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Implicações translacionais de uma nova ferramenta de detecção de células tumorais circulantes no monitoramento do câncer de próstata

Leandro Alves de Oliveira

Uberlândia
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FOLHA DE APROVAÇÃO

Leandro Alves de Oliveira

Avaliação do aptâmero A4 selecionado por 3D Cell SELEX no rastreamento do câncer de próstata

Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Faculdade de Medicina da Universidade Federal de Uberlândia, como requisito parcial para a obtenção do título de Mestre em Ciências da Saúde.
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Banca examinadora

Presidente da banca (orientador): Prof. Dr. Luiz Ricardo Goulart Filho

Titular: Prof. Dr. Omar Pacheco Simão.
Instituição: FAMED-UFU.

Titular: Prof. Dr. Marcelo Takeo Rufato Okano
Instituição: FACULDADE DE MEDICINA-USP.

DEDICATÓRIA

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Epígrafe

" *O objetivo da ciência é substituir as palavras por fatos e as impressões por demonstrações."*

François Magendie, 1783-1855, fisiologista francês.

RESUMO

Introdução: O diagnóstico precoce de câncer de próstata (CaP) é essencial para aumentar a sobrevida dos pacientes, mas os marcadores e métodos atuais não possuem sensibilidade e especificidade suficientes, tornando o diagnóstico ainda muito impreciso. Recentemente, as células tumorais circulantes (CTCs) têm surgido não como método de rastreio do CaP, mas sim como marcadores de prognóstico utilizando um arsenal de diversos alvos para a captura dessas células. Contudo, a busca por um método ou marcadores comuns para o rastreio, diagnóstico, prognóstico e monitoramento da doença ainda se apresenta com um dos principais objetivos técnico-científicos a ser alcançado. **Objetivo:** apresentar um novo marcador, o aptâmero A4 selecionado previamente por 3D-Cell SELEX na linhagem PC3, e avaliar sua capacidade de detectar CTCs por citometria de fluxo no sangue de pacientes com CaP virgens de tratamento e sob diferentes regimes terapêuticos. **Material e métodos:** o estudo avaliou 34 homens com CaP e 16 homens sem alterações prostáticas. Foi coletado o sangue em tubo com EDTA, e após proceder a lise de hemácias, as células nucleadas de cada paciente foram incubadas com o aptâmero A4 conjugado à biotina, e em seguida lavadas e incubadas com estreptoavidina-FITC para posterior análise em citometria de fluxo. Os percentuais de CTCs foram comparados entre os dois grupos de pacientes e correlacionados com idade, níveis de PSA, estadiamento e procedimentos terapêuticos adotados (bloqueio hormonal, radioterapia e cirurgia). O limite de detecção acima de 1% de CTCs foi considerado positivo, utilizando como base o percentual observado em todos os 16 controles negativos. **Resultados:** todos os pacientes foram diagnosticados como positivos independentemente do tempo de terapia ou do estadiamento, exceto um paciente sob bloqueio hormonal que não apresentou CTCs. O percentual de CTCs apresentou alta correlação com idade (R=0,75) e com os níveis de PSA (R=0,80)

de forma exponencial, embora seis pacientes com altos índices de células circulantes apresentaram PSA<0,02ng/mL, considerados como falha bioquímica. **Conclusão:** nossos resultados preliminares indicam uma acurácia elevada de 98% e demonstra um grande potencial de aplicação dessa nova tecnologia diagnóstica tanto no rastreamento, quanto no monitoramento do tratamento do CaP, o qual deverá ser melhor investigado em população de risco.
Palavras-chave: Palavras-chave: Câncer de próstata, diagnóstico, aptâmero, citometria de fluxo, células tumorais circulantes.

ABSTRACT

Introduction: prostate cancer (PCa) early diagnosis is essential to boost patients' life expectance. Although, current biomarkers and diagnosis methods do not present reliable sensibility and specificity, making the diagnosis rather imprecise. Recent methodologies have been using circulating tumor cells (CTCs), not for screening of PCa, but as prognosis indicators, employing a vast array of techniques to capture those cells. However, the search for a new biomarkers or diagnosis methods able to screen, diagnosis, assist in prognosis and in the disease monitoring still one of the major technical and scientific objectives to be achieved. **Objective:** To present a new biomarker for PCa, the aptamer A4, previous screened in the prostate cancer cell line PC3, using 3D-Cell SELEX. And to able to detect, by flow cytometry, CTCs in blood samples of PCa patients undergoing various treatment regimen. **Material and methods:**

the study evaluated 34 PCa patients and 16 health controls. Blood samples were collected in EDTA tubes, and after erythrocytes lysis, nucleated cells were incubated with A4 aptamer conjugated with biotin, them the cells were washed and incubated with streptavidin-FITC for later flow cytometer analysis. Percentage of CTCs were compared between patient's groups and correlated against age, PSA levels, staging and treatment regimen (hormonal blockade, radiotherapy and surgery). Detection limit above 1% of CTCs was considered positive, based on the percentage observed on all of the 16 negative controls. **Results:** all patients were positively diagnosed independently of therapy time or staging, except for one patient undergoing hormonal blockade therapy, which does not present detectable CTCs. CTCs percentage presented high correlation against age (R=0.75) and with PSA levels (R=0.80) with exponential behavior, although, six patients with high CTCs count presented PSA levels <0.02 ng/mL, and were considered was biochemical errors. **Conclusion:** Our preliminary results indicated high accuracy (98%) and demonstrate a potential application of this technology for diagnosis and screening, as well as in the monitoring of PCa evolution, which should be better investigated in the risk population.

Keywords: Prostate cancer, diagnosis, aptamer, flow cytometry, circulating tumor cells

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LISTA DE ABREVIATURAS E SÍMBOLOS

CaP	Câncer de Próstata
CEP	Comitê de Ética em Pesquisa
CTC	Célula Tumoral Circulante
DRE	Exame Retal Digital
EDTA	Ácido Etilenodiamino Tetra-Acético
FITC	Isotiocianato de fluoresceína
HPB	Hiperplasia Prostática Benigna
INCA	Instituto Nacional do Câncer
SELEX	Evolução Sistemática de Ligantes por Enriquecimento Exponencial
PBS	Tampão fosfato salino
PCA3	Gene 3 do Antígeno do Câncer de Próstata
PCR	Reação em cadeia de polimerase
PSA	Antígeno Específico Prostático
RT	Transcriptase reversa

SUMÁRIO

1 INTRODUÇÃO

O Câncer de Próstata (CaP) é um grande problema de saúde pública mundial, devido à sua alta incidência e mortalidade [1, 2]. Quando não tratado o CaP pode se tornar mais agressivo, possibilitando sua progressão para hormônio resistente e culminando com a ocorrência de metástases [3, 4]. A sobrevida de pacientes com CaP está relacionada a muitos fatores, especialmente à extensão do tumor no momento do diagnóstico [5]. Atualmente, o acompanhamento das alterações prostáticas é realizado em 4 etapas: toque retal (DRE), dosagem do antígeno prostático específico (PSA), ultrasonografia transretal e biópsia [6, 7]. Contudo, a complexidade do manejo da doença está cada vez mais aumentado devido à progressão heterogênea e dos subgrupos com diferentes prognósticos [8].

A utilidade do PSA é bastante limitada em distinguir entre os estágios do CaP e as condições benignas como a HPB e prostatites, e sua utilização no rastreamento do CaP não tem sido recomendada [9, 10]. Portanto, torna-se evidente a necessidade de se identificar novas ferramentas que possam ter maior especificidade e sensibilidade para a detecção do CaP [9, 11]. Vários alvos biológicos têm sido propostos como marcadores para o diagnóstico do CaP, mas apenas o RNA não-codificante *PCA3* tem demonstrado utilidade, sendo detectado tanto em sangue periférico [12] quanto em urina [13], bem como combinado ao PSA [12]. Contudo, os estudos apresentam sensibilidades e especificidades moderadas, com acurácia aproximada de 70%. Recentemente, uma nova tecnologia diagnóstica baseada em aptâmeros para captura e amplificação do *PCA3* por RT-PCR aumentou a sensibilidade do diagnóstico para 100% [14], mas ainda requer a purificação de RNAs totais e logística para coleta da amostra devido à alta instabilidade do RNA.

2 FUNDAMENTAÇÃO TEÓRICA

Historicamente, a detecção de células tumorais circulantes (CTCs) da próstata foi primeiramente realizada por meio da RT-PCR com diversos marcadores e combinações, tais como PSA (prostate specific antigen), PSMA (prostate specific membrance antigen), PSCA (prostate stem cell antigen), CK19 (cytokeratin 19) e PTHrP (Parathyroid hormone–related protein), dentre os quais o PSMA e PSCA são altamente expressos em tumores metastáticos [15]. Estima-se que milhares de CTCs são liberadas na circulação sanguínea diariamente [16] e que elas sobrevivem durante 1-2,5h [17].

Recentemente, a captura de CTCs, usadas como biomarcadores e também chamadas de biópsias líquidas, tem sido aplicada com sucesso no câncer de próstata com potencial uso no diagnóstico e rastreamento [18-20]. Uma extensa revisão recente aborda a importância clínica da detecção e análise de CTCs, especialmente nos estágios iniciais do câncer [21].

Embora níveis altos de CTCs sugerem uma associação com a progressão da doença, o valor preditivo para o monitoramento terapêutico é ainda inconclusivo [22]. Sistemas microfluídicos mais avançados associados à imunofluorescência foram subsequentemente desenvolvidos para a identificação da origem tumoral por meio da captura de CTCs em sangue periférico contra os antígenos prostáticos PSA (prostate specific antigen) e PSMA (prostate specific membrane antigen), utilizando como prova-de-conceito o sangue normal contaminado com linhagens de células tumorais (DU145, PC3 e LNCaP) [23]. Contudo, o mesmo sistema foi testado em 5 pacientes metastáticos e em 12 controles, o qual detectou CTCs em apenas 3 pacientes (60%) e em 1 indivíduo controle (8%), não atingindo a sensibilidade e especificidade necessárias. Por outro lado, embora metástases estejam associadas às CTCs e à um pior prognóstico, 35% dos pacientes metastáticos são indetectáveis pelo sistema

CellSearch® [20]. Este teste baseia-se na seleção positiva de CTCs utilizando anticorpos contra o antígeno da molécula de adesão celular epitelial (EpCAM) com detecção complementar com os anticorpos anti-CD45, e anti-citoqueratinas 8, 18, e 19 [24]. Semelhantemente à CellSearch, o AdnaTest também utiliza o anti-EpCAM para a identificação de CTCs, mas complementa sua detecção com amplificação de mRNAs dos genes PSA, PSMA e EGFR (epidermal growth factor receptor), resultando em detecção de CTCs em somente 67% dos pacientes metastáticos antes do tratamento [25]. Contudo, a grande limitação desses testes está no uso de marcadores de células mesenquimais em um tumor altamente heterogêneo, o que restringe a detecção à uma população que provavelmente esteja associada à apenas alguns fenótipos agressivos do CaP [26-30]. Nesse contexto, foi desenvolvida uma outra plataforma de imagem com alto desempenho, a FAST (fiberoptic array scanning technology), em que 3 milhões de células são espalhadas em lâminas de microscopia e detectadas simultaneamente com DAPI, anti-CD45 e anti-citoqueratinas, resultando em uma positividade de aproximadamente 93% [31], teste este ainda em estudos de validação. Duas revisões extensas recentes sobre todos os testes desenvolvidos atualmente para detecção de CTCs foram apresentados [22], e todas as tecnologias estão associadas à imunocaptura, imunohistoquímica e/ou a microfluídica e eletroforese, e apenas um ensaio (Vita-Assay) reivindica o uso da citometria de fluxo com uma série de marcadores (EpCAM, CK, CD44, CD34, CD45, vimentin).

Portanto, utilizamos um novo aptâmero (A4) ligante de alta afinidade para linhagens de células tumorais da próstata recentemente desenvolvido por 3D-Cell Selex [32], mas devido a sua grande especificidade para linhagens tumorais hipotetizamos que o marcador poderia ser usado como sistema de captura de CTCs em sangue periférico e usado para quantificar células tumorais circulantes por citometria de fluxo. Neste trabalho, avaliou-se o percentual de CTCs marcadas com o aptâmero A4 e detectados por citometria de fluxo em pacientes

virgens de tratamento e em pacientes sob diferentes regimes terapêuticos como prova-de-conceito, e o potencial uso da tecnologia é discutido nessa pesquisa.

3 OBJETIVOS

3.1 Objetivo geral

Aumentar o desempenho no diagnóstico e monitoramento do CaP através da biologia molecular.

3.2 Objetivos específicos

Avaliar o uso do aptâmero A4 e citometria de fluxo na detecção de células tumoras circulantes no CaP.

Title: Translational Implications of a Novel Circulating Tumor Cells Detection Tool in Prostate Cancer Monitoring

One sentence summary:

A novel aptamer-based flow cytometry tool was developed to detect circulating tumor cells in the peripheral blood of prostate cancer patients, which has been successfully applied for tracking and treatment monitoring.

Authors: Leandro A. Oliveira[1,2], Aline G. Souza[1], Vivian A. Goulart[1], Luiz R. Goulart[1*]

Affiliations:

[1] Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, MG, Brasil.

[2] School of Medicine. Graduate Program in Health Sciences, Federal University of Uberlândia, Uberlândia, MG, Brasil.

***Corresponding author**

Luiz R. Goulart. Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia (UFU), 38400-902, Uberlândia-MG, Brazil. Phone: +55 34 3225-8440. E-mail: lrgoulart@ufu.br.

ABSTRACT

Circulating tumor cells (CTCs) have been used as prognostic markers in prostate cancer (PCa). We present a novel aptamer-based flow cytometry tool to detect CTCs in the peripheral blood of PCa patients with and without treatment and healthy controls. Percentage of CTCs were compared between patients' groups and correlated with age, serum PSA levels, staging and therapeutic procedures adopted. The cutoff limit >1% of CTCs was considered positive. PCa patients were diagnosed as positive irrespective of time of therapy or staging. CTCs increased exponentially and showed high correlation with age (R = 0.75) and PSA levels (R = 0.80), although six patients with PSA <0.02 ng/mL presented high CTCs%, and were considered biochemical failure. Our results presented an accuracy of 98%, and demonstrated a potential application of this new diagnostic tool for PCa tracking and treatment monitoring, which should be further investigated in men at risk.

Key words: Prostate cancer, diagnosis, aptamer, flow cytometry, circulating tumor cells.

INTRODUCTION

Prostate cancer (PCa) is a major public health problem worldwide because of its high incidence and mortality (*1, 2*). When untreated, PCa may become more aggressive, allowing its progression to hormone resistance, culminating with the occurrence of metastases (*3, 4*). The PCa patients' survival is related to many factors, especially the tumor extent at the time of diagnosis (*5*). Currently, monitoring of prostatic changes is performed in four stages: rectal examination (DRE), prostate specific antigen (PSA) dosage, transrectal ultrasonography, and biopsy (*6, 7*). However, the complexity of the cancer management is increasingly higher due to the heterogeneous progression of the disease and the differential prognoses for patients' subgroups (*8*).

The utility of PSA is quite limited in distinguishing between stages of PCa and benign conditions, such as BPH and prostatitis, and their use for PCa screening has not been recommended (*9, 10*). Therefore, it is evident the urgent need to identify new tools that may present greater specificity and sensitivity for PCa diagnosis (*9, 11*). Alhtough several biological targets have been proposed as diagnostic markers, only the PCA3 non-coding RNA has shown utility, which can detected both in the peripheral blood (*12*) and in urine (*13*), as well as in combination with PSA (*12*). However, the studies present moderate sensitivities and specificities, with approximate accuracy of 70%. Recently, a new aptamer-based diagnostic technology that includes the magnetic capture of the circulating PCA3 RNA followed by amplification through RT-PCR has increased the diagnostic sensitivity to 100% (*14*). Nevertheless, the sample size was limited, and the new tool still requires purification of total RNA and logistics for sample collection due to greater instability of RNA molecules.

Historically, detection of RNA from circulating tumor cells, namely CTCs of the prostate, was first performed by RT-PCR with various markers and combinations, such as the prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), PSCA (prostate specific stem cell antigen), CK19 (cytokeratin 19) and PTHrP (Parathyroid hormone-related protein), among which PSMA and PSCA are highly expressed in metastatic tumors (*15*). It is estimated that thousands of CTCs are released into the bloodstream daily (*16*), which are able to survive for 1-2.5h (*17*).

Recently, the capture of CTCs as biomarkers, also called liquid biopsies, has been successfully applied to prostate cancer with potential use in diagnosis and screening (*18-20*). An extensive review addresses the clinical importance of the detection and analysis of CTCs, especially in the early stages of cancer (*21*).

Although high levels of CTCs suggest an association with disease progression, the predictive value for therapeutic monitoring is still inconclusive (*22*). More advanced microfluidic systems associated with immunofluorescence has been subsequently developed to identify the tumor origin by capturing CTCs in the peripheral blood against PSA and PSMA as a proof-of-concept, using healthy blood contaminated with tumor cell lines (DU145, PC3 and LNCaP) (*23*). However, the same system was tested in five patients with metastases and in 12 controls, but CTCs could be detected in only three patients (60%) and on control individual (8%), without achieving the necessary sensitivity and specificity. On the other hand, although metastases are associated with CTCs and a worse prognosis, 35% of metastatic patients are undetectable by the CellSearch® system (*20*). This assay is based on the positive selection of CTCs using antibodies against the epithelial cell adhesion molecule antigen (EpCAM) with complementary detection of anti-CD45 antibodies, and anti-cytokeratins CK-8, CK-18, and CK-19 (*24*). Similar to CellSearch, AdnaTest also uses anti-EpCAM to identify CTCs, but complements its detection with mRNAs of the PSA, PSMA and EGFR genes, resulting in detection of CTCs in only 67% of the cells in pre-treatment metastatic patients (*25*). However, the major limitation of these tests is the use of mesenchymal cell markers in highly heterogeneous tumors, which restricts detection to a population likely to be associated with only a few aggressive PCa phenotypes (*26-30*). In this context, another high-performance imaging platform, FAST (fiberoptic array scanning technology), was developed in which three million cells are scattered on microscopy slides and detected simultaneously with DAPI, anti-CD45 and anti-cytokeratins, resulting in a positivity of approximately 93% (*31*), although this test is still under validation. An extensive review of all current CTC detection tests have recently been presented (*22*), and all technologies are associated with immunocapture, immunohistochemistry and/or microfluidic and electrophoresis, and only one assay (Vita-Assay) claims the use of flow cytometry with a series of markers (EpCAM, CK, CD44, CD34, CD45, Vimentin).

In this investigation, we hypothesized that a novel aptamer with high affinity to prostate tumor cells lines could be used as a CTCs' biomarker in the peripheral blood of prostate cancer patients. We evaluated by flow cytometry the percentage of CTCs labeled with the A4-aptamer in treatment-naive patients and in patients under different therapeutic regimens as a proof-of-concept, and the potential use of this technology is discussed herein.

RESULTS

Establishment of the new aptamer-based flow cytometry for liquid biopsies, and patients' characteristics.

We have previously selected the A4-aptamer against a tumor cell line by 3D-Cell Selex (*32*), but its clinical application was not demonstrated. In order to understand its behavior as a CTC marker in clinical settings, we have established a diagnostic methodology based on flow cytometry, which involves a very simple, fast and straightforward procedure, as described in **Figure 1**.

The clinical-laboratory characterization of the groups of patients and controls is presented in **Table 1**. Regarding the age, PCa patients presented ages greater than 55 years old, whereas the control group presented an age range between 28 to 44 years. Patients with PCa also presented highly significant frequencies for both smoking and alcoholism. Regarding skin color, 79.4% of PCa patients were classified as black or mulatto, which were significantly different from controls. Among PCa patients, approximately 80% presented Gleason ≤ 7, while 38.4% were staged as advanced cancer (T3b and T4). CTCs were counted in both control and patient groups, and the cutoff for CTCs established as negative result was 1% based on results observed for all controls (**Figure 2**). The distribution of PCa patients according to their disease staging, regardless the treatment status, showed great variability without any correlation with the clinical-laboratory staging.

In a single case, one patient originally classified as T4, and then re-staged to T1, under hormonal blockage had CTC index of less than 1%; however, a reanalysis of the patient. For T2-staged patients, CTC levels were widely distributed, ranging from 1.2% to 25.8%, variability that was also observed in the other stages.

CTC levels are correlated with age and serum PSA levels

In relation to age, there was a high correlation of CTCs with increasing age ($R = 0.75$) with an exponential behavior as the age advanced, regardless the patients' staging (**Figure 3**). The highest levels of CTCs were observed in patients over 65 years of age.

In relation to PSA levels, two analyzes were performed, one with all values, and another without outliers, considered as biochemical failure. In the latter analysis, a high correlation ($R = 0.80$) was observed with an exponential behavior as PSA increased, independent of the treatment regimen (**Figure 4**).

CTC levels are not correlated with different therapeutic interventions or with treatment period

Another important finding was the large variation of CTC levels for different types of treatment (**Figure 5**). There was no correlation of therapeutic success with CTC levels, since all therapeutic combinations showed highly variable levels of CTCs. However, among the two most frequently used therapeutic options, 6 out of 9 patients undergoing hormonal therapy (66.6%), and 7 out of 14 patients undergoing radical prostatectomy (50%) presented reduced percentages of CTCs (<4.2%). The first therapeutic option was the most effective when staging was considered, since the largest number of patients was in the T4 stage.

Regarding the analysis of minimal residual disease in PCa patients, according to the time of treatment (**Figure 6**), we have demonstrated that both CTCs% and serum PSA levels showed a low positive correlation with the period of therapy (R=0.34). Therefore, it was not possible to associate both markers with disease progression post-treatment. However, in relation to PSA levels recommended for relapse (<0.2 ng/dL), 15 patients still had lower levels and were considered disease-free, although CTCs ranged from 1.2% to 25.8%. For patients with recurrent disease, the percentages of CTCs ranged from 3.33% to 24.66%. It is important to note that among those 15 patients with PSA <0.2 ng/dL, 9 patients had CTCs% between 1.2% and 3.35% and the treatment time ranged from 9 to 25 months, which led us to establish a cutoff for CTCs% below 5% that coincided with the period of 25 months post-treatment as indicative of a stable disease with no progression. The serum PSA levels in most patients in this condition were below 0.2 ng/dL (9/15). It is important to observe that the remaining six patients (40%), with PSA levels below the cutoff for tumor recurrence, presented CTCs% from 14.59 to 25.8, which were considered biochemical failure.

CTC levels detected by the aptamer-based flow cytometry method presents excellent diagnostic parameters

Table 2 presents the diagnostic parameters of the A4-aptamer in detecting CTCs, indicating high performance, with an accuracy of 98%. Although the sample size was small, the three treatment-naive patients presented positive results for the marker. Another important observation was that all negative controls had mean levels of CTCs below 1%.

DISCUSSION

This research describes a new methodology based on flow cytometry with a new biomarker that is able to detect circulating tumor cells (CTCs) in the peripheral blood of patients without treatment and under different therapeutic regimens. Although prostate cancer is a heterogeneous, multifocal, and highly complex disease with different stages in its evolution involving the cascade of testosterone activation, we have demonstrated that the use of CTCs as biomarkers throughout the disease progression was possible due to their capture by a common biological target mediated by the novel A4-aptamer developed by 3D-Cell Selex (*32*). The new tool allowed the detection of all positive patients in all stages and treatments, except for one patient undergoing hormonal treatment that was re-staged from T4 to T1, who was later characterized as Paget's disease. Although these may be considered preliminary results due to the small sample size, the importance of these findings is discussed herein.

The ideal characteristics of a excellent biomarker are high specificity and sensitivity, good viability, minimally invasive, reproducibility and low cost, and in the case of PCa, the marker should also be a molecule that may be applied for disease tracking, diagnosis, prognosis, and treatment monitoring *(33)*. PSA was the main tool applied to the PCa screening in the recent past, but it has been shown to be of low specificity, promoting overdiagnosis with unnecessary biopsies, and also leading to overtreatment *(5, 10, 34, 35)*. However, PSA levels still allow assessment of treatment response, and may help to detect relapse cases following local treatment of the tumor *(33, 34, 36)*. Among various technologies for CTCs detection, the CellSearch capture system, which includes the immunohistochemistry evaluation of several markers, was named liquid biopsy, and its use is restricted to prognosis *(20)*.

Currently, there is only one report that has claimed the use of flow cytometry, which has used FACS (flow cytometry cell sorting) to sort cells with the CD45-/ALDH1+ markers that were both EpCAM+ and EpCAM- from breast cancer; however, the authors did not perform the same procedure for healthy donors *(37)*, serving only as a methodology for phenotypic identification of tumor cells and not for diagnosis. Similarly, the system called Vita-Assay *(38)* was developed, which captures CTCs via FACS to enrich and propagate cells for subsequent drug sensitivity studies, through injection into animal models followed by large-scale sequencing. Also aiming *in situ* drug screening tests, a microfluidic system has

been developed to allow robust formation of CTC clusters without pre-enrichment *(39)*, which can provide a noninvasive and inexpensive assessment of drug development or personalized therapy. The last three strategies were used to better characterize the molecular aspects of CTCs without any diagnostic pretension.

Our new diagnostic platform based on flow cytometry and aptamer labeling has surprisingly achieved an excellent precision (98%), much higher than those presented by existing epithelial markers (around 65%), such as EpCAM and markers associated with epithelial-mesenchymal transition (CK, CD44, CD34, CD45, vimentin). Interestingly, some technologies are now directed to the analysis of larger blood volumes, particularly in early tumors, where the presence of CTCs is even more infrequent *(40)*. In our investigation, using only 5 mL of peripheral blood, we were able to detect CTCs in all PCa patients, with and without treatment, except in a patient who was later diagnosed as Paget's disease, with CTCs below 1%. This detection ability may also be associated with labeling of many non-epithelial cell types other than epithelial ones, but with similar size, since no epithelial markers have been included, such as EpCAM. We are currently attempting to use a panel of markers to phenotype all A4+ CTCs by flow cytometry, with greater sample size, mainly to justify the significant correlation of CTCs detected by the A4-aptamer with age and the post-treatment PSA levels, suggesting that this marker may actually represent an advance in the diagnosis.

Interestingly, in addition to improving PCa diagnosis, the marker may also be used for PCa monitoring. The lack of correlation between the positive A4-aptamer detection with different stages and different treatment regimens, and the medium correlation with the time of treatment suggest the need for new therapeutic approaches in order to reduce the minimal residual disease. The various therapeutic regimens in the arsenal against PCa confirmed the current literature regarding the disease relapse after local treatment and putative cure, and also as palliative treatments in patients with systemic disease, since there was great fluctuation of PSA levels (*35*).

The significant exponential correlations observed between the percentages of CTCs with age and PSA levels also indicate an intimate relationship of CTCs with advanced age, which is also associated with post-treatment PSA levels and recurrence (*9*). However, it is important to note that six patients (40%) presented PSA <0.2 ng/mL (*41*) and very high levels of CTCs, which was an indication of biochemical failure and advanced metastatic disease. Probably, during the progression of the disease in these patients CTCs have reached higher levels of non-differentiating cells, which may lead to difficulties in the disease management

(*42*). This hypothesis has yet to be substantiated by additional molecular and immunological studies in these CTCs through cell sorting by FACS.

The correlation of post-treatment serum PSA levels with the percentage of CTCs becomes even more significant when taking into account that PSA has been advocated as a marker of response to prostate cancer treatment in both radical prostatectomy and radiotherapy, and hormone therapy (*41*). After radical surgery, the lowest PSA value, defined as PSA nadir (PSAn), should be less than 0.2 ng/dL. However, PSA levels above this value after 60 to 90 days post-surgery suggest residual disease, requiring further treatment. Thus, to define prognosis it is used the time it takes to double PSA values, and the time it takes for biochemical relapse (*42*). For radiotherapy, the most reliable way to monitor PSA after nadir, which can take up to 18 months to achieve, is to observe an increase of at least 2 ng/dL to have a diagnosis of recurrence (*43*). For PCa monitoring after hormonal blockade and chemotherapy, there is still no consensus regarding the PSA pattern, because during hormonal refractoriness cells are undifferentiated and do not produce PSA as a naive-treatment tumor cell. Still in relation to PSA levels after therapy in a large study, patients who had radiotherapy associated with androgenic suppression had a significantly higher chance of being disease-free in 5 years than those who had only radiation therapy, with odds of 80% and 62%, respectively (*44*). An extensive analysis of 32 studies with therapeutic regimens has been presented in a comprehensive review without reaching a consensus (*45*). However, radiotherapy remains one of the few therapeutic interventions that offers a potential cure for patients who have PSA-based recurrence after radical prostatectomy. The response rate of PSA to the rescue radiation therapy, defined as any incremental decrease in PSA in response to radiotherapy, has reached up to 90%. A high PSA response rate to radiotherapy suggests that the majority of patients with post-operative PSA relapse may be due either to the local tumor recurrence in the prostate bed or to the distant occult metastasis simultaneously (*45*). However, despite the different therapeutic options adopted in this investigation, the percentage of CTCs observed in the various patients indicates that there is still no option defined as more successful, although hormone suppression was more successful than radiotherapy based on the reduced percentage of CTCs.

With regard to prognosis and/or relapse, it was not possible to associate both serum PSA levels and CTC percentage with disease evolution, since both markers showed a low correlation with time after treatment. However, our preliminary data suggest that the percentage of CTCs below 5% detected in a period of up to 25 months post-treatment may be

indicative of a stable disease with no progression, since most patients (60%) in this condition had serum PSA levels below 0.2 ng/dL. Therefore, patients' monitoring data in this investigation also suggest that the detection and quantification of CTCs can anticipate treatments or avoid unnecessary procedures by creating new algorithms based on CTC levels. It is important to emphasize that the future of CTC detection lies in the molecular characterization of these cells, since simple enumeration is not enough. Specific molecular signature is expected to provide critical information about the tumor's biology of the patient, which will allow the identification of new personalized therapies (*46*).

The performance of the A4-aptamer as diagnostic test was excellent, as it provided an accuracy of 98%, with sensitivity of 97% and specificity of 100%, and opens new diagnostic possibilities, although these results must be seen with caution due to the small sample size and because BPH patients were not included. The exclusion of BPH patients was due to the high probability of having a non-detectable tumor by conventional methods. But, these parameters become more impacting, even with the reduced sampling, because the performance of the A4-aptamer analyzed by flow cytometry was much higher than those presented by other reported CTC markers (*33*), which also used smaller sample sizes, and required an arsenal of markers and tools that barely reached sensitivities close to 65%. The potential presented by this new technology can really impact not only the epidemiology of the disease, but the early diagnosis, and the most adequate monitoring, which must be further demonstrated in a larger sampling, already in the collection phase.

MATERIAL AND METHODS

Collection of biological samples and the inclusion and exclusion criteria

Biological samples (peripheral blood) were obtained from 34 patients with histologically confirmed prostate carcinoma and from 16 patients without prostatic abnormalities as the control group at the Clinics' Hospital of the Federal University of Uberlândia (UFU), with the approval of the Research Ethics Committee under the number CEP 562,678, and patients' consents were obtained. The clinical-laboratory parameters obtained of all individuals were: age, serum PSA levels, familial history, skin color classification, smoking and alcohol drinking behaviors, Gleason score, disease staging, and therapeutic regimens. Patients above 45 years of age and classified with benign prostatic hyperplasia (BPH) were excluded from the study due to the higher probability of the

coexistence of both BPH and PCa, and the increased chances of having localized PCa (*1, 47, 48*). We have adopted this exclusion criterion also because inflammation is a well-established tumor promoter that contributes to cancer growth, angiogenesis, and resistance to apoptosis (*49, 50*), and induction of cell proliferation under conditions of inflammation dramatically increases predisposition of cells to cancer (*51*). So, in order to prevent increased false positive rates in this proof-of-concept, we have decided to exclude BPH patients from this study, although a thorough investigation in this group of patients is highly desired.

Characterization of the Aptamer A4 as a biomarker and its synthesis

The A4-aptamer was previously selected using the 3D Cell SELEX technique and tested for prostate carcinoma, as described by Souza et al. (2016) (*32*). For this work the A4-aptamer was synthesized and biotin-conjugated at the Integrated DNA Technology (IDT, USA).

Analysis of CTCs by Flow Cytometry

CTCs were detected in both PCa and control groups by the A4-aptamer through flow cytometry. After collection of the blood in EDTA tube, plasma separation was performed by centrifugation at 1500 rpm for 10 minutes. 200 µL of the leukocyte layer was then collected and lysed from the red blood cells using 10X lysis buffer (BD Pharm Lyse) as recommended by the manufacturer. The samples were then centrifuged at 200xg for 5 minutes. The supernatant was discarded and the pellet of cells formed was washed 2X with 1X PBS buffer. After centrifugation, the supernatant was discarded, cells were incubated with 10 µL of the A4-aptamer (100 pmol/µL) diluted in 100 µL of 1X PBS buffer for 1 hour at 37ºC. Cells were then washed twice with 1X PBS, and further incubated with streptavidin-FITC (1 µg/mL) diluted in 100 µL of 1X PBS for 40 minutes at 37°C. Thereafter, the wash was repeated twice and cells were resuspended in 100 µL of 1X PBS for flow cytometry analyses (Accuri C6®, Becton Dickinson).

Statistical Analysis

The power analysis for a Wilcoxon signed-rank test was conducted in G*Power to determine a sufficient sample size using an alpha of 0.05, a power of 0.80, a large effect size (dz = 0.8), and two tails (*52*). Based on the aforementioned assumptions, the desired sample size was at least 15 observations for each group. The PCa group sampling of 34 patients was

performed to increase the power of the test, and also to have a better representation of different therapeutic regimens.

The analysis performed on the Accuri C6 Flow Cytometer indicated the percentage of CTCs labeled with the A4-aptamer for both control and PCa groups. The comparison of means was performed by the Mann-Whitney test (continuous variables and non-parametric analyses). Receiver operating characteristics (ROC) curve was calculated with a 95% confidence interval and the estimated diagnostic parameters (sensitivity, specificity and accuracy) according to the cutoff limits for the CTC values of the control group. Correlations and regression analyzes were performed between percentages of CTCs, age and PSA levels. All analyzes were performed in GraphPad Prism software version 7.0, using probability values below 5% as significant.

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Conflict of Interests

The A4-aptamer marker described in this investigation is claimed in a patent application filed on Jan 24th, 2017 by LRG, VAG e AGS under the number BR1020170015637.

Tables:

Table 1. Laboratory and clinical characterization of prostate cancer patients and controls and the percentage of circulating tumor cells (CTCs) detected by flow cytometry.

Characteristics	Control (N=16)	Patients (N=34)	P Value
Age in years – mean (range)	38.0 (28-44)	72.26 (55-92)	0.001
PSA (ng/mL) – mean (range)	0.98 (0.3-1.7)	0.76 (0.01-3.0)	0.35
Patients with Familial History	4 (25.0)	09 (26.47)	---
Classification of the Skin Color:			
• **Black – N (%)**	0 (0)	16 (47.05)	0.001
• **Brown – N (%)**	0 (0)	11 (32.35)	0.001
• **White – N (%)**	16 (100)	07 (20.60)	0.001
Smokers – N (%)	0 (0)	09 (26.47)	0.001
Alcohol Drinkers - N (%)*	0 (0)	08 (23.52)	0.001
% CTCs – Mean (Range)	0.51 (0.07-0.90)	8.64 (0.13-25.8)	0.001
% CTCs em Smokers – mean (sd)	---	11.05 (8.82)	0.61
% CTCs em Non-Smokers – mean (sd)	---	7.77 (6.66)	
% CTCs de Alcohol Drinkers – mean (sd)	---	5.47 (5.57)	0.16
% CTCs de Non-Alcohol drinkers – mean (sd)	---	9.61 (7.58)	
Naïve-Treament Patients (N)	---	03	---
Patients under Different Therapeutic Regimens (N)	---	31	---
Gleason Score – N (%):			
• **6 (3+3)**	---	14 (41.17)	---
• **7 (3+4)**	---	08 (23.53)	---
• **7 (4+3)**	---	05 (14.70)	----
• **8 (4+4)**	---	03 (8.82)	---
• **9 (4+5 e 5+4)**	---	04 (11.76)	---
Disease Staging – N (%):			
• **T1**	---	02 (5.88)	---
• **T2a**	---	05 (14.70)	---
• **T2b**	---	06 (17.65)	---
• **T3a**	---	08 (23.53)	---
• **T3b**	---	04 (11.76)	---
• **T4**	---	9 (26.64)	---

* Alcohol Drinking (daily habit);

Table 2. Diagnostic parameters for detection of circulating tumor cells (CTCs) in prostate cancer according to the A4-aptamer labeling and detection by flow cytometry, using a *cutoff* of 1% of CTCs.

Diagnostic Parameters	*Value (%)*
Sensitivity	97%
Specificity	100%
Positive Predictive Value	100%
Negative Predictive Value	93.75%
Accuracy	98%

Figure Legends

Figure 1. Procedure for detection of circulating tumor cells (CTCs) in the peripheral blood of patients using the A4-aptamer-based flow cytometry analysis. A. Sample preparation and processing. B. Examples of positive (tumor patient) and negative (control) results of the flow cytometry analyses.

Figure 2. Percent of circulating tumor cells (CTCs) detected by the A4-Aptamer through flow cytometry for prostate cancer (PCa) diagnosis. The cutoff establishment based on results observed in controls. The *cutoff* was considered positive for PCa when CTC levels were greater than 1.0%.

Figure 3. Correlation analysis between the percentage of circulating tumor cells (CTCs) and patients' age according to the A4-aptamer detection by flow cytometry. The regression equation and the tendency line are presented. The age ranges observed for prostate cancer patients (PCa) and controls were respectively, 55 - 92 years and 28 - 44 years.

Figure 4. Correlation analyses between serum PSA levels and the percentage of circulating tumor cells (CTCs) according to the detection of the A4-aptamer by flow cytometry. Regression equations and tendency lines are presented. A. Correlation analysis with all patients, including *outliers* (red circle), considered as biochemical failure of serum PSA. B. Correlation analysis excluding *outliers*.

Figure 5. Analysis of the minimal residual disease based on detection of circulating tumor cells (% CTCs) in prostate cancer patients under different therapeutic regimens. (Y=yes, and N-no).

Figure 6. Analysis of minimal residual disease according to the percentage of circulating tumor cells (CTCs) and PSA levels, according to treatment time (months). (A) Percent

CTCs, and (B) serum PSA levels of prostate cancer patients. Red-dotted lines in both graphics represent the cutoff values, which are 5% for CTCs and 0.2ng/dL for serum PSA levels.

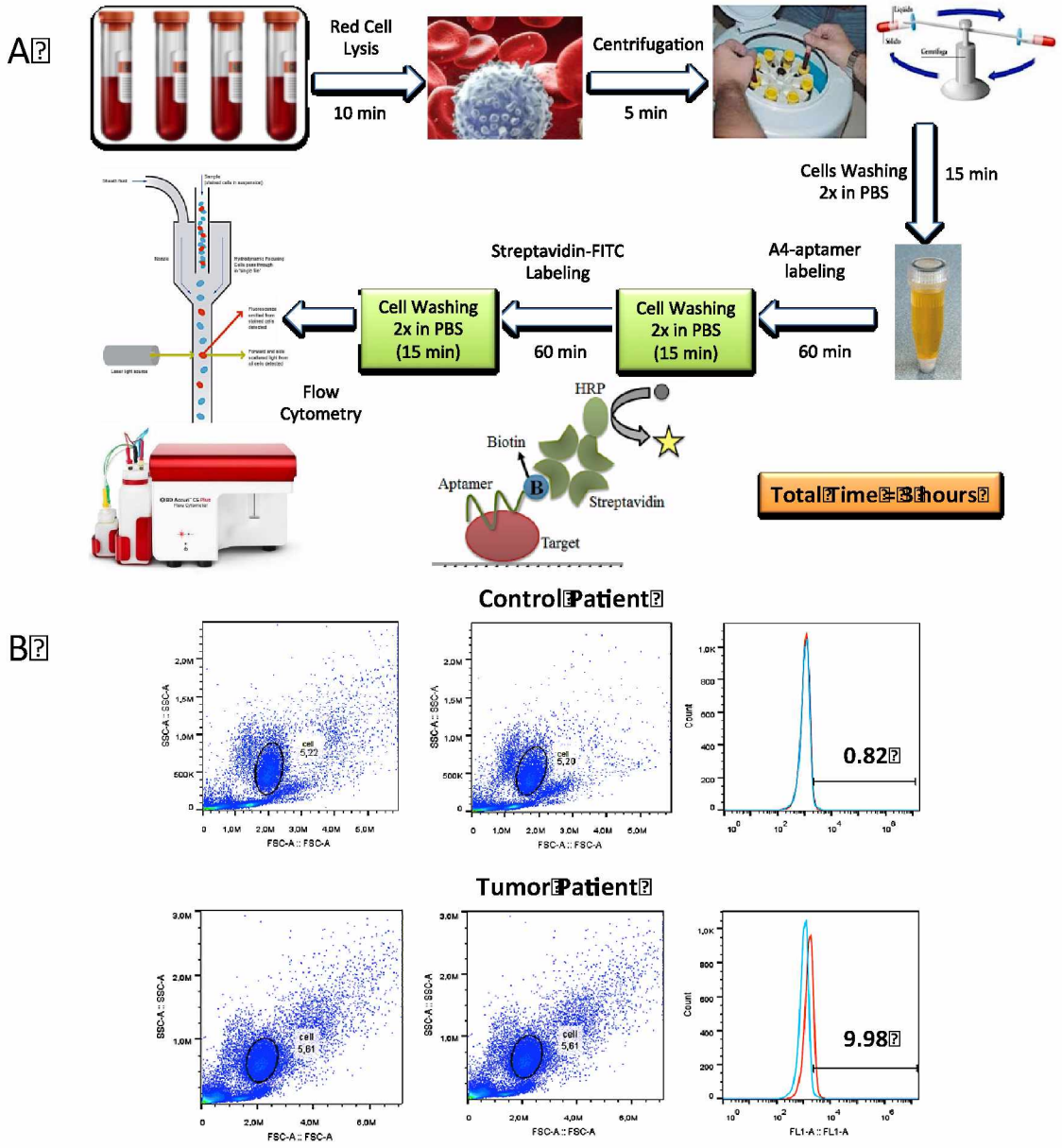
A
Red Cell Lysis
10 min
Centrifugation
5 min
Cells Washing 2x in PBS
15 min
A4-aptamer labeling
60 min
Cell Washing 2x in PBS (15 min)
Streptavidin-FITC Labeling
60 min
Cell Washing 2x in PBS (15 min)
Flow Cytometry
HRP
Biotin
Aptamer
Streptavidin
Target
Total Time = 3 hours
B
Control Patient
0.82
Tumor Patient
9.98

Figure 1

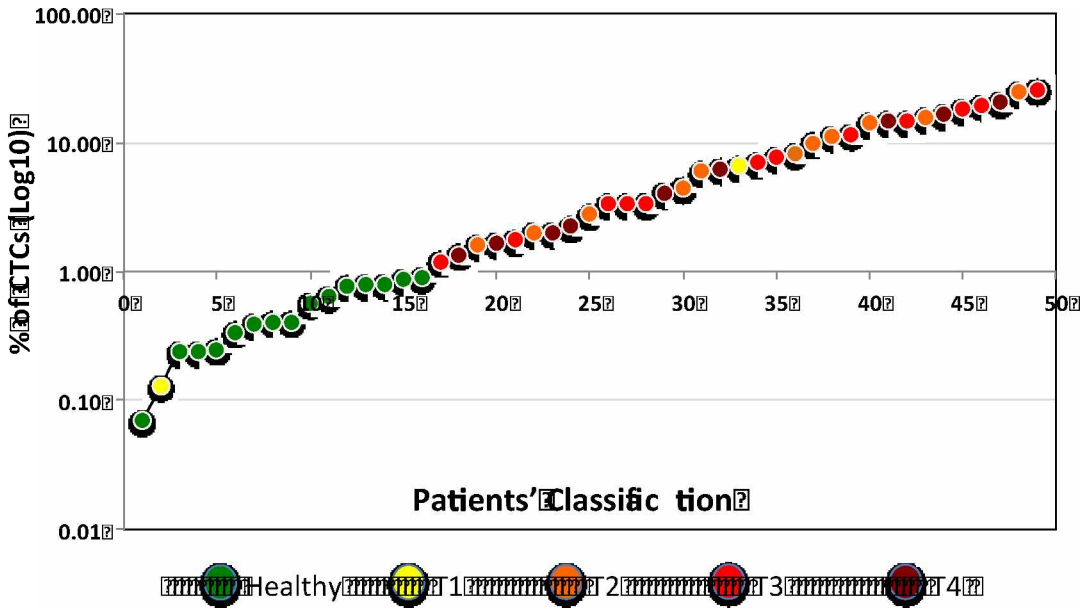
100.00
10.00
1.00
0.10
0.01
0
5
10
15
20
25
30
35
40
45
50
Patients' Classification
Healthy
T1
T2
T3
T4

Figure 2

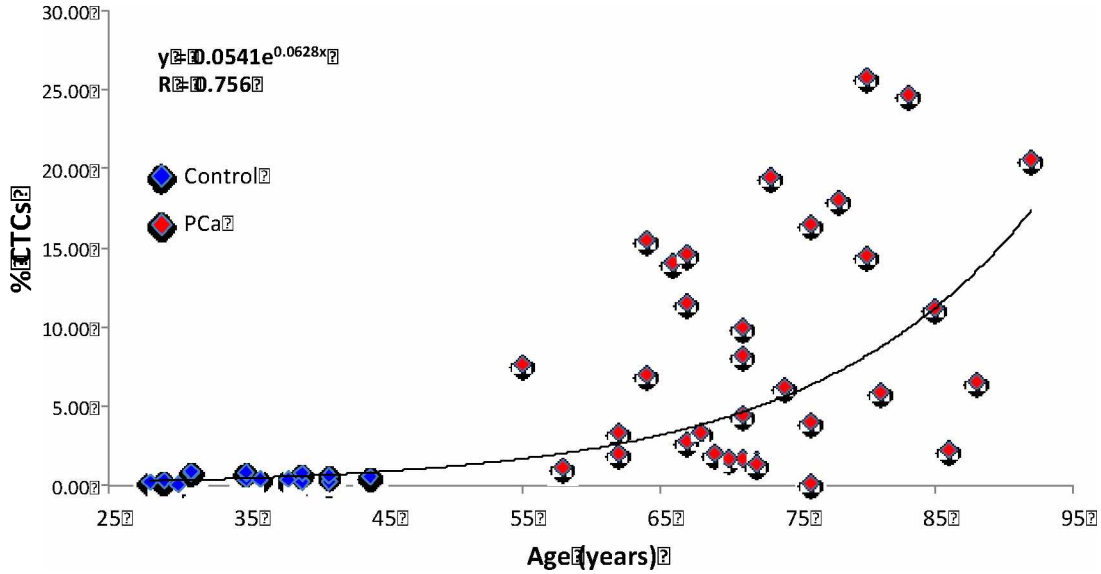
y = 0.0541e^0.0628x
R = 0.756
Control
PCa
% CTCs
Age (years)
30.00
25.00
20.00
15.00
10.00
5.00
0.00
25
35
45
55
65
75
85
95

Figure 3

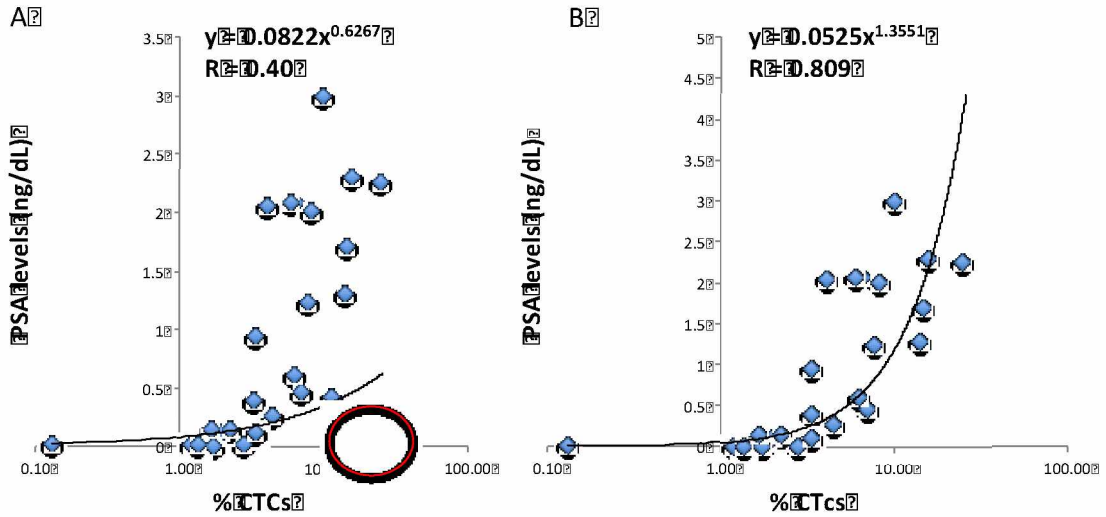
A
y = 0.0822x^0.6267
R² = 0.40
PSA levels (ng/dL)
% CTCs
0.10
1.00
10
100.00
B
y = 0.0525x^1.3551
R² = 0.809
PSA levels (ng/dL)
% CTcs
0.10
1.00
10.00
100.00

Figure 4

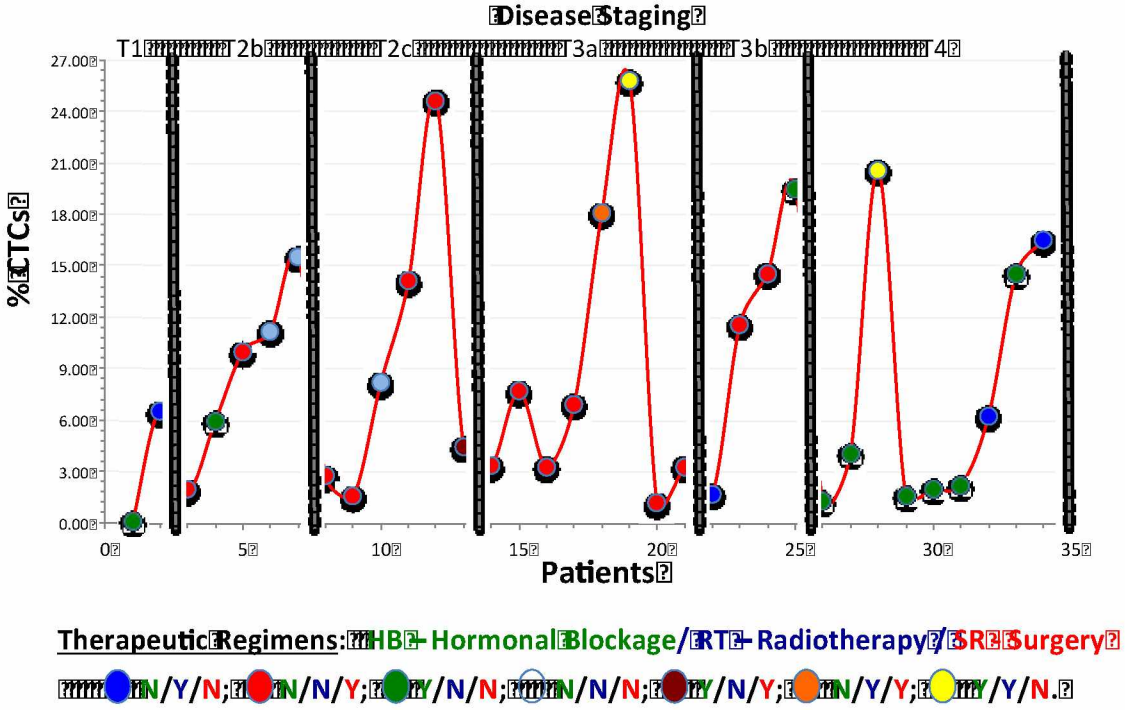
Disease Staging
T1
T2b
T2c
T3a
T3b
T4
27.00
24.00
21.00
18.00
15.00
12.00
9.00
6.00
3.00
0.00
0
5
10
15
20
25
30
35
Patients
Therapeutic Regimens: HB - Hormonal Blockage/ RT - Radiotherapy/ SR Surgery
N/Y/N; N/N/Y; Y/N/N; N/N/N; Y/N/Y; N/Y/Y; Y/Y/N.

Figure 5

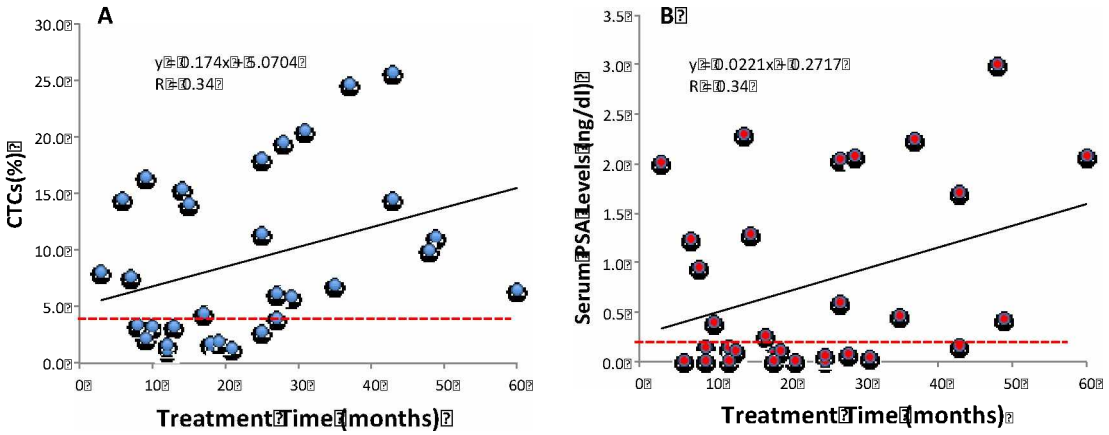
A
y = 0.174x + 5.0704
R² = 0.34
CTCs(%)
Treatment Time (months)
B
y = 0.0221x + 0.2717
R² = 0.34
Serum PSA Levels (ng/dl)
Treatment Time (months)

Figure 6

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