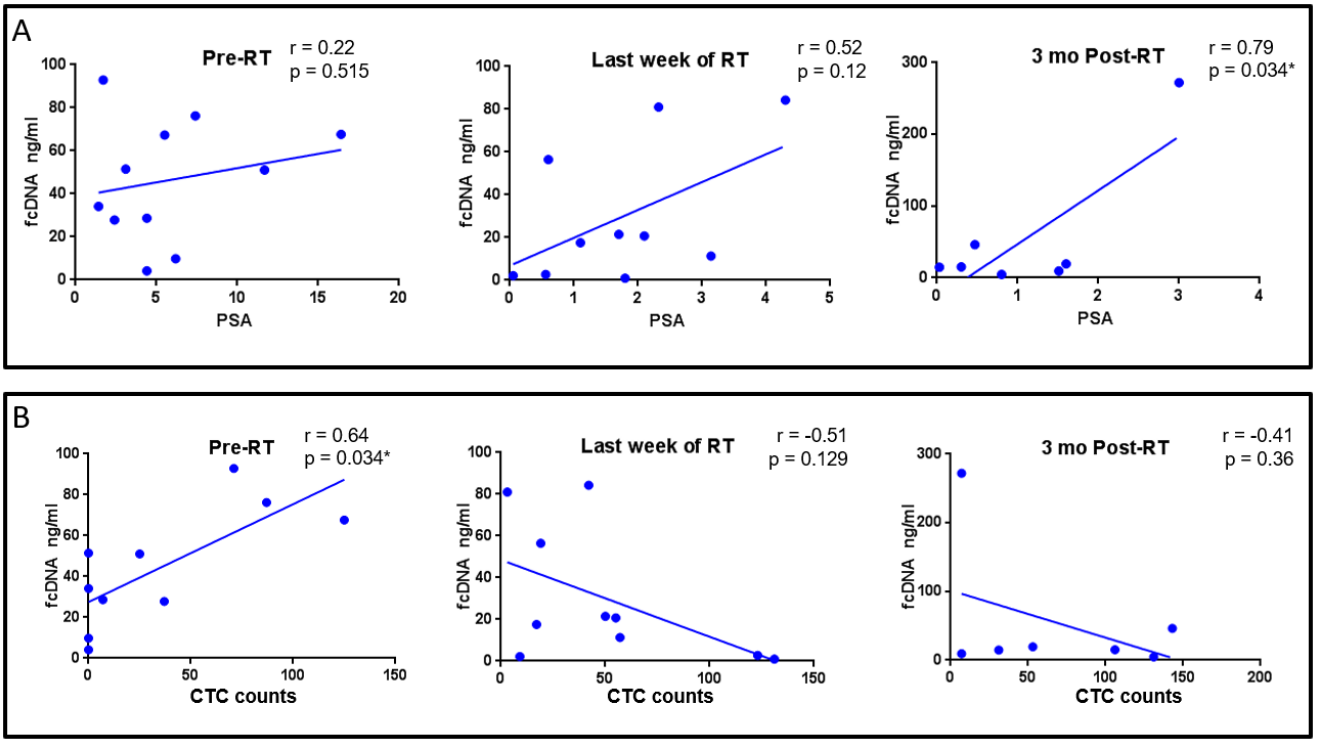


**Figure 5:** Association between **(A)** fcDNA and PSA, and **(B)** fcDNA and CTC in patients from primary treatment trial at 3 different time points (pre-RT, last week of RT and 3 mo post-RT. (*denotes statistical significance).

### V Relationship Between CTC and fcDNA Values and PSA

In Figure 4**,** the relationship between CTCs and PSA at each experimental time point (pre-and post-RT) are shown. Ostensibly, an opposite trend between primary and salvage treatment is shown. The direct correlation with CTC counts and PSA pre-RT in the primary treatment patients changes to a reciprocal relationship 3 months post-treatment. Nonetheless, the relation did not show any statistical significance. There is no correlation between PSA and CTCs pre-RT for salvage patients; but, at the later time points, (last week of RT and at 3 mo post-RT), significant direct correlations are observed. The relationship between fcDNA and PSA in patients who received primary treatment are shown in Figure 5A. There is no significant relationship seen between fcDNA and PSA pre-RT; however, 3 mo post-RT measurements reveal a significant direct correlation. Interestingly, there was signification correlation between CTCs and Gleason Score (Supplemental Figure 2).

### VI Relationship Between CTC and fcDNA

The relationship between fcDNA values and CTC counts at different experimental time-points in patients treated primarily are illustrated in Figure 5B**.** A significant association between CTCs and fcDNA was seen pre-RT, whereas no correlation was observed in the post- treatment series.

### Discussion

In this paper we describe for the first time the changes in CTC and fcDNA levels pre- and post-RT in men enrolled in two prospective clinical trials for primary and salvage radiation treatment. We found that CTC counts increase significantly after primary RT treatment in those whose biochemical response was more robust. Two pilot studies report CTCs detection rates of 21% (in localized intermediate and high-risk prostate cancer) and 10% [23, 24]. In Lowes *et al*. a 73% CTCs detection rate in post-prostatectomy patients prior to salvage radiotherapy is reported and 8.3% in patients with biochemical recurrence after primary treatment [11, 25]. Comparatively, in our study, higher CTCs detection rates were reported for both primary (64%) and salvage patients (100%), respectively. This is most likely due to the higher sensitivity of the platform used in our study [16]. To the best of our knowledge, there are no reports describing CTCs changes following primary external beam RT. A recent study from Japan, reported elevation in CTC numbers after brachytherapy for prostate cancer [26]. However, this was attributable to surgical manipulation of the gland. Similarly to our observation for overall increase of CTCs counts with RT, a study of patients with Non-Small Cell Lung Cancer (NSCLC) by Martin *et al.* also reports increase of CTCs post-RT [21]. In addition, this study revealed the tendency of CTCs to form cell clusters or circulating tumor microemboli (CTMs) comprised of ≥ 2 cells especially during RT. Our findings support this data that CTCs of prostate cancer tend to form clusters of more than 2 cells [21, 22].

The significant negative correlation between CTC counts and PSA values at 3 months post-RT can be explained by the release of tumor cells that do not express or produce PSA. This negative correlation indicates that detected CTCs may not be fully comprised of viable cells and may be in an apoptotic or quiescent state. A study published recently in NSCLC used a viral detection method to demonstrate a decrease in live CTCs after RT, adding to our hypothesis about the decrease in

viability of detected CTCs after RT [27]. Furthermore, these data lends support to the hypothesis that RT can cause disruption of the tumor microenvironment and can cause a continuous shedding of tumor cells into the peripheral blood circulation [21]. It is known that tumor vasculature can be disrupted by RT and that this results in an increase in vessel leakage from the primary tumor [28]. In particular, endothelial cell damage has been reported often with high radiation doses [29]. Further analysis of CTCs after RT in prostate cancer is needed to help determine the percentage of viable cells within detected CTC counts. Additional characterization of these CTCs could possibly uncover more aggressive phenotypes which may provide more prognostic information, analogous to reported findings in breast cancer [30].

In our study, patients who received salvage RT were classified as favorable responders using a cutoff PSA value of 0.1 ng/ml [20]. In these patients, the pre-RT CTC counts showed no significant differences between the biochemical favorable responder and unfavorable responder groups, which agrees with another study by Lowes et al. [11]. They showed that there were no statistically significant differences in CTC levels following RT between patients with biochemical response vs those with biochemical failure. The significant correlations between CTC counts and PSA in the post-RT series shown in our salvage treatment data indicate that a higher PSA value was positively correlated with higher CTC counts. This can be explained by prostatic tumor cells, expressing PSA, leaving the salvage RT site and contributing to higher CTC counts. This lends support to the use of CTC counts to determine if patients are responding to treatment. The combination of CTC counts and PSA values may help us determine if patients post-prostatectomy are responding to the salvage RT. For patients not receiving RT, many studies have reported that fcDNA released into the blood stream can be associated with early detection and prognosis of prostate cancer [31, 32]. The distinct differences observed in the correlation between CTCs and fcDNA could be interpreted as CTCs being the source of fcDNA; therefore, the higher number of structurally intact CTCs, the lower levels of fcDNA. There are previous studies that show a significant relationship between the concentration of CTCs and fcDNA in prostate cancer patients [33]. It is presumed that the fcDNA comes from the lysis of cancer cells that get detached from the primary tumor and enter the bloodstream as disseminated tumor cells during the process of epithelial-mesenchymal transition [34]. Independently of fundamental courses, both markers have shown to correlate with a phenotype of an aggressive tumor [35-37].

In conclusion, despite the low sample size and short-term follow-up, this is a unique study that analyzed CTCs/fcDNA dynamics pre- and post-RT in prostate cancer patients treated with primary and salvage RT and also looked at correlations of CTCs/fcDNA with PSA. Our group is working on studying the disruption of tumor vasculature and microenvironment after RT that causes the release of CTCs/fcDNA into the blood stream and also looking at single cell analysis and genomic profiling of CTCs in prostate cancer. We continue collecting blood from patients on these and other institutional clinical trials; a larger cohort will give us increased statistical power in order to associate with more recognized and clinically meaningful end points.

## Conflicts of Interest

R.D. and R.C. are co-inventors of Circulating Tumor Cells (CTC) microfiulters used for CTC analysis in the manuscript. R.D., R.C and A.W. are part of the leadership team of Circologix Inc that markets the automated CTC capture system.

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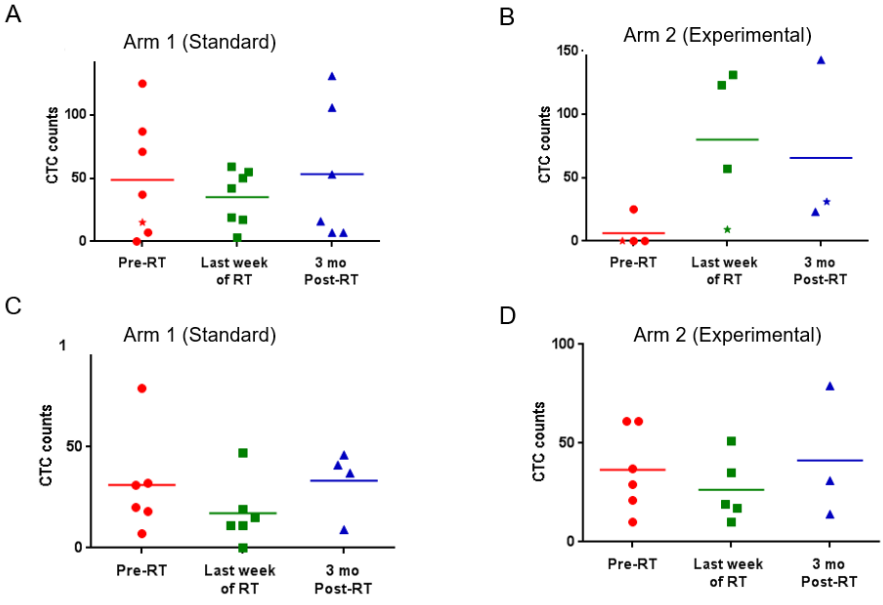
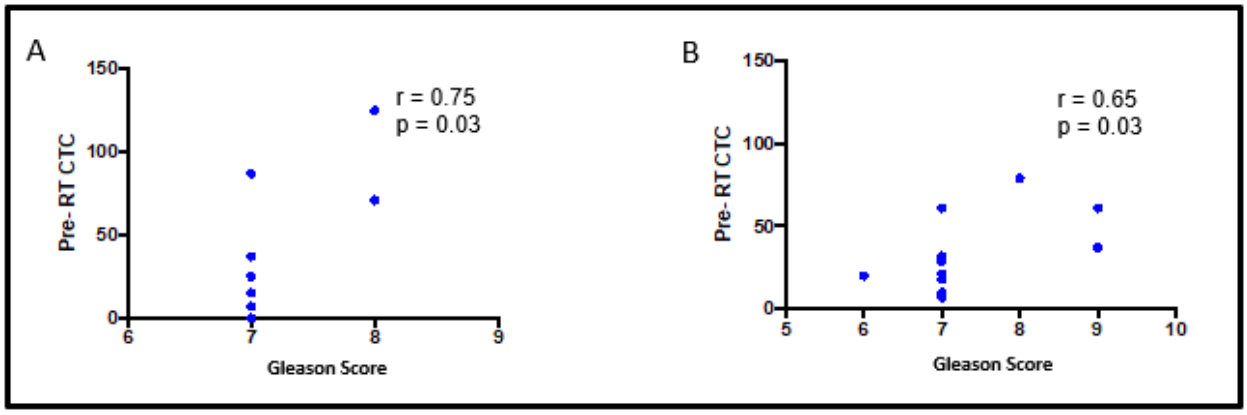



**Supplemental Figure 1:** Distribution of CTCs by treatment arms in patients from **(A&B)** Primary treatment and **(C&D)** Salvage treatment trials pre-RT, last week of RT and 3 mo post-RT. Horizontal lines represent the mean. The stars indicate patients on ADT.




**Supplemental Figure 2:** Correlation between pre-RT CTC counts and Gleason score for patients in the **(A)** Primary treatment; and **(B)** Salvage treatment trials.

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