



**UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA**

**CARACTERIZAÇÃO DOS APTÂMEROS D4 E R4 COMO FERRAMENTAS
TERANÓSTICAS NO CÂNCER DE PRÓSTATA**

Aluna: Esther Campos Fernández

Orientadora: Profa. Dra. Vivian Alonso Goulart

UBERLÂNDIA - MG

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ALUNA: Esther Campos Fernández

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Aos vinte e cinco dias do mês de novembro de dois mil e vinte e dois, às 08:00 horas, reuniu-se via web conferência pela Plataforma Cisco Webex, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Resolução de nº06/2020 e Resolução nº 19/2022 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Dr. João Bosco Pesquero, Dr^a. Adriana Freitas Neves, Dr^a. Thaise Gonçalves de Araújo, Dr. Nilson Nicolau Junior e Dr^a. Vivian Alonso Goulart, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Dr^a. Vivian Alonso Goulart apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

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Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do

Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



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DEDICATÓRIA

Ao meu pai Dani (†): a minha primeira saudade..., o meu primeiro porquê..., o meu impulso..., a minha força diária..., o meu companheiro inconsciente de tantos e tantos dias...

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À minha família: minha mãe Basi, meu pai Rober, minha irmã Julia, meus avós Víctor (†) y Julia, meus tios Gerardo y Elvira, Carmina y José Domingo e meus primos Víctor, Andrea y Raquel. O que dizer? Eles já sabem!

Aos meus amigos daqui, dali e de lá por dividirem momentos intensos e simples, conversas banais e profundas, afinal experiências irrepetíveis; e por me acolherem em outros tantos momentos não tão idílicos.

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APRESENTAÇÃO

O câncer de próstata (CaP) é uma das neoplasias malignas mais incidentes e a sobrevida dos pacientes depende do estadiamento da doença no momento que é feito o diagnóstico. Quase 90% dos pacientes diagnosticados com CaP localizado sobrevivem após cinco anos. Entretanto, menos de 30% dos pacientes diagnosticados com CaP metastático sobrevivem nesse período. Portanto, um diagnóstico precoce reduziria a mortalidade e melhoraria a qualidade de vida dos pacientes com CaP.

A suspeita da presença de CaP parte de dois procedimentos: a detecção de nódulos indicativos de tumores malignos pelo toque retal e a dosagem sérica >4 ng/mL do antígeno prostático específico ou PSA (do inglês *prostate-specific antigen*). Esses exames podem ainda ser complementados por análises de imagem, como a ultrassonografia. Desse modo, a suspeita de CaP é confirmada pela análise histopatológica de uma biópsia tecidual que determina o diagnóstico.

Contudo, o diagnóstico atual conta com uma elevada taxa de falsos negativos. No caso dos procedimentos de toque retal e biópsia, eles não avaliam o volume total da próstata. No toque retal apenas é examinada a parte posterior da glândula prostática. Na biópsia, os fragmentos extraídos podem apresentar um erro de amostragem no qual as células cancerígenas não se encontram no tecido biopsiado. Indivíduos com dosagem normal de PSA também podem apresentar a doença, não sendo diagnosticados e contribuindo à taxa de falso-negativos. Consequentemente, o diagnóstico desses pacientes acontece posteriormente, em um estado mais avançado do câncer. Esse diagnóstico tardio diminui a efetividade do tratamento e a sobrevida do paciente.

Por outra parte, o PSA não é específico do CaP, pois outros órgãos afetados ou não por tumores produzem PSA. Além disso, outras condições da próstata como prostatites ou hiperplasias prostáticas benignas podem aumentar os níveis de PSA sérico. Por conseguinte, níveis elevados podem apresentar resultados falso-positivos, sendo indicativos de CaP na ausência dessa doença. Assim, muitos pacientes podem ser sobrediagnosticados, o que leva ao seu sobretratamento. O sobretratamento costuma acarretar procedimentos cirúrgicos agressivos como a prostatectomia radical, que pode provocar disfunção erétil, incontinência urinária e infecção, entre outras consequências.

Ainda, alguns homens que passam pela cirurgia desenvolvem recidiva bioquímica durante os primeiros 10 anos, apresentando valores de PSA >0,20 ng/mL. Antes ou durante a cirurgia, células tumorais da próstata podem espalhar-se desde o local primário do tumor a outras partes do corpo. Essas células tumorais são transportadas através do sistema circulatório ou linfático e são conhecidas como células tumorais circulantes (CTCs). As CTCs são capazes de extravasar e invadir diferentes órgãos. Nesse momento, as CTCs são chamadas de células tumorais disseminadas (DTCs, do inglês *disseminated tumor cells*). As DTCs podem ficar dormentes por um tempo indeterminado ou darem lugar a células iniciadoras de metástase (MICs, do inglês *metastases-inducing cells*), que proliferam originando uma micrometástase que produz PSA. A presença dessas micrometástases é conhecida como doença residual mínima.

Devido às falhas no diagnóstico e tratamento dos pacientes com CaP, essa doença causa grandes perdas econômicas aos sistemas de saúde. Assim, novas estratégias estão em contínuo desenvolvimento para resolver esse problema de saúde pública e melhorar a sobrevida e a qualidade de vida dos pacientes com CaP. Nesse sentido, sistemas mais específicos para o reconhecimento de biomarcadores de CaP possibilitariam um diagnóstico mais precoce e um estadiamento mais preciso, diminuindo a mortalidade e morbidade dos pacientes.

Partindo do fato de que os tumores liberam componentes como células, ácidos nucleicos, proteínas e vesículas extracelulares na linfa e no sangue, o uso desses componentes como biomarcadores tumorais detectados em biópsias líquidas seria uma proposta plausível para a melhora do manejo clínico de pacientes oncológicos.

As biópsias líquidas são amostras de fluídos corporais que requerem procedimentos de coleta menos invasivos por se tratar de amostras mais acessíveis, o que favorece a preferência dos pacientes por esse tipo de técnica. Por se basearem na detecção de componentes liberados pelo tumor e não de áreas teciduais acometidas, as biópsias líquidas representam melhor o estado global do câncer no indivíduo, podendo detectar o início dos processos metastáticos de tumores primários e inclusive de secundários.

Entre as biópsias líquidas, a amostra de sangue é a mais usada na prática clínica atual e as CTCs são o componente tumoral sanguíneo mais estudado até a data no CaP. A citometria de fluxo é uma das técnicas de detecção de CTCs mais usada e é baseada

na análise de expressão de antígenos de superfície que serve tanto para quantificar as CTCs, quanto para medir o nível de expressão dos antígenos de superfície. As CTCs já têm sido estudadas como biomarcadores tumorais de diagnóstico, prognóstico, monitoramento de resposta a determinados tratamentos, e rastreamento de doença residual mínima para prever recidivas em vários tipos de câncer. A citometria de fluxo utiliza comumente anticorpos marcados com agentes fluorescentes que ligam especificamente a antígenos das células de interesse.

Porém, existe uma alternativa ao uso de anticorpos que é o uso de aptâmeros. Os aptâmeros apresentam uma função equivalente, pois também são capazes de ligar especificamente a um determinado antígeno. A diferença dos anticorpos é que são glicoproteínas, enquanto os aptâmeros são geralmente oligonucleotídeos ou também peptídeos. Assim, devido ao menor tamanho dos aptâmeros, a afinidade de ligação é maior porque apresentam uma maior acessibilidade aos seus alvos e ainda esses alvos podem ser de menor tamanho. Além disso, os anticorpos são produzidos a partir dos linfócitos B de animais e apresentam um custo maior para síntese. Já os aptâmeros são sintetizados quimicamente a partir de bibliotecas randômicas de oligonucleotídeos expostos ao alvo em um processo iterativo chamado de SELEX (do inglês *Systematic Evolution of Ligands by EXponential enrichment*). Pelo fato dos aptâmeros não serem de origem animal, eles não são imunogênicos e sua produção é mais rápida, apresenta uma grande reprodutibilidade e permite modificações químicas que podem melhorar seu desempenho e ampliar suas funções. Por exemplo, um aptâmero específico de CaP acoplado a uma droga ou a um agente de contraste pode não apenas identificar seu alvo, mas também administrar um tratamento ou permitir a visualização *in vivo* dos tumores quando administrado a um paciente. Consequentemente, os aptâmeros têm um grande potencial para serem usados em sistemas de reconhecimento de biomarcadores que possibilitem uma melhor teranóstica, neologismo originário da junção das palavras terapia e diagnóstico.

Aptâmeros de RNA ligantes de células de CaP foram selecionados contra a linhagem humana de células prostáticas tumorais PC-3 por nosso grupo de pesquisa em 2016. A partir de um dos aptâmeros selecionados, o aptâmero A4, foram desenhados aptâmeros menores de DNA e RNA e denominados D4 e R4, respectivamente. Com o intuito de

validar o uso dos aptâmeros D4 e R4 em sistemas de detecção de CTCs prostáticas para auxiliar sobretudo no diagnóstico de CaP a partir de amostras de sangue periférico, esse trabalho apresenta a caracterização funcional de D4 e R4 e a identificação dos seus potenciais alvos moleculares.

Desta maneira, esta tese está dividida em três capítulos. O Capítulo I apresenta uma revisão sistemática com uma descrição abrangente das aplicações de aptâmeros na área de CaP durante a última década para compreender melhor seus avanços e perspectivas. A revisão sistemática já foi publicada em 2021. O Capítulo II apresenta o manuscrito de um artigo científico sobre a caracterização *in silico*, *in vitro* e *ex vivo* dos aptâmeros D4 e R4 para determinar suas utilidades clínicas no contexto do CaP. O Capítulo III inclui o conteúdo da patente intitulada SISTEMA APTAIMUNOLÓGICO PARA A DETECÇÃO DE CÂNCER DE PRÓSTATA, que foi publicada em 2022 (BR102020013625-9 A2 e PTC/BR2021/050290) e desenvolvida durante esse doutorado. A patente foi apresentada para a homologação da qualificação desse doutorado.

CAPÍTULO I – ARTIGO DE REVISÃO

Título

O uso de aptâmeros no câncer de próstata: uma revisão sistemática das aplicações teranósticas

Resumo

Uma vez que o câncer de próstata (CaP) depende de diagnósticos e terapias limitados, alternativas mais eficazes são necessárias. Os aptâmeros são ferramentas versáteis que podem ser aplicadas para um melhor manejo clínico dos pacientes com CaP. Esta revisão mostra as tendências nas aplicações baseadas em aptâmeros para CaP para entender seu futuro desenvolvimento. Buscamos artigos sobre aptâmeros aplicados em CaP nas bases de dados *Pubmed*, *Scopus* e *Web of Science* publicados na última década. Quase 80% dos artigos usaram aptâmeros previamente selecionados em novas abordagens. No entanto, o cell-SELEX foi a técnica mais aplicada para a seleção de novos aptâmeros permitindo sua ligação a alvos em sua configuração nativa. Os aptâmeros de ssDNA foram 24% mais comuns do que os aptâmeros de RNA. Os aptâmeros específicos de PCa mais estudados foram o aptâmero de DNA específico de PSA PSap4 # 5 e os aptâmeros de RNA específicos de PSMA A10 e A9, sendo PSA e PSMA os alvos mais relatados. Portanto, os pesquisadores ainda preferem a facilidade de uso dos aptâmeros de DNA. As biópsias líquidas de sangue representaram 24% de todas as amostras, sendo as amostras clínicas mais promissoras. Especialmente notáveis, os métodos eletro-analíticos foram responsáveis por mais de 40% das técnicas de diagnóstico e os tratamentos com sistemas de liberação de drogas ou modificadores transcricionais foram relatados em 70% dos artigos. Embora todos esses artigos tenham mostrado aptâmeros clinicamente relevantes para CaP e haja boas perspectivas para seu uso, o desenvolvimento dessas estratégias estava em seus estágios iniciais. Assim, os aptâmeros não estão totalmente validados e prevemos que a conclusão dos estudos clínicos permitirá a implementação dessas tecnologias baseadas em aptâmeros na prática clínica do CaP.

Palavras chave: câncer de próstata; aptâmeros; teranóstica; diagnóstico; biossensores; nanotecnologia.

Title

The use of aptamers in prostate cancer: a systematic review of theranostic applications

Abstract

Since prostate cancer (PCa) relies on limited diagnosis and therapies, more effective alternatives are needed. Aptamers are versatile tools that may be applied for better clinical management of PCa patients. This review shows the trends on aptamer-based applications for PCa to understand their future development. We searched articles reporting aptamers applied in PCa on the Pubmed, Scopus and Web of Science databases over the last decade. Almost 80% of the articles used previously selected aptamers in novel approaches. However, cell-SELEX was the most applied technique for the selection of new aptamers allowing their binding to targets in their native configuration. ssDNA aptamers were 24% more common than RNA aptamers. The most studied PCa-specific aptamers were the DNA PSA-specific aptamer PSap4#5 and the PSMA-specific RNA aptamers A10 and A9, being PSA and PSMA the most reported targets. Thus, researchers still prefer the ease of use of DNA aptamers. Blood-based liquid biopsies represented 24% of all samples, being the most promising clinical samples. Especially noteworthy, electro-analytical methods accounted for more than 40% of the diagnostic techniques and treatment approaches with drug delivery systems or transcriptional modifiers were reported in 70% of the articles. Although all these articles showed clinically relevant aptamers for PCa and there are good prospects for their use, the development of all these strategies was in its early stages. Thus, the aptamers are not completely validated and we foresee that the completion of clinical studies will allow the implementation of these aptamer-based technologies in the clinical practice of PCa.

Keywords: prostate cancer; aptamers; theranostics; diagnostics; biosensors; nanotechnology.

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Title

The use of aptamers in prostate cancer: a systematic review of theranostic applications

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Abstract

Since prostate cancer (PCa) relies on limited diagnosis and therapies, more effective alternatives are needed. Aptamers are versatile tools that may be applied for better clinical management of PCa patients. This review shows the trends on aptamer-based applications for PCa to understand their future development. We searched articles reporting aptamers applied in PCa on the Pubmed, Scopus and Web of Science databases over the last decade. Almost 80% of the articles used previously selected aptamers in novel approaches. However, cell-SELEX was the most applied technique for the selection of new aptamers allowing their binding to targets in their native configuration. ssDNA aptamers were 24% more common than RNA aptamers. The most studied PCa-specific aptamers were the DNA PSA-specific aptamer PSap4#5 and the PSMA-specific RNA aptamers A10 and A9, being PSA and PSMA the most reported targets. Thus, researchers still prefer the ease of use of DNA aptamers. Blood-based liquid biopsies represented 24% of all samples, being the most promising clinical samples. Especially

noteworthy, electro-analytical methods accounted for more than 40% of the diagnostic techniques and treatment approaches with drug delivery systems or transcriptional modifiers were reported in 70% of the articles. Although all these articles showed clinically relevant aptamers for PCa and there are good prospects for their use, the development of all these strategies was in its early stages. Thus, the aptamers are not completely validated and we foresee that the completion of clinical studies will allow the implementation of these aptamer-based technologies in the clinical practice of PCa.

Keywords: prostate cancer, aptamers, theranostics, diagnostics, biosensors, nanotechnology.

1. Introduction

Prostate cancer (PCa) is the second most frequent malignancy after lung cancer among men worldwide. In 2018, 1,276,106 new cases of PCa and 358,989 deaths related to PCa were reported. This mortality rate of PCa corresponds to 3.8% of all deaths caused by cancer in men in that year [1].

Incidence and mortality of PCa across populations and over time provide information about individual risk factors and population screening [2]. Risk factors for PCa are age, family history, ethnicity and inherited genetic factors. The incidence rates for African-American men are higher when compared to Caucasian men, with 158.3 new cases diagnosed per 100,000 men and their mortality is approximately twice as much [3,4].

Currently, PCa diagnosis is made by measuring serum prostate specific antigen (PSA), which is a serine protease also known as kallikrein-3 (KLK3) and secreted by cells in the prostatic epithelium in different situations such as PCa, hyperplasia, prostatitis, at advanced age, after surgical interventions, ejaculation or hormone treatments [5]. PSA measurement is applied in PCa screening and monitoring of this disease progression, considering positive values for PCa the ones above 4.0 ng/mL [6]. This cutoff usually leads to false positives that may bring about unnecessary biopsies, overdiagnosis and overtreatment [7].

Since PSA is not exclusive of the PCa condition, other biomarkers are being used in tests to determine whether a biopsy is necessary after finding high levels of PSA, helping to reduce unnecessary biopsies. Prostate health index is a test that combines three forms of PSA: total PSA, free PSA and an inactive proenzyme form ([-2] pro-PSA). Similarly, the 4K score test measures four prostate-derived biomarkers: total PSA, free PSA, inactive PSA and human kallikrein 2. Both tests are performed on blood samples. Other assays have been introduced into the clinical practice, such as prostate cancer antigen 3 (PCA3), TMPRSS2-ERG, ExoDx and SelectMDx [8]. Some tests are applied after biopsy. For instance, Oncotype DX, which searches for 12 cancer-related genes; ProMark, which measures the expression of eight proteins for prognosis and ConfirmMDx, which investigates DNA methylation. Few tests utilize circulating tumor cells (CTCs). The CellSearch system uses magnetic nanoparticles and antibodies to target epithelial

adhesion molecules on CTCs for their enumeration and the Adna test analyzes CTC gene expression by reverse transcription polymerase chain reaction (RT-PCR) [7,9].

Advances in our understanding of the epidemiology, diagnosis, grading, staging, imaging and management of PCa have changed clinical practice and influenced guideline recommendations [10]. The heterogeneous nature of PCa has encouraged more personalized treatment strategies to boost positive clinical outcomes [9]. However, serum PSA and histopathological examination of tumor biopsies are still the gold standards for PCa diagnostics and monitoring response to treatment.

As the search for more specific biomarkers and more precise techniques that improve diagnosis, prognosis and prediction of treatments for PCa continues, targeted theranostic (diagnostic and therapeutic) agents with PCa-specific affinity molecules are promising approaches. Monoclonal antibodies are widely available affinity molecules in the healthcare sector. Nonetheless, chemical antibodies known as aptamers show advantages compared to them. They both have a comparable affinity in the nanomolar range, but aptamers show no immunogenicity, being safer for clinical practice. Aptamers are more easily, quickly and economically produced due to their chemical synthesis and modification, which also yield no inter-batch variability. Aptamers are highly stable in extreme conditions, which guarantees a higher shelf life and easier storage and transport. Their nanometric size allows them to interact with a wide variety of targets, from inorganic molecules to whole cells and to penetrate tissues and internalize into cells. Furthermore, they can be screened in the original biological conditions without prior knowledge of the target molecule, thus allowing the discovery of unknown biomarkers [11].

Aptamers are short single-stranded DNA (ssDNA) or RNA molecules, or short chains of amino acids that bind to target molecules with high affinity thanks to their particular three-dimensional structures, which allow them to form stable and specific complexes with their target molecules [12]. Thirty years ago, Tuerk and Gold published a paper where they selected the first nucleic acids by the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technique [13]. Shortly after, Ellington and Szostak also published a paper where they selected nucleic acids by this technique and called them aptamers [14]. SELEX consists of sequential rounds of selection and amplification steps. An initial synthetic oligonucleotide or peptide library of millions of random sequences is screened

after iterative incubations with the target of interest in increasingly stringent conditions. Unbound sequences are removed after every round resulting in the amplification of increasingly specific binding molecules that are sequenced at the end of the process [15]. Although aptamers have been known for three decades and aptamer-based drugs have been evaluated in clinical trials, Pegaptanib is the only aptamer commercialized until now. Macugen is its commercial name and its use is approved for the treatment of wet age-related macular degeneration in the United States, Europe, Canada, Brazil and Australia [16]. Therefore, there are some impediments that prevent aptamers from reaching the market. The need for better clinical approaches for PCa makes aptamers very promising candidates as theranostic tools in this field. Thus, we aimed to identify trends in the use of aptamers for the clinical practice of PCa to understand them and predict future tendencies in this area.

2. Materials and methods

2.1 Literature search in PubMed, Scopus and Web of Science

We performed a bibliographic search on three databases: PubMed, Scopus and Web of Science. The search in each database was made using the same keywords and filters. The key words used for these searches were “aptamer” AND “prostate cancer” AND (“prognosis” OR “diagnosis” OR “treatment”). The searches were limited by the type of articles that were journal articles (and clinical trials when available); the data, from 2010 to August 29th, 2019; and the language that was English. The articles obtained from these three databases were retrieved for further analysis.

2.2 Exclusion and inclusion criteria

Duplicate articles in the databases were excluded and then, titles and abstracts were analyzed to verify whether they met our filters for type of articles, data and language, and our eligibility criteria: articles published in journals with an impact factor greater or equal to two, evaluating aptamer performance and reporting aptamers specific for PCa biomarkers. Papers that did not meet the criteria were excluded and the remaining ones were further assessed from their full texts to ensure that our criteria were met. The selected papers were read by two authors who filled in an excel table with data from the

articles. Discrepancies between these authors were solved by means of a discussion with a third one.

2.3 Questions for the quality assessment of retrieved articles

The articles included from PubMed, Scopus and Web of Science databases were analyzed according to their quality standards by nine questions defined by the CASP Qualitative Checklist (questions 1, 2, 6, 7, 8 and 9) [17], the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) (question 4) [18] and the Cochrane Handbook for Reviews of Diagnostic Test Accuracy (questions 5 and 9) [19]. The third question (3) was added by us to appraise the methodologies used in the articles. For each retrieved article, the questions were answered by two authors who filled in an excel table with the answers “no”, “yes” or “unclear”. Discrepancies between these authors were solved by means of a discussion with a third one.

The questions are listed as follows: 1. Was there a clear statement of the aims of the research? 2. Was the research design appropriate to address the aims of the research? 3. Did the study use appropriate and relevant materials such as biological samples or *in vivo* models? 4. Was the execution of the index test described in sufficient detail to permit replication of the test? 5. Did the study provide a clear definition of what was considered to be a positive result? 6. If necessary, have ethical issues been taken into consideration? 7. Was the data analysis sufficiently rigorous? 8. Is there a clear statement of findings? 9. Was the study free of commercial funding?

2.4 Data extraction

The following data were extracted from the articles that we included in our analysis for their descriptive analysis and comparison in terms of percentage: name and type of aptamers, aptamer refinements, origin of aptamers, target biomarkers, clinical applications, samples and assays used, and diagnostic techniques and type of treatments with their corresponding administration route (Table 1).

3. Results and discussion

3.1 Retrieved articles

Initially, 258 articles were found. After eliminating duplicates and reading and analyzing the other ones, 199 articles were found not to fit our eligibility criteria and were excluded (Figure 1). The remaining 59 articles were analyzed to extract data for their evaluation (Table 1).

3.2 Quality of the selected articles

Taking into account the total number of answered questions about the quality of the selected studies, almost three quarters of them were answered positively, 20% of questions received an unclear answer and only 2% a negative answer to the established criteria. Four percent of the answers were not applicable due to question number five (5), which was specific to articles reporting aptamers for diagnostic approaches. Regarding the answers for each quality question, around 45% of the articles did not state clearly the aims of the research, but all the articles showed an appropriate experimental design. Forty-two percent of the articles did not use relevant materials such as biological samples to test their hypotheses or theranostic platforms. Most of the articles explained the index test in detail. When we analyzed the definition of a positive result for those articles testing diagnostic approaches, more than a half failed to report a cut off value and a few of others showed a value, but did not specify it properly. More than one in five studies did not consider ethical issues when biological samples were used. We considered that around 23% of the papers reported insufficiently rigorous analyses of their data and that in 12% of them, the results section was poorly described, resulting in an uncertain analysis of data. The majority of the articles displayed clearly its findings. However, more than 10% of the articles were funded by companies being susceptible to a potential conflict of interests (Figure 2).

3.3 Origin of aptamers

Seventy-eight percent of the analyzed studies reported chemical synthesis of aptamers, since they were previously selected and reported in other papers (Figure 3). The Cell-SELEX method, which was reported by eight percent of the papers [20–24], involves a cell target instead of a protein target and may be used to identify aptamers that internalize into specific cells for targeted therapy. Conventional SELEX (protein-based SELEX) appeared in seven percent of the research studies [25–28]. Two articles used a proprietary SELEX technique called SOMAscan [28,29]. One article applied the yeast

two-hybrid method to select a peptide aptamer that bound to and suppressed the androgen receptor (AR) protein [30]. Another one used complementarity of sequences to design an aptamer against the genomic androgen response element sequence [31]. Computational techniques such as genetic algorithms may be included after the selection process to optimize aptamers in a kind of maturation process, as in the case of the aptamer PSap4#5 [32]. Our results corroborate the fact that the most common aptamer selection methods are protein- and cell-SELEX techniques [11]. The fact that there is no need to know the target molecule and its more native conformation – the one found in nature – on the cell-SELEX process may have made this technique more common in theranostic aptamer-based applications in general and particularly in PCa. In addition, most of the aptamers from the retrieved studies were not selected in the reported articles. That indicates that aptamers are being used as targeting molecules in more sophisticated tools that are under development.

3.4 Types of aptamers

In the selected articles, the use of oligonucleotide aptamers is predominantly 61% and 37% of DNA and RNA, respectively. Both types of aptamers have similar characteristics, but differ in terms of target accessibility and chemical stability. RNA aptamers are chemically unstable due to the presence of a reactive hydroxyl group, which is deprotonated in alkaline solutions triggering their hydrolysis. DNA aptamers are less reactive and relatively more stable because of the absence of this group in deoxyribose sugar of DNA nucleotides, making their storage, handling and processing easier and more affordable. In addition, studies proved that DNA aptamers are more stable than RNA aptamers in serum samples, facilitating their use in clinical settings [33]. In terms of production and selection, DNA aptamers are faster and cheaper because the SELEX of RNA aptamers requires extra caution due to chemical instability and to the fact that amplification of RNA libraries requires extra steps after every round. For these reasons, DNA aptamers may be preferred in aptamer-based applications, including those for PCa.

One of the RNA aptamers was modified with a ssDNA fragment and could hence be considered as a hybrid DNA-RNA aptamer. This type of hybrid aptamer is used for the treatment against PCa because this modification allows RNA aptamers to bind to their targets as well as load nanoparticles (NPs) for drug delivery at the same time [34]. A

peptide aptamer was also used in the analyzed studies (2%). Researchers selected a short amino acid stretch that binds specifically to the human AR in yeast and mammalian cells and is fused to a silencing domain. This aptamer (PAB-CoR) exhibited corepressor activity by inhibiting both the AR-mediated transactivation and the expression of its target gene *PSA* [30].

Among the most reported aptamers, PSap4#5 was found in 21 articles [25,32, 35–53], followed by A10, in 16 articles [22,34, 54–66,79], A9, in five articles [67–71] and SOMAmers, in two articles [28,29]. PSap4#5 is a well-characterized DNA aptamer ligand of PSA. A10 and A9 are RNA aptamer ligands of the extracellular domain of the prostate specific membrane antigen (PSMA) that have been also used in some of their truncated forms such as A10-3, A10-3.2 and A9g, respectively [72]. PSMA is a specific biomarker for PCa that is overexpressed in PCa tissues. Its ability to internalize bound ligands has aroused an exceptional interest for PCa imaging and therapy [73]. SOMAmers are slow off-rate modified aptamers that form the affinity reagents of the proprietary assay SOMAscan for biomarker discovery [74]. Therefore, these aptamers may be the most promising ones for PCa due to their high characterization and wide application in several systems that are working on their improvement. Considering the natural occurring condition of RNA aptamers, the relevance of PSMA as a PCa target, and the improved affinity of A10 and A9 aptamers after their truncations, A10 and A9 aptamers stand out due to their performance in PCa theranostics.

3.5 Types of ligands and biomarkers

During the analysis of the selected articles for this review, we detected that most of them reported a target protein, while only 7% [20,21,24,27] reported target cells, namely LNCaP, 22Rv1, PC3 and DU-145, which are well-established human prostate cancer cell lines. The first one has a high androgen sensitivity, the second one has a low androgen sensitivity and the others are androgen non-reliant cell lines [20]. When it comes to studies that had a target protein, PSA was the most reported biomarker (46%) [8,25,26,32,37–59], maybe due to its extensive use in the current clinical practice of PCa (Table 1). Some of these studies combined PSA with other biomarkers such as epithelial cell adhesion molecule (EpCAM) expressed on the surface of exosomes [78] and vascular endothelial growth factor (VEGF), which promotes the formation of blood vessels

and has been studied to improve the precision of PCa diagnosis [45,50]. Other biomarkers combined with PSA were *P53* and *BRCA1* genes identified by probes. These genes are important in tumor suppressing and when mutated can be indicators of prostate tumor evolution [42].

PSMA was observed in 34% of the articles as a target protein [22,34,54,56–71,80]. PSMA is an integral membrane protein that became an attractive target for PCa after succeeding in the detection of metastatic PCa tumors in patients via immunoscintillography. Furthermore, PSMA exhibits a prominent extracellular domain and naturally internalizes bound ligands into the cells where it is expressed [72]. Three articles (5%) used the nuclear steroid receptor AR as a PCa biomarker, considering the important function of this receptor in the development of PCa [30,31,55]. Nucleolin is another nuclear biomarker, involved in cell proliferation and overexpressed in tumor cells, that was reported by one article [81]. Besides PSMA, other studies reported membrane proteins such as MUC1, an important biomarker for cancer detection related to metastasis mechanisms and overexpressed in blood [82], and integrin $\alpha 6\beta 4$, a receptor involved in tumor progression, invasion and metastasis [23]. As we mentioned before, two articles used the multiplex approach SOMAscan to detect a protein array as PCa biomarkers. This technique enabled the detection of a large number of proteins [28,29]. These two studies together with other two [67,78] used proteins expressed on exosomes as biomarkers (Figure 4).

3.6 Aptamer refinements: attachments and modifications

Aptamers displayed a high variety of refinements including modifications to their sequence and attachments to increase their functions and enhance their performance in theranostic applications. For example, tumor-associated endothelial cells display an enhanced permeability and retention effect for nanosized materials due to their poor alignment around tumor-associated blood vessels. Therefore, aptamers conjugated to nanomaterials are able to escape from blood circulation and accumulate into tumor tissues where they may also exert their theranostic action because of the ineffective lymphatic drainage of these tissues [83]. We divided this complex set of improvements into ten categories to facilitate their understanding: reporters, polymers, modified nucleobases, spacers, gold, macromolecules, hybridized oligonucleotides, solid oxides,

carbon allotropes and drugs. We further explain these aptamer refinements below, in order from the most to the least abundant according to the selected articles (Figure 5).

3.6.1 Reporters

Reporters for aptamer visualization and biomarker detection were the most common refinements (14%). They were mainly used in cell-based assays for preclinical diagnostic tests. Among them, fluorescent dyes were by far the most frequent, accounting for more than 70%. 6-Carboxyfluorescein (6-FAM) was the most used fluorescent dye. Redox probes represented 20% of the reporters [39,47,50,55,82] and were used for diagnostic strategies in aptasensors with methylene blue as the most used probe. Other types of dyes appeared only in one of the selected articles. For example, guanines were used as a chemiluminescent dye [45] and 1, 4-aminothiophenol (4-ATP) was used as a reporter in Raman spectroscopy [56] (Figure 6A).

3.6.2 Polymers

When we analyzed the aptamer refinements, polymers appeared in a high percentage (13%), which are macromolecules of repeated monomers linked together in a three-dimensional network. From our selected articles, we can state that polymers were able to decrease problems like toxicity, solubility, non-specific binding and colloidal, photothermal and serum instability of aptamer-targeted nanomaterials.

Polyethylene glycol (PEG) was the most reported polymer (41%) found in our study [24,27,54,58,59,63–65,80]. PEG is attached to the surface of nanocarriers to avoid nonspecific serum protein adsorption, which impairs the recognition of the specific target and promotes their clearance by the mononuclear phagocyte system. Therefore, this polymer increases both the circulation time of nanocarriers in blood and the chances of target identification [84].

Biodegradable polymers such as polylactic acid (PLA), polylactic-co-glycolic acid (PLGA) [24,54] and polycaprolactone (PCL) [63,65] were combined with PEG to form NPs with higher half-lives to encapsulate and control the release of therapeutic compounds, making 18% of the total of polymers. Since they are amphiphilic polymers, they spontaneously assemble into core-shell structures in water, which facilitates their production. Furthermore, their hydrophobic core allows a high load of insoluble drugs and

their hydrophilic shell provides steric protection [85]. Natural polymers such as the chitosan polysaccharide [21,25,49] and the silk fibroin protein [48] also represented 18% of the polymeric modifications and were used due to their biocompatible, biomechanical, biodegradable and loading properties.

Two studies (9%) used the polyamidoamine (PAMAM) dendrimer as a macromolecular vector for high loading of therapeutic agents in delivery systems [64] or high loading of reporters in diagnostic systems for signal amplification [25]. One study used polystyrene beads called Dynabeads as polymeric nanocarriers [45], another study applied quinone-based polymers as electrode coating [35], and another one applied a polyelectrolyte multilayer of polystyrene sulfonate (PSS) and polyallylamine hydrochloride (PAH) on silicon nanowires [44] (Figure 6B).

3.6.3 Modified nucleobases

Various chemical modifications have been introduced to prepare nucleic acids for biomedical applications, making 12% of the aptamer refinements in our study. When thinking about aptamer modifications, special attention is required to aptamers of RNA. RNA nucleotides are more prone to nuclease degradation and spontaneous hydrolysis due to their highly reactive 2'-hydroxyl group. Several modified nucleotides have been developed to stabilize RNA aptamers and still mimic their native counterparts [86].

Almost two thirds of the modified nucleotides had 2'-fluoro groups [20,22,26,34,57–59,62–66,68]. Fluorination is a particularly attractive modification because it can dramatically broaden the structural diversity of aptamers and enhance their affinity to targets [87]. Other 2'-hydroxyl substituents reported in the selected articles were 2'-O-methyl groups [24,68] and modified deoxyuridines (dU) such as 5-benzylaminocarbonyl-dU (BndU), 5-naphthylmethylaminocarbonyl-dU (NapdU), 5-tryptaminocarbonyl-dU (TrpdU) and 5-isobutylaminocarbonyl-dU (IbdU) [28,29]. Both of these substituents represented 10% of the modified nucleobases. The modified dUs are part of the SOMAmers, which have improved binding affinity and selectivity [88].

Inverted deoxythymidines are another type of chemical modifications of nucleotides that are incorporated at the ends of aptamers forming 5'-5' or 3'-3' -linked nucleotide caps. They are used mainly in therapeutic systems because they lead to 5'-5' or 3'-3' linkages,

which avoid their degradation by exonucleases in blood [88]. The selected articles only described 3' caps of inverted deoxythymidines, being 15% of the modifications [58,62,65] (Figure 6D).

3.6.4 Spacers

Nanocomposites need physical spacers of appropriate length to reduce the steric hindrance from surrounding molecules or nanomaterials. Thus, spacers allow adequate recognition of the target by aptamers attached to nanocomposites [89]. Among aptamer refinements, spacers represented 12% of them. Eighty-five percent of the spacers were alkanes of 6, 12 or 18 carbons [20,27,32,35,39,40,43,46,50,51,55,56,58,63–65,68] and 15% were oligonucleotides that were usually made of consecutively repeated nucleotides [59,71,79] (Figure 6C).

3.6.5 Gold

Gold (Au) is a versatile component of nanocomposites that appeared in the selected articles in several ways, including NPs (55%) [25,32,42,43,47,48,51,52,55,56,69], nanorods (NRs) (20%) [27,49,58,77] and as a component of electrodes (25%) [39,40,46,50,82]. Au nanomaterials are highly biocompatible and offer good conductive, colorimetric and nonlinear optical properties that enable the development of sensing probes for their application in biosensors, diagnostic imaging and therapeutic systems. In fact, 12% of the aptamer refinements were made with an Au nanostructure (Figure 6E).

Au nanomaterials act as fluorescence quenchers through fluorescence resonance energy transfer (FRET) based on the target-induced conformational change of aptamers [90]. Furthermore, Au NPs display tunable optical properties depending on their sizes and shapes. Their shapes have the most influence on their optical properties affecting the brightness of the nanocomposites. In this way, conjugation of aptamers to Au NPs enables the quantification of biomarkers providing a sensitive technique with an additional high spatial resolution for imaging [56]. In colorimetric assays, Au NPs have a size-dependent color change property from red (disperse particles) to blue (aggregates) that has been used to develop aptasensors [91]. Au NRs act in a very similar way to AuNPs and are usually applied to enhance electron transfer in aptasensors. In the case of Au

electrodes, their efficient electron transfer with aptamers triggers signal gains or losses after their target binding that allow biomarker detection [39].

3.6.6 Macromolecules

Macromolecules derived from natural sources are an excellent choice in terms of biocompatibility. Indeed, they were among the most used refinements for aptamers used in the PCa field (11%). Here, we considered as macromolecules those that were not included in organic polymers or oligonucleotides.

The coenzyme biotin was one of the two most applied types of macromolecules in our included articles, representing 32% of them [25,27,34,38,44,45]. Biotin interacts non-covalently with streptavidin tetramers. This high-affinity interaction has allowed the use of this complex in biotechnological applications to enhance targeted delivery of loads. Streptavidin-biotin complexes conjugated to aptamers preserve the integrity of aptamers in degrading environments [92]. For instance, when attached to the 5' or 3' end of aptamers in applications for human health solutions, they may avoid the activity of exonucleases decreasing the clearance rate of aptamers in blood circulation.

Lipids were the other most applied type of macromolecules (32%) in our study [21,24,58,59,63,65]. They were usually combined with aptamers to design targeted nanostructures for diagnostic imaging and therapeutic delivery such as phospholipid micelles or liposomes to encapsulate lipophilic drugs or imaging agents for their parenteral delivering and cellular uptake, improving the stability of the carrier system and slowing down its clearance rate.

Proteins were more than a quarter of the modifications of macromolecules. However, they formed a very diverse group. Each protein offered different functions. For example, atelocollagen [57] and antibody anti-EpCAM [70] were combined with different aptamers to enhance the targeting of bones and tumor epithelial cells, respectively. In order to inhibit castration-resistant PCa cell proliferation in a treatment strategy, a peptide aptamer was attached to other three protein modifications: a transduction domain to internalize into cells, a silencing domain to inhibit both the AR-mediated transactivation and the expression of its target gene *PSA*, and a transitory glutathione-S-transferase tag to allow its chromatographic purification [30].

Metal-organic compounds represented 10% of the macromolecules. They are large self-assembled conjugate units made of highly ordered crystalline materials with high porosity and large surface area. They bind to aptamers through non-covalent interactions and contain metal ions that offered redox, optical, electronic and magnetic characteristics suitable for biosensors. Their large volume allowed them to act as mass amplifiers that proportionally amplified detection signals, while their photo-induced electron transfer allowed them to be explored as fluorescence nanoquenchers [41,42], (Figure 6F).

3.6.7 Hybridized oligonucleotides

Of the total of aptamer refinements in our selected articles, 7% were additional hybridized oligonucleotides partially complementary to the aptamers. Forty-six percent of these hybridized oligonucleotides were non-coding RNAs applied in therapeutic platforms and 54% of them were ssDNAs acting as linkers of nanocomposites and aptamers or as key components of enzyme-free assays (Figure 6G).

Non-coding RNAs were hairpin-structure molecules delivered into target cells thanks to their conjugation to internalizing aptamers, whose binding to molecular targets enabled their endocytosis. Then, non-coding RNAs regulated the expression of target genes by the RNA activation process with small activating RNAs (saRNAs) [61], or the RNA interference process with small interfering RNAs (siRNAs) [22,62,66] or microRNAs (miRNAs) [57,64] in therapeutic strategies.

ssDNA linkers attached to nanocomposites were combined with aptamers through partial hybridization with an elongated part of the aptamers that did not impair the recognition of their target molecules. For RNA aptamers, ssDNA linkers provided higher stability, protected them from chemical modifications [34] and served as an intercalation site for the loading of therapeutic agents [71]. In the case of ssDNA aptamers, a three-way junction pocket DNA was reported. This nanostructure was composed of three strands of AS1411 aptamer, which acted as a therapeutic agent with two activities: antiproliferative activity due to its nucleolin targeting after internalization into target cells and cytotoxic activity due to its high drug loading in its duplex regions and its acidic pH-triggered drug release [81].

ssDNAs as key components of enzyme-free assays were used in different strategies, where the competition between the hybridization of the aptamer with a partially complementary ssDNA and the binding of this aptamer to its target allowed biomarker quantification and even signal amplification. For example, in colorimetric and photothermal aptasensors [38] and in fluorescence nanoquenchers [67,77] alone or combined with a hybridization chain reaction [79].

3.6.8 Solid oxides

Low-cost bulk fabrication and resistance of a wide range of pH and temperatures are properties that make solid oxides attractive nanomaterials. Solid oxides constituted 7% of the aptamer refinements. They can be combined with other nanomaterials to design lab-on-a-chip devices such as portable aptasensors. Most of the solid oxides found in the included articles were metallic oxides such as superparamagnetic iron oxide (Fe_3O_4) NPs (46%) and titanium oxide (TiO_2) NPs and nanosheets (15%). However, metalloid oxides such as silicon oxide (SiO_2) nanofilms and nanowires represented 39% of the total (Figure 6H).

Due to their biocompatibility, high surface-area-to-volume ratio and easy-to-conjugate targeting ligands, dyes and drugs, Fe_3O_4 NPs have multifunctional theranostic capabilities. Fe_3O_4 NPs allowed quantifications of tumor components such as CTCs [70] and exosomes [67,78] from body fluids. They exhibited optical properties for fluorescence *in vitro* and *in vivo* imaging, peroxidase-like activity for color change detection, magnetic properties for biomarker capture and *in vivo* magnetic resonance imaging, and signal amplification mediated by their good light-heat conversion for simultaneous multicolor and photothermal sensing [34,38,71].

Nanosized TiO_2 is a chemical and photochemically stable nanomaterial with biocompatible properties, highly available and affordable [93]. TiO_2 was used for signal amplification to enhance the sensitivity of electrochemical aptasensors due to its high electron transfer ability, great surface area and compatibility to attach to biomolecules such as aptamers and enzymes [48,76].

Nanoscaled SiO_2 is a semiconductor nanomaterial that may be used as an electrode substrate or covering in aptasensors. SiO_2 provided an easy surface modification, which

resulted in a larger sensing area for greater aptamer loading and target detection [8,39,46,50]. Furthermore, SiO₂ improved the photothermal stability of other nanomaterials avoiding their melting under high intensity and extended periods of therapeutic laser irradiation thanks to their thickness and fast heat dissipation, which in addition avoided aggregation-induced spectral shifting [58].

3.6.9 Carbon allotropes

Carbon is a chemical element able to form molecular structures of only carbon atoms called carbon allotropes. Classical carbon allotropes are amorphous carbon, diamond and graphite. However, the most promising carbon allotropes in theranostic applications are the novel ones involving nanomaterials derived from graphitic sheets such as graphene, carbon nanotubes (CNTs), graphene quantum-dots (GQD) and fullerenes [94].

Among the aptamer refinements found in our study, 6% consisted of carbon allotropes. The retrieved articles exploited the features of graphene (73%), CNTs (18%) and GQD (9%) (Figure 6I), with 80% of the articles combining these carbon allotropes with metallic NPs to enhance the performance of aptasensors with higher surface area, catalytic properties and lower limit of detection of PCa biomarkers. Among the metallic NPs, Au NPs were the most used (63%), followed by Fe₃O₄ NPs (25%) and TiO₂ NPs (12%).

Graphene is a planar graphitic sheet with a large and easily functionalizable surface consisting of an sp² hybridized carbon network with available π electrons, which enables a high loading of other nanomaterials. Thus, it may act as a substrate material in aptasensors. Half of the articles reported the use of graphene as graphene oxide (GO) [38,42,70,79] and the other half as reduced graphene oxide (rGO) [47,51,55,76]. GO is the oxide form of graphene and rGO is derived from GO through chemical or thermal reduction. GO displays a surface rich in oxygen-containing functional groups as compared to graphene. The removal of these groups during reduction gives rise to rGO, which also exhibits a red-shifted absorption spectrum and higher electrical conductivity. GO showed a high optical transmittance, strong near-infrared (NIR) absorbance, efficient fluorescence quenching, great electron mobility and strong affinity to ssDNA compared to dsDNA [95].

CNTs are hollow cylinders consisting of rolled-up graphitic sheets with high surface-to-volume ratio for a good loading and high electrical conductivity. The articles included in our study reported multi-walled (MWCNTs), which comprised several concentrically graphitic sheets interlinked via van der Waals forces [51,80].

GQDs are one or few graphitic layers with one lateral dimension structure up to 100nm. They preserve a large surface area and available π electrons, which provided an excellent photoluminescence with resistance to photo-bleaching, electrocatalytic properties, electron transfer and biocompatibility [49].

3.6.10 Drugs

Six percent of the refinements on aptamers were drugs used as antineoplastics in articles that described therapeutic platforms. The cytostatic agent doxorubicin was the most reported drug (40%). Its red fluorescence enables its detection through fluorescent microscopy or flow cytometry. Since this chemotherapeutic compound may be uptaken by membrane-permeable non-target cells, it was carried by a targeted delivery system and administered at the minimum effective dose to avoid side effects. Thus, doxorubicin was able to intercalate into the double helix of nucleic acids and establish hydrophobic interactions with nanomaterials, which allowed a pH-sensitive delivery in acidic conditions such as those of cancer cells and a higher drug loading [21,34,71,81].

Another drug that was reported in our study was the chemotherapeutic agent TGX221, which achieved 20% of the reported drugs. TGX221 is a synthetic inhibitor of phosphatidylinositol 3-kinase (PI3K), which promotes tumorigenesis and cancer progression of tumor cells. TGX221 displays cell membrane permeability, poor solubility in water and narrow therapeutic index that were overcome by a targeted delivery system that specifically reached target tissues with minimal adverse effects [63,65].

Docetaxel is another chemotherapeutic drug applied in the clinical treatment of PCa that appeared twice in our selected articles (20%). Due to its hydrophobic nature, it needed to be encapsulated to internalize target cells [24,54]. It was simultaneously co-delivered with the hydrophilic drug cisplatin, which appeared once in our study (10%). Since they possess different solubility and mode of action, their temporally controlled co-delivery achieved a synergistic cytotoxic effect [54].

Auristatin derivatives monomethyl auristatin E (MMAE) and F (MMAF) are highly cytotoxic microtubule inhibitors. They were reported just once (10%) and needed a target delivery system to get into the cell cytoplasm and cause apoptosis [20] (Figure 6J).

3.7 Types of assays

The development of diagnostic, therapeutic or preventive agents for a particular disease may take 12 years, mainly spent on the first phases (early and late discovery) and cost around a billion dollars, mainly spent on the latest phases (preclinical and clinical studies). Only one of 10,000 discovered agents is able to reach the market. Generally, the others drop from development due to efficacy and toxicity problems during preclinical safety studies and clinical studies. Cell-based *in vitro* assays are used to optimize these agents and identify human specific risks regarding their permeability, pharmacokinetics, bioavailability, metabolic stability and interactions with other drugs. Thus, *in vitro* assays enable the selection of candidates for more successful preclinical animal safety studies [96]. *In silico* studies usually complement or precede *in vitro* assays, as they need experimental validation. Bioinformatics algorithms run these assays to predict the interaction of aptamers with their targets offering their secondary structures, stability parameters and complementarity in the case of nucleic acid targets. While *in silico* and *in vitro* assays are developed in the early and late discovery phase, *in vivo* assays are developed in the preclinical phase. Consequently, the use of *in vivo* assays would indicate a further development of these agents. Even though *in vivo* assays better mimic physiological conditions, we should bear in mind that studies performed in animals such as rodent or non-rodent species may fail to represent human responses because they may differ significantly in the enzymes involved in drug metabolism, gut physiology and pharmacokinetics [96].

Therefore, our included articles showed an earlier phase of development, as 76% reported only *in vitro* assays such as cell viability, serum stability, targeting assays and so forth. *In vivo* assays such as inhibition of tumor growth, survival and tissue distribution assays were reported by 24% of the selected articles with cell line-derived xenograft tumor mouse models [20,22,57,59,61–66,68,71,79,80] together with *in vitro* assays. *In silico* assays of our selected studies used the software Mfold [97], which was reported by six articles [20, 26, 34, 39, 50, 66], and RNAstructure [98], which was reported by two

articles [61, 68], to characterize the corresponding aptamers. Only the Gene Runner software (Hastings Software Inc. Hastings, NY, USA, <http://www.generunner.net>), which was reported by one article [31], characterizes and additionally selects an aptamer from partially complementary sequences to the DNA binding domains of the AR receptor.

The standard method for aptamer selection is the *in vitro* technique SELEX. Nevertheless, less laborious techniques with higher output have appeared over the last years [99]. Rather than alternative techniques to SELEX, these *in silico* techniques are complementary techniques. In this way, they are combined approaches with *in vitro* and *in silico* methods. *In silico* techniques based on computational methods such as docking, molecular dynamics and even machine learning allow a faster and more affordable selection of aptamers. These algorithms did not appear in more articles because they reported aptamers previously selected and their *in silico* modeling was reported in preceding articles reporting their selection.

Clinical trials using theranostic application for PCa were not found in the retrieved articles, which highlights the early development of these technologies in the clinical practice of PCa. However, we expect the assessment of some of the most studied PCa-specific aptamers such as PSap4#5, A10 and A9, shortly in clinical trials.

3.8 Clinical applications

More than a half of the selected articles were diagnostic research studies, while treatment research ones accounted for almost 34% of them. Thus, the fewest articles reported diagnostic and prognostic research studies and theranostic research studies with 7% and 5%, respectively (Figure 7). All these studies showed clinically relevant targets and potential clinical applications that may be implemented in the medical practice of PCa. However, there are obstacles that prevent aptamers from reaching their space in the general clinical practice. Despite the advantages of aptamers against antibodies, the resistance of professionals to replace antibodies as tools in standard techniques is one of the main obstacles. The ignorance of their existence and the lack of a full validation for these aptamers are other obstacles [100]. These last ones depend directly on the aptamer industry and researchers and they could overcome that easily with more investments in scientific communication and validation assays.

3.8.1 Biological samples

The majority of the analyzed articles used prostate cell lines as biological materials to test the clinical applications of aptamer-based systems, followed by blood samples, mouse xenograft models, antigens in buffer, tissue sections, urine and saliva (Figure 8). Regarding the cell lines used, we only reported the prostate cell lines (Table 1), but other cell lines were used in some articles as controls. Among tumor prostate cell lines, PC-3, LNCaP, DU-145, C4-2, 22Rv1, LAPC-4 and VCaP appeared in the selected articles based on their biomarker expression levels, mainly PSMA and AR. Nonetheless, some of these cell lines were modified to change the expression of these biomarkers. Non-tumor prostate cell lines RWPE-1, PrEC, PNT2 and BPH-1 were also applied as control cell lines.

Within blood-derived samples, either serum spiked with the target antigen (47%) or pure serum from human blood samples (37%) was applied in diagnostic platforms. In addition, two articles (11%) reported human plasma [29,78] and only one (5%) reported whole rabbit blood spiked with LNCaP cells, which imitated CTCs found in the blood of PCa patients [70]. Whole blood is highly degradable and requires a fast processing to avoid hemolysis. Plasma corresponds to the liquid portion of blood after centrifugation that has been treated with an anticoagulant to avoid clotting, whereas serum has the same composition but lacks fibrinogen and coagulation factors because it has not been treated [101]. The fact that whole blood is hardly used may be explained because of its instability. In the case of plasma, its low use may be due to the presence of anticoagulants that interfere in the recognition of targets by their corresponding aptamers.

The selected articles reported murine xenograft models for *in vivo* studies. These models were designed from immunocompromised mice transplanted with cells from human PCa cell lines in the following order from most to least used: PC-3 [59,62,71,80,81], LNCaP [57,62,63,71], C4-2 [22,59,63], 22Rv1 [20,63] and LAPC-4 [63]. Thanks to xenograft models, researchers were able to outline the pharmacodynamics and pharmacokinetics of aptamer-based nanoformulas regarding tissue distribution, maximum tolerable dose and antitumor activity.

Antigens in buffers were used as mocked body fluids to mimic liquid biopsies that may load PCa-specific antigens, mainly PSA. Among body fluids, blood, seminal fluid, urine

and stool are body fluids that may carry a high load of PCa components in patients affected by this disease due to their proximity to the prostate gland [102]. In fact, urine was the second body fluid most reported by the selected studies [29,67,79]. However, saliva also appeared in the selected articles as a source of liquid biopsy for PCa [79]. The reason for this may be the ease of obtaining saliva that makes this procedure a very patient-friendly one. In contrast to liquid biopsies that are easily accessible samples acquired by non-invasive procedures, tissue sections or tissue biopsies are obtained after a small surgery, an invasive procedure that was also reported and that is currently used in the clinical practice of PCa.

3.8.2 Route of administration

According to the articles analyzed, the most used route of administration was intravenous, representing 75% of the studies [20,57,59,62,63,65,66,68,71,79,80]. The intravenous route consists of the administration of substances through injection in the vein. This method allows a fast systemic circulation of the products applied and a lower concentration of them. In addition, it provides a total bioavailability of pharmaceuticals and a direct distribution to all tissues of the circulatory system [103,104]. Most studies used the mice's tail vein for injection of the aptamer-targeted system [20,57,62–66,68,71,79,80]. Only one study [59] used the retro-orbital venous sinus of mice that is located behind the eyes, in the lateral and medial corners [105]. This sinus is a site of safe, easy and fast application of substances because of its easy access to numerous blood vessels. It shows high absorption rate and tolerance to high concentrations of solutions, as well as non-physiological pH [106].

The intraperitoneal injection was used in 25% of the articles [22,61,66], and consists of a technique of easy learning and execution, besides being safe and triggering little stress to the animals. This technique consists of the injection of substances into the peritoneal cavity, which allows contact between the administered product and the systemic circulation. Although this route exhibits fast absorption of drugs, the pharmacokinetics of the administered drugs is similar to the oral route, occurring a high rate of metabolism of first passage. With the loss of a portion of the injected product before reaching the bloodstream, intraperitoneal injections would require larger quantities of the injected

product [107]. Therefore, this issue may be the main reason for the limited use of the intraperitoneal injection and the preference for the intravenous one.

3.8.3 Diagnostic techniques

Most of the selected papers used electro-analytical techniques in aptasensors, which are biosensors including aptamers for target detection (Figure 9). Nowadays, biomarkers may be detected from invasive tissue biopsies analyzed by immunohistochemistry (IHC) in the case of proteins or fluorescence *in situ* hybridization (FISH) in the case of RNA or DNA. An alternative for these techniques is the non-invasive liquid biopsies analyzed by enzyme-linked immunosorbent assays (ELISA). However, biomarkers are detected with limited sensitivity in ELISA compromising the analysis of those with low concentrations. Biosensors are reliable, fast, affordable and portable devices composed of a highly sensitive receptor able to recognize a specific analyte and a transducer able to convert the detection of the analyte into a signal providing quantitative or semi-quantitative information [108]. The sensitivity of these biosensors is usually enhanced through the use of emerging nanomaterials such as metal nanoparticles, polymers, carbon allotropes and so on, which increase the surface area improving the conductivity of electrodes to amplify electrochemical signals [76].

Forty-four percent of the electro-analytical methods reported by the selected articles were based on voltammetry [35,39,47,50,52,82] (Figure 9). This technique measures electric current values after varying its potential in different ways, which results in different types of voltammetry. The most used types were in the following order: cyclic voltammetry (CV), square wave voltammetry (SWV), differential pulse voltammetry (DPV) and differential pulse stripping voltammetry (DPSV). Interestingly, a DNA amplification technique called rolling circle amplification (RCA) was combined with DPSV. A ssDNA primer was elongated to form a long ssDNA with the assistance of a circular DNA template and polymerases. This ssDNA contained thousands of tandem repeats that were complementary to the circular template and served as a template for the formation of metal NPs, which acted as signal reporters and amplifiers. The release of metal ions in acid solutions were detected by DPSV to quantify a single PSA molecule after its recognition by a specific aptamer attached to a nanocomposite together with the primer [52].

Thirty-seven percent of the electro-analytical methods were impedimetric ones and used electrochemical impedance spectroscopy (EIS) [40,43,44,46,48,76]. Compared with voltammetric methods, EIS uses a low excitation voltage that allows long-term monitoring and avoids electrode heating and damage preserving the biological sample [109]. Although voltammetry and impedance were used in several articles to characterize and analyze the fabrication of aptasensors, only 19% of the articles combined them simultaneously for the detection of PCa biomarkers [25,49,51] (Figure 9).

Besides electro-analytical techniques, researchers explored other options. Fluorescence spectrometry appeared in 28% of the studies [28,29,32,36,41,42,67,70,74,76,77], including two articles applying this technique to microarray chips [28,29] and another one adding a polarized filter to measure anisotropy [41]. Imaging techniques were represented in 15% of the papers. Photoacoustic imaging was the most common in the selected articles [60,69], followed by magnetic resonance [71], ultrasound [80] that also appeared combined with fluorescence [59] and surface-enhanced Raman scattering [56] (Figure 9).

Imaging techniques offer *in vivo* information about not only the detection and level of expression of molecular biomarkers, but also about the anatomy and physiology of the tumors [110]. However, these techniques may be considered as a little more invasive techniques. They require the uptake of the aptamer by the patients, needing pharmacokinetic and pharmacodynamic analyses. Comparing with imaging techniques, electro-analytical techniques and fluorescence spectrometry perform their analyses *ex vivo*, which makes them more patient-friendly techniques and facilitates their implementation in PCa clinical practice. In addition, they do not need large equipment and infrastructure. These may be the reasons for the greater interest in electro-analytical techniques for PCa diagnosis.

Less common techniques included chemiluminometry [37,45,53] and colorimetry [38,78], in which absorbance or transmittance may be measured in a spectrophotometer. Colorimetry was also combined with photothermal sensing [38]. Real-time apta-PCR was reported once for the detection of PSA, where aptamers without labeling acted as biomolecular recognition elements, but also as reporter molecules for PCR amplification [26] (Figure 9).

3.8.4 Treatment approaches

The majority of treatments tested aptamers conjugated with chemotherapeutic drugs (39%) such as doxorubicin [21,34,71,81], docetaxel [24,54], cisplatin [54], auristatin derivatives [20] and TXG122 [63,65] (Figure 10). Chemotherapy is a potent treatment, but as chemotherapeutic agents have side effects and interfere in patients' quality of life [34], target drug delivery systems are needed. Indeed, our selected therapeutic studies evaluated aptamers conjugated with drugs commonly used in PCa treatment that are toxic to other cells.

Thirty-one percent of our retrieved articles assessed aptamers conjugated to transcriptional modifiers being most of them non-coding RNAs such as saRNAs, siRNAs and miRNAs and including the small ubiquitin-related modifier (SUMO) protein (Figure 10). Transcriptional modifiers associated with aptamers increase, decrease or even silence gene expression. One article tried a saRNA to increase *DPYSL3v2* gene expression, which was considered as a metastatic suppressor in PCa [61]. Differently, two siRNAs were used to silence *EGFR* and *survivin* oncogenes inhibiting multiple signaling pathways [22]. Other two studies used a specific siRNA to knockdown *DNAPK* gene, which led to the inhibition of a related double-strand DNA break (DSB) repair pathway and to the consequent sensitization of target PSMA-positive cells to ionizing radiation [62,66]. Another transcriptional silencer is the corepressor SUMO, whose mutant SUMOG97A was used to suppress the AR, known as one of the principal promoters of PCa proliferation [30]. Unlike the other studies, two opted for miRNAs, once they can target multiple genes reducing PCa activity [57,64].

Treatments with the direct application of an aptamer, without conjugation to cytotoxic agents, appeared in 17% of the treatment research studies analyzed (Figure 10). In these studies, aptamers bound specific targets to control cancer cell progression by inhibition of the target molecule. One of the aptamers inhibited, decreased and interfered in the expression of cancer progression genes by competing against the hormone response element (HRE) in its binding to the AR [31]. A9g aptamer inhibited PSMA activity reducing metastasis [68] and the 2'F-modified aptamers R3 and S2 moderated PSA activity [26]. Another one called IDA inhibited the binding of $\alpha 6\beta 4$ integrin to laminin, which activates signaling pathways associated with PCa metastasis [23].

Three studies (13%) used direct NIR laser treatment, which decreased cell viability (Figure 10). Since radiation therapy has been limited in PCa patients due to the low tolerance of the surrounding tissues, the conjugation of aptamers to radiosensitizing agents reduced their side effects in PCa patients. Two articles used aptamers attached to AuNRs targeting PSMA+ cells [58] and cancer stem cells [27], as these NPs can transform photon energy into thermal energy inducing hyperthermia in cells. The other one utilized an aptamer attached to GO bound to the photosensitizer ICG to induce the formation of reactive oxygen species (ROS) inside PSMA+ cells after exposure to laser irradiation [70].

Most treatment approaches for PCa conjugated the aptamers to therapeutic agents, evidencing that just a small portion of them interact directly with therapeutic targets and inhibit them. Although chemotherapy was the most common approach, the development of transcriptional modifiers seems to be a growing area in aptamer-based treatments for PCa.

4. Conclusions

Current epidemiology of PCa indicates that new PCa diagnostic and therapeutic modalities are necessary. Studies worldwide that used aptamers for clinical applications in PCa over the last 10 years were identified. They showed the tendencies for the use of aptamers in this area. In general, well-established aptamers for PCa were modified with a high variety of refinements including modifications to their sequence and attachments to increase their functions and enhance their performance in theranostic applications. In this way, newly selected aptamers for PCa were scarce. The target molecules of aptamers for PCa were predominantly proteins. The PSA protein was the most reported target. However, its use in the management of PCa patients is susceptible to both false positive and negative results and alternative biomarkers are under study. Although DNA aptamers were more reported probably because they are easier to handle, PCa-specific RNA aptamers were remarkable due to their high affinities and more relevant targets, for example PSMA. The use of the cell-SELEX technique was outstanding for the selection of new PCa-specific aptamers. Most of the selected studies reported *in vitro* assays, mainly for diagnostic proposals such as aptasensors that identified PCa biomarkers through electro-analytical techniques. After the use of PCa cell lines as biological material

in the retrieved studies, human blood was the preferred choice for liquid biopsies of PCa patients. Majority of aptamer-based therapeutic tools were attached to drugs where the aptamer-targeted system was injected intravenously in mice models.

Considering all of these findings, very diverse approaches display a promising emerging development of aptamer-based theranostic methodologies for PCa. Nonetheless, there is still a gap in the last phases of their development as theranostic agents. This gap prevents them from being widely available and consumed by the healthcare industry. Thus, clinical trials and studies with a larger cohort of patients are critical to completely validate the use of aptamers in the clinical practice of PCa. Furthermore, other barriers to overcome are those intrinsic to aptamers. Essentially, they remain unknown to healthcare professionals, causing resistance to their use despite their virtues against antibodies.

Figure and table captions

Table 1. List of selected articles reporting aptamers for prostate cancer applications over the last decade. *No underlining: DNA aptamer; Simple underlining: RNA aptamer; Double underlining: peptide aptamer. [‡]*In vivo* assays reported in addition to *in vitro* assays that were used in all the articles. ^{||}*In silico* characterization. ^{ip}Intraperitoneal administration. ^{iv}Intravenous administration. [†]●: Dye; ●: Polymer; ●: Spacer; ●: Modified nucleobases; ●: Gold; ●: Organic nanomaterials; ●: Hybridized oligonucleotide; ●: Solid oxide; ●: Carbon-based nanomaterial; ●: Drug. AR, androgen receptor; DBD, DNA-binding domain; EpCAM, epithelial cell adhesion molecule; MRI, magnetic resonance imaging; MUC1, Mucin ; NIR, near-infrared; PSA, prostate specific antigen; PSMA, prostate specific membrane antigen; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SELEX, systematic evolution of ligands by exponential enrichment; SERS, surface-enhanced Raman scattering; VEGF, vascular endothelial growth factor.

Figure 1. Study flow diagram during the selection process. n, number of records.

Figure 2. Quality assessment of the retrieved studies.

Figure 3. Origin of aptamers. SELEX, systematic evolution of ligands by exponential enrichment.

Figure 4. Biomarkers. AR, androgen receptor; MUC1, Mucin; PSA, prostate specific antigen; PSMA, prostate specific membrane antigen.

Figure 5. Categories of aptamer refinements.

Figure 6. Aptamer refinements. A. Reporters. B. Polymers. C. Spacers. D. Modified nucleobases. E. Gold. F. Macromolecules. G. Hybridized oligonucleotides. H. Solid oxides. I. Carbon allotropes. J. Drugs. PAMAM, polyamidoamine; PEG, polyethylene glycol; RNA, ribonucleic acid; SOMAmers, slow off-rate modified aptamers; ssDNA, single-stranded deoxyribonucleic acid.

Figure 7. Clinical applications.

Figure 8. Biological samples.

Figure 9. Diagnostic techniques. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SERS, surface-enhanced Raman scattering.

Figure 10. Treatment approaches. NIR, near infrared.

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Table 1

Name of original aptamer*	Refinements [†]	Origin of aptamer	Biomarker	Clinical applications	Biological samples and assays	Diagnostic techniques	Treatment approach
apt [75]	-	Chemical synthesis	PSA	Diagnosis	Antigen in blood	Fluorescence spectrometry	-
A9g [67]		Chemical synthesis	Exosomal PSMA	Diagnosis	Urine	Fluorescence spectrometry	-
Nameless [76]		Chemical synthesis	PSA	Diagnosis	Antigen in blood	Impedance	-
<u>A10-3.2</u> [80]		Chemical synthesis	PSMA	Diagnosis	Cell lines and mouse xenograft model [‡]	Ultrasound imaging ^{iv}	-
AS2 and EpApt [78]		Chemical synthesis	Exosomal PSA and EpCAM	Diagnosis and prognosis	Cell lines and blood	Colorimetry	-

PSap4#5 and V7t1 [45]		Chemical synthesis	PSA and VEGF	Diagnosis	Antigen in buffer	Chemiluminometry	-
PSap4#5 [47]		Chemical synthesis	PSA	Diagnosis	Antigen in blood	Voltammetry	-
<u>E3</u> [20]		Cell-SELEX	LNCaP, 22Rv1, PC-3 and DU-145 cells	Treatment	Cell lines and mouse xenograft model [‡] , Mfold [¶]	-	Drug ^{iv}
PSap4#5 [48]		Chemical synthesis	PSA	Diagnosis	Blood	Impedance	-
PSap4#5 [49]		Chemical synthesis	PSA	Diagnosis	Blood	Voltammetry and impedance	-
PSap4#5 and V7t1 [50]		Chemical synthesis	PSA and VEGF	Diagnosis	Cell lines, Mfold [¶]	Voltammetry	-
apt 1 [21]		Cell-SELEX	LNCaP cells	Treatment	Cell lines	-	Drug

PSap4#5 [51]		Chemical synthesis	PSA	Diagnosis	Blood	Voltammetry and impedance	-
<u>A10-3.2</u> [59]		Chemical synthesis	PSMA	Diagnosis	Cell lines and mouse xenograft model [‡]	Ultrasound and fluorescence imaging ^{iv}	-
<u>A10-3.2</u> [22]		Cell-SELEX	PSMA	Treatment	Cell lines and mouse xenograft model [‡]	-	Non-coding RNA ^{ip}
<u>A10-3.2</u> [60]		Chemical synthesis	PSMA	Diagnosis and prognosis	Cell lines	Photoacoustic imaging	-
AMH [31]		Complementarity	DBD of AR	Treatment	Cell lines, Gene Runner ^l	-	Direct
SOMAmers [29]		Proprietary SELEX	Exosomal protein array	Diagnosis	Blood and urine	Fluorescence spectrometry	-

<u>A10-3.2</u> [61]		Conventional SELEX	Extracellular portion of PSMA	Treatment	Cell lines and mouse xenograft model [‡] , RNAstructure	-	Non-coding RNA ^{ip}
<u>A10-3-J1</u> [34]		Chemical synthesis	Extracellular portion of PSMA	Treatment	Cell lines, Mfold	-	Drug
IDA [23]	-	Cell-SELEX	Integrin $\alpha 6\beta 4$	Treatment	Cell lines	-	Direct
PSap4#5 [52]		Chemical synthesis	PSA	Diagnosis	Antigen in blood	Voltammetry	-
PSap4#5 [53]		Chemical synthesis	Free PSA	Diagnosis and prognosis	Antigen in buffer	Chemiluminometry	-
<u>A10-3</u> [62]		Chemical synthesis	PSMA	Treatment	Cell lines, mouse xenograft model		Non-coding RNA ^{iv}

					and tissue sections [‡]		
PSap4#5 [25]		Conventional SELEX	PSA	Diagnosis	Blood and tissue sections	Voltammetry and impedance	-
<u>A10</u> [63]		Chemical synthesis	Extracellular portion of PSMA	Treatment	Cell lines, mouse xenograft model and tissue sections [‡]	-	Drug ^{iv}
PSap4#5 [35]		Chemical synthesis	PSA	Diagnosis	Antigen in buffer	Voltammetry	-
<u>A10-3.2</u> [64]		Chemical synthesis	PSMA	Treatment	Cell lines and mouse xenograft model [‡]	-	Non-coding RNA ^{iv}
PSap4#5 [36]		Chemical synthesis	PSA	Diagnosis	Antigen in blood	Fluorescence spectrometry	-

PSap4#5 [37]		Chemical synthesis	PSA	Diagnosis	Antigen in blood	Chemiluminometry	-
<u>A9g</u> [68]		Chemical synthesis	PSMA	Treatment	Cell lines, mouse xenograft model and tissue sections [‡] , RNAstructure	-	Direct ^{iv}
<u>A9</u> [69]		Chemical synthesis	PSMA	Diagnosis	Cell lines	Photoacoustic imaging	-
<u>S2</u> and <u>R3</u> [26]		Conventional SELEX	PSA	Diagnosis and treatment	Antigen in buffer, Mfold	RT-qPCR	Direct
CSC1 and CSC13 [27]		Conventional SELEX	DU-145 cells and DU-145 stem cells	Treatment	Cell lines	-	NIR irradiation
<u>A10</u> [65]		Chemical synthesis	PSMA	Treatment	Cell lines [‡]	-	Drug ^{iv}

<u>A10-3</u> [66]		Chemical synthesis	PSMA	Treatment	Cell lines, mouse xenograft model and tissue sections [‡] , Mfold	-	Non-coding RNA ^{ip}
<u>PAB-CoR 524</u> [30]		Two-hybrid screening	AR	Treatment	Cell lines	-	Corepressor
<u>A10</u> [54]		Chemical synthesis	PSMA	Treatment	Antigen in buffer	-	Drug
PSap4#5 [38]		Chemical synthesis	PSA	Diagnosis	Antigen in buffer	Colorimetry and photothermal sensing	-
A10 [55]		Chemical synthesis	AR	Diagnosis	Cell lines	Voltammetry	-
H1 [79]		Chemical synthesis	PSA	Diagnosis	Antigen in buffer, blood, urine and saliva	Fluorescence spectrometry	-

<u>A10-3.2</u> [56]		Chemical synthesis	PSMA	Diagnosis (prediction and monitoring)	Cell lines and tissue sections	SERS imaging	-
PSap4#5 and nameless [39]		Chemical synthesis	PSA and free PSA	Diagnosis	Cell lines, Mfold ^{ll}	Voltammetry	-
PSap4#5 [40]		Chemical synthesis	PSA	Diagnosis	Antigen in buffer	Impedance	-
AS1411 [81]		Chemical synthesis	Nucleolin	Treatment	Cell lines, mouse xenograft model and tissue sections [‡]	-	Drug ^{iv}
PSap4#5 [41]		Chemical synthesis	PSA	Diagnosis	Blood	Fluorescence spectrometry	-

PSap4#5 [42]		Chemical synthesis	PSA	Diagnosis	Antigen in blood	Fluorescence spectrometry	-
S3.1/S2.2 [82]		Chemical synthesis	MUC1	Diagnosis	Antigen in buffer and cell lines	Voltammetry	-
PSap4#5 [43]		Chemical synthesis	PSA	Diagnosis	Antigen in buffer	Impedance	-
PSap4#5 [44]		Chemical synthesis	PSA	Diagnosis	Antigen in buffer	Impedance	-
<u>A10-3.2</u> [57]		Chemical synthesis	PSMA	Treatment	Cell lines, mouse xenograft model and non-human primates [‡]	-	Non-coding RNA ^{iv}
DNA1 [77]		Chemical synthesis	PSA	Diagnosis	Blood	Fluorescence spectrometry	-

<u>A9</u> [70]		Chemical synthesis	PSMA on CTCs	Diagnosis and treatment	Cell lines and cells in blood (rabbit)	Fluorescence spectrometry	NIR irradiation
PSap4#5 [32]		Chemical synthesis	PSA	Diagnosis	Blood	Fluorescence spectrometry	-
<u>XEO2 mini</u> and <u>XEO6 mini</u> [24]		Cell-SELEX	PC-3 and LNCaP cells	Treatment	Cell lines	-	Drug
PSap4#5 [46]		Chemical synthesis	PSA	Diagnosis and prognosis	Antigen in blood	Impedance	-
SOMAmers [28]		Proprietary SELEX	Exosomal protein array	Diagnosis	Cell lines	Fluorescence spectrometry	-
<u>A9 CGA</u> [71]		Chemical synthesis	PSMA	Diagnosis and treatment	Cell lines and mouse xenograft model [‡]	MRI ^{iv}	Drug ^{iv}

<u>41-base</u> <u>A10</u> [58]		Chemical synthesis	PSMA	Treatment	Cell lines	-	NIR irradiation
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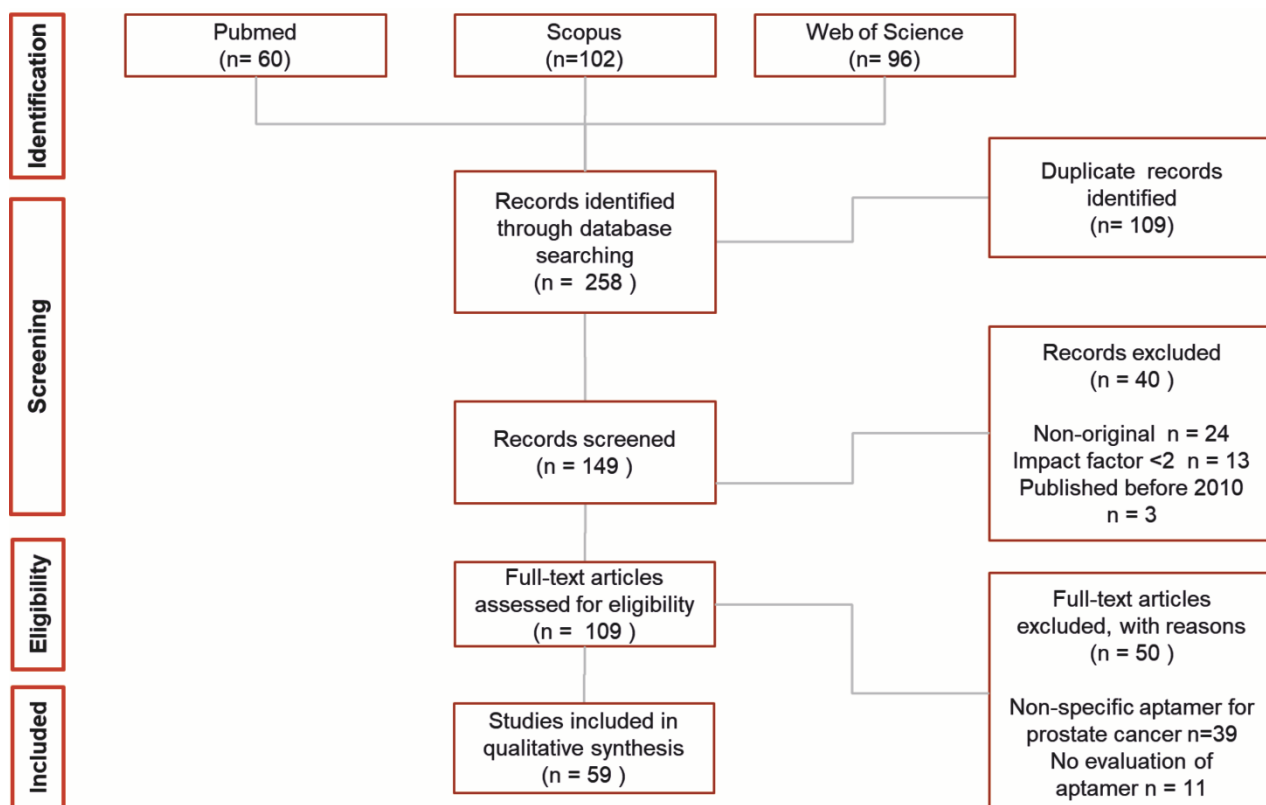


Figure 1

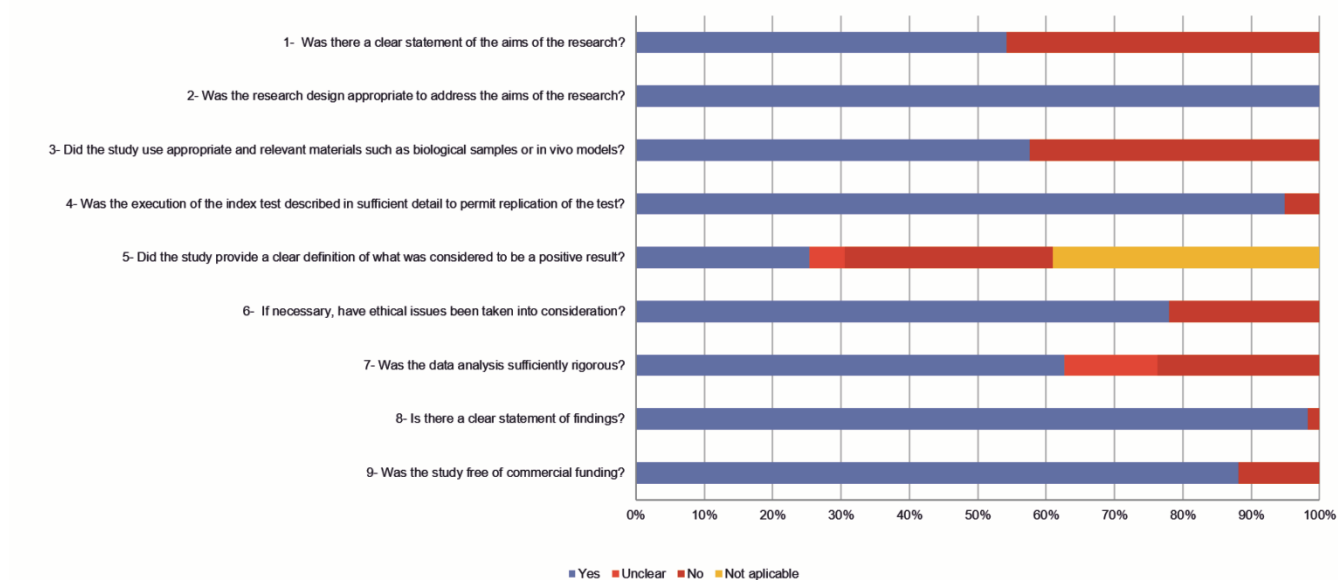


Figure 2

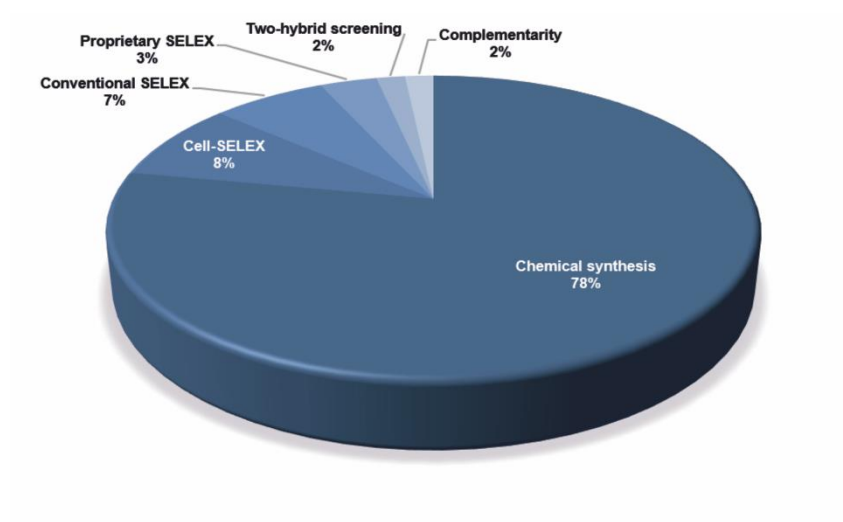


Figure 3

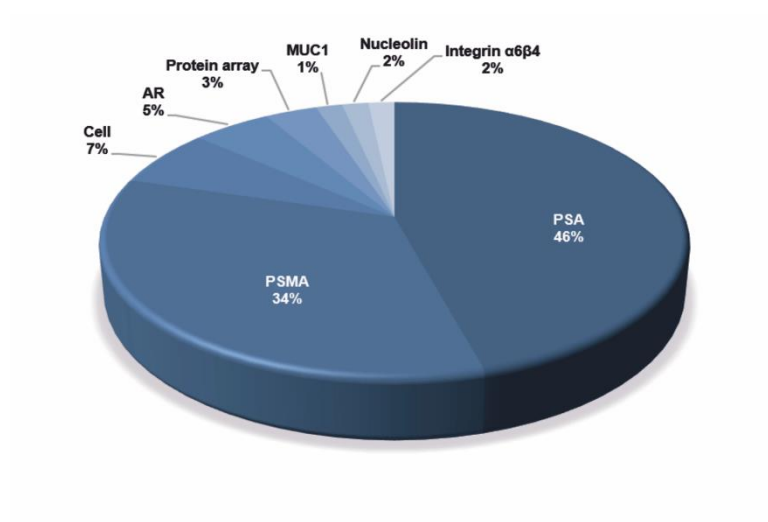


Figure 4

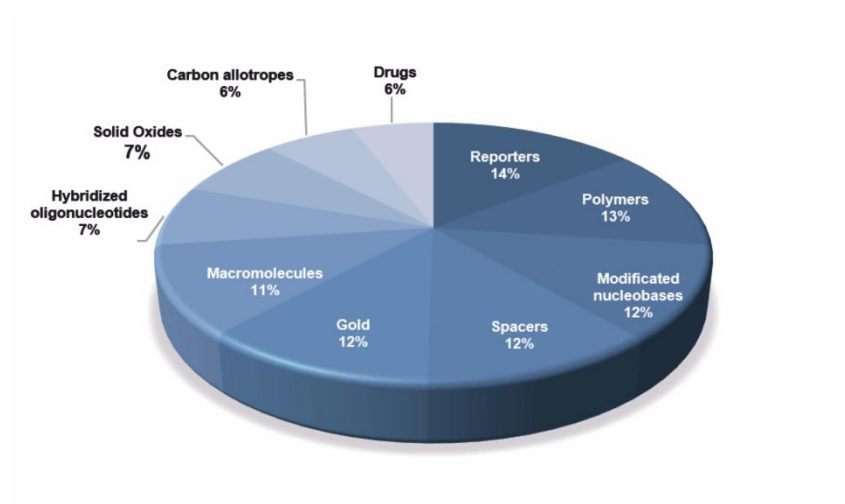


Figure 5

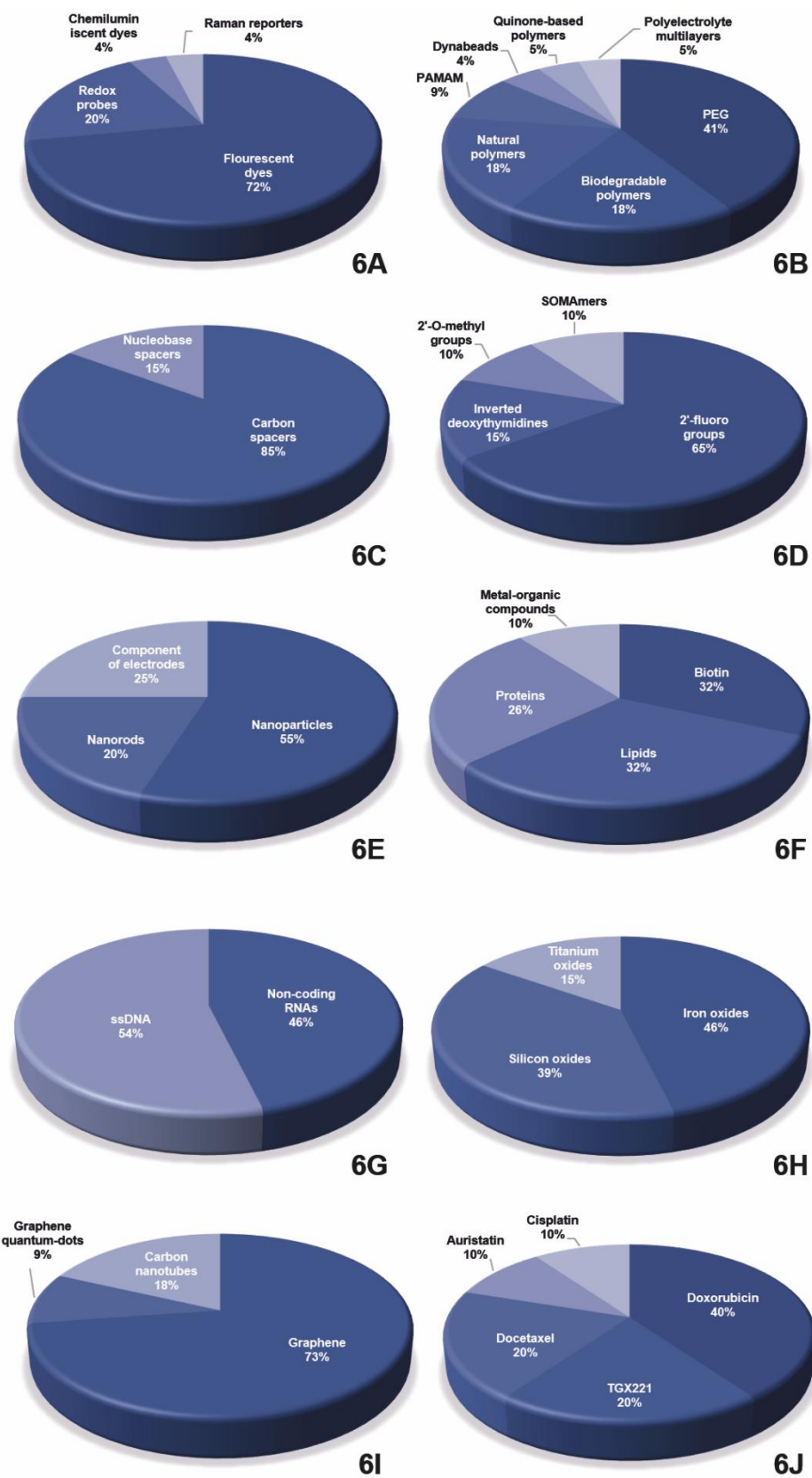


Figure 6

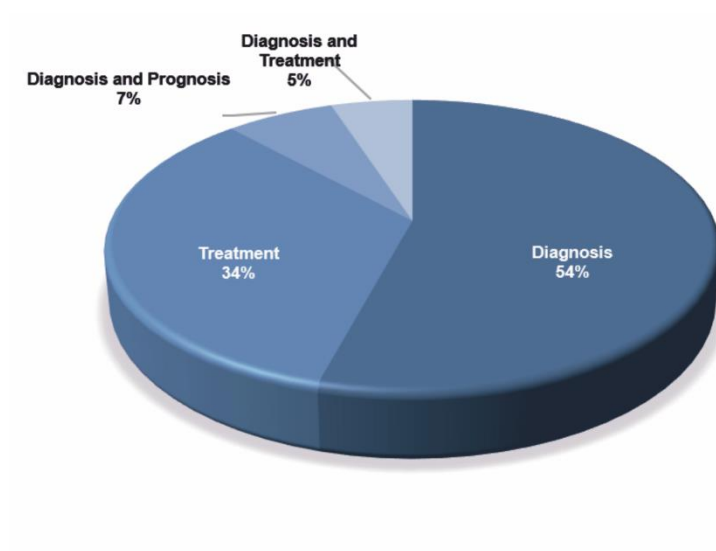


Figure 7

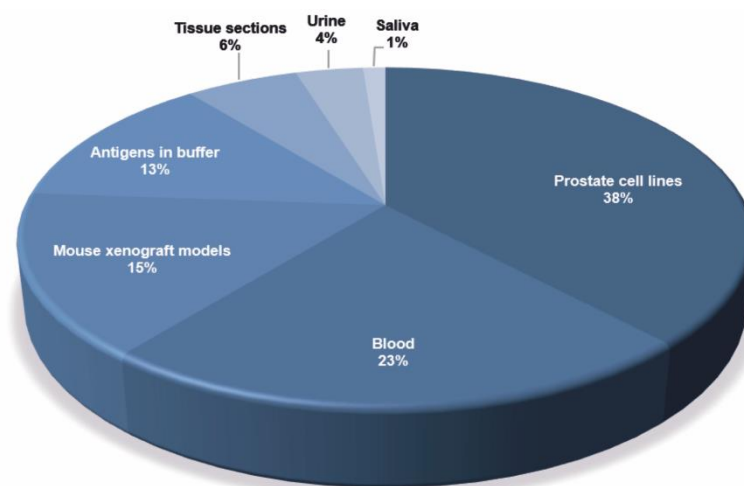


Figure 8

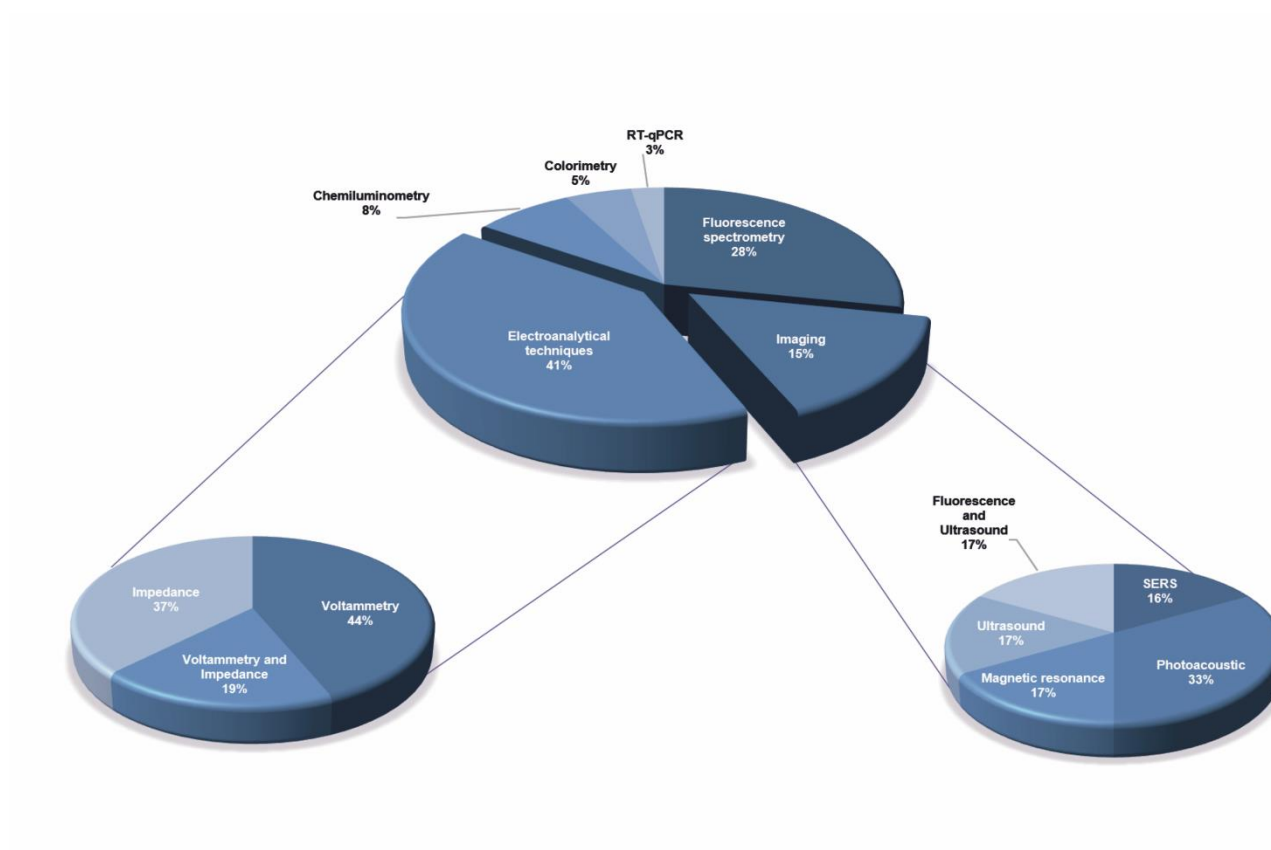


Figure 9

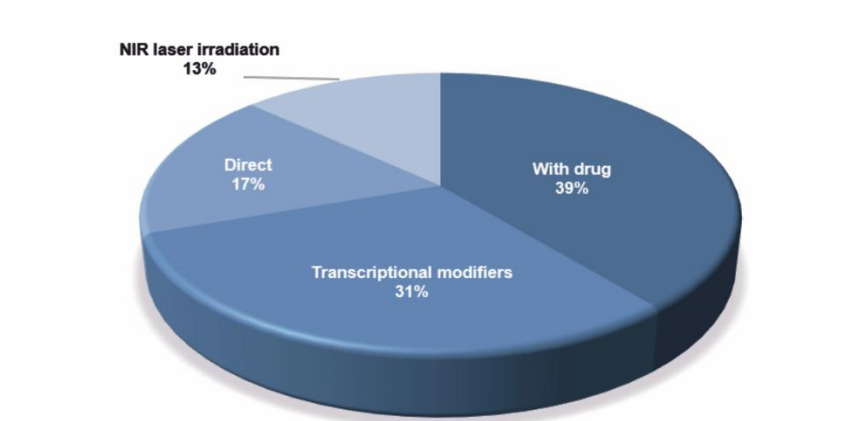


Figure 10

CAPÍTULO II – ARTIGO CIENTÍFICO

Título

Decifrando o potencial dos aptâmeros D4 e R4 para a prática clínica no câncer de próstata

Resumo

Os aptâmeros D4 e R4, de DNA e RNA respectivamente, surgiram a partir da modificação do aptâmero A4 ligante de células PC-3. Nosso objetivo foi caracterizar e comparar esses aptâmeros para avaliar seus desempenhos como ferramentas teranósticas no câncer de próstata. Representamos suas estruturas mediante algoritmos computacionais. Obtivemos sua resistência contra nucleases de soro humano. Avaliamos sua ligação a linhagens celulares da próstata RWPE-1, PNT-2, PC-3 e DU-145 e os efeitos da sua presença na viabilidade e migração das mesmas linhagens. Determinamos sua força de ligação às células PC-3 e DU-145 a partir da constante de dissociação. Incubamos os aptâmeros com a camada leucocitária do sangue de pacientes e identificamos o perfil mesenquimal e epitelial das células ligantes com os anticorpos anti-EpCAM, anti-CD45, anti-CD133 e anti-CD44. Finalmente, precipitamos as proteínas alvo dos aptâmeros e simulamos seu acoplamento *in silico*. Os aptâmeros descritos apresentam estruturas similares e se ligam especificamente a células tumorais da próstata sem modificar os parâmetros celulares estudados, mas com diferentes afinidades. R4 apresenta uma resistência a nucleases menos favorável para o seu uso clínico. As células ligantes de ambos aptâmeros são CD44+, mas as ligantes de D4 são também EpCAM+, o que indica que poderiam identificar diferentes etapas do processo metastático. As possíveis proteínas alvo NXPE2, ADAM30 e MUC6 precisam ser mais estudadas para compreender melhor sua interação com os aptâmeros e seu envolvimento na progressão do câncer de próstata. Esses resultados apoiam a futura aplicação dos aptâmeros D4 e R4 na teranóstica do câncer de próstata.

Palavras chaves: aptâmeros, biópsia líquida, câncer de próstata, células tumorais circulantes.

Title

Deciphering the potential of aptamers D4 and R4 for the clinical practice in prostate cancer

Abstract

The DNA and RNA aptamers D4 and R4, respectively, emerged from the modification of the PC-3 cell-binding aptamer A4. Our objective was to characterize and compare these aptamers to assess their performance as theranostic tools in prostate cancer. We represented their structures through computational algorithms. We obtained its resistance against human serum nucleases. We evaluated its binding to RWPE-1, PNT-2, PC-3 and DU-145 prostate cell lines and the effects of their presence on the viability and migration of the same cell lines. We determined its binding strength to PC-3 and DU-145 cells from the dissociation constant. We incubated the aptamers with the buffy coat from the blood of patients and identified the mesenchymal and epithelial profile of the ligand cells with anti-EpCAM, anti-CD45, anti-CD133 and anti-CD44 antibodies. Finally, we precipitated the target proteins of the aptamers and simulated their *in silico* docking. The described aptamers present similar structures and bind specifically to prostate tumor cells without modifying the cellular parameters studied, but with different affinities. R4 has a less favorable nuclease resistance for its clinical use. The ligand cells of both aptamers are CD44+, but the D4 ligands are also EpCAM+, which indicates that they could identify different stages of the metastatic process. The possible target proteins NXPE2, ADAM30 and MUC6 need to be further studied to better understand their interaction with aptamers and their involvement in the progression of prostate cancer. These results support the future application of D4 and R4 aptamers in prostate cancer theranostics.

Keywords: aptamers; liquid biopsy; prostate cancer; circulating tumor cells.

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Title: Deciphering the potential of aptamers D4 and R4 for the clinical practice in prostate cancer

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Running head: Aptamers D4 and R4 for prostate cancer theranostics

Abbreviations: BSA, bovine serum albumin; BPH, benign prostatic hyperplasia; CD, cluster of differentiation; CTC, circulating tumor cell; ELASA, enzyme linked aptasorbent assay; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; FBS, fetal bovine serum; HRP, horseradish peroxidase; *K_d*, dissociation constant; MET, mesenchymal-epithelial transition; OPD, o-phenylenediamine; PBS, phosphate-buffered saline; PCa, prostate cancer; PEG, polyethylene glycol; PSA, prostate specific antigen; SAv, streptavidin; *T_m*, melting temperature.

Abstract

The DNA and RNA aptamers D4 and R4, respectively, emerged from the modification of the PC-3 cell-binding aptamer A4. Our objective was to characterize and compare these aptamers to assess their performance as theranostic tools in prostate cancer. We represented their structures through computational algorithms. We obtained its resistance against human serum nucleases. We evaluated its binding to RWPE-1, PNT-2, PC-3 and DU-145 prostate cell lines and the effects of their presence on the viability and migration of the same cell lines. We determined its binding strength to PC-3 and DU-145 cells from the dissociation constant. We incubated the aptamers with the buffy coat from the blood of patients and identified the mesenchymal and epithelial profile of the ligand cells with anti-EpCAM, anti-CD45, anti-CD133 and anti-CD44 antibodies. Finally, we precipitated the target proteins of the aptamers and simulated their *in silico* docking. The described aptamers present similar structures and bind specifically to prostate tumor cells without modifying the cellular parameters studied, but with different affinities. R4 has a less favorable nuclease resistance for its clinical use. The ligand cells of both aptamers are CD44+, but the D4 ligands are also EpCAM+, which indicates that they could identify different stages of the metastatic process. The possible target proteins NXPE1, ADAM30 and MUC6 need to be further studied to better understand their interaction with aptamers and their involvement in the progression of prostate cancer. These results support the future application of D4 and R4 aptamers in prostate cancer theranostics.

Key words

Aptamers; liquid biopsy; prostate cancer; circulating tumor cells.

INTRODUCTION

Estimates for cancer in 2020 revealed that prostate cancer (PCa) is the most frequently diagnosed cancer in men. In terms of overall incidence, PCa is the fourth type of cancer with the highest incidence for both sexes and the second one in men. In addition, PCa is the second leading cause of cancer mortality in men and delayed diagnosis and treatment related to the COVID-19 pandemic are expected to influence negatively PCa statistics according to the International Agency for Research on Cancer (IARC).¹

PCa mortality is strongly associated with the stage of disease progression at the time of diagnosis. Patients diagnosed at an early stage, with local PCa show better survival rates compared to those diagnosed at a more advanced stage, with metastatic PCa. Indeed, 79% of patients with local PCa show a 10-year progression-free survival, while only 12% of patients with regional metastases show it.²

Since the eighties, urologists and oncologists have used levels of serum prostate-specific antigen (PSA) for PCa screening, prognosis, assessment of recurrence and monitoring responses to treatment. However, elevated levels of serum PSA may also indicate other prostatic pathologies underlining a low accuracy for PCa detection and a limited clinical use. Therefore, the use of PSA as a PCa biomarker remains controversial. Overdiagnosis and resulting overtreatment of indolent tumors or other non-malignant pathologies such as prostatitis or benign prostatic hyperplasia (BPH) may cause more harm than benefit to the patients.³

These data stress the need for earlier and more precise diagnosis strategies to provide more effective treatments for PCa patients, prevent advanced stages of this disease and reduce its mortality rates. Consequently, we developed an alternative

method to assist in the diagnosis of PCa using the aptamers D4 and R4 as prostate circulating tumor cells (CTC) detecting agents by flow cytometric analysis.⁴

Tumor cells have altered regulation abilities that support their continuous proliferation and lead to invasion of other tissues where they form secondary tumors –also known as metastases. Cells from the primary tumor are shed into lymphatic and blood circulations, where they are called CTCs.⁵ Prostate CTCs in blood samples offer the chance to perform liquid biopsies to analyze PCa progression repeatedly and in a comprehensive way, from all tumors present in a patient. Accordingly, liquid biopsies avoid the expensive and invasive procedures of tissue biopsies. Flow cytometry allows the detection of CTCs through the binding of affinity molecules to antigens expressed on the surface of CTCs.

Aptamers are oligonucleotides or peptides that specifically bind to biological targets due to their three-dimensional conformation. Hence, they are affinity molecules able to bind to other molecules on CTCs. Detection of CTCs by aptamers may allow an early diagnosis of metastatic cancer through their enumeration or the expression analysis of their surface markers and/or genes.⁶ In addition, CTCs are related to cancer survival and indicate minimal residual disease (MRD) allowing recurrence and therapy monitoring.⁷ They may guide decision making on treatment strategies and even act as therapeutic targets.⁸

Although monoclonal antibodies are also affinity molecules that may detect CTCs, aptamers show several superior characteristics. Aptamers are not immunogenic and exhibit a high stability under harsh conditions, avoiding expensive transportation and storage requirements. They bind to specific targets with higher affinity due to their great structural stability when forming complexes and their size within the nanoscale that improves their accessibility to a varied range of targets. They are screened by the

systematic evolution of ligands by exponential enrichment (SELEX), an *in vitro* technique of iterative rounds of selection and amplification from a large oligonucleotide library. Their chemical synthesis confers high reproducible and low variable batches and allows further modifications with functional chemical groups that enhance their properties. Moreover, their economical and fast screening may be automatized.⁹

As aptamers may recognize cancer cell markers, aptamer-based drugs and diagnostic agents are potential tools that may revolutionize the clinical practice of cancer. Thus, our research group analyzed two versions of the A4 aptamer selected by three-dimensional (3D)-Cell SELEX:¹⁰ the single-stranded DNA aptamer D4 and the RNA aptamer R4. Our aim was to characterize and compare these two aptamers according to their molecular targets, structure, nuclease stability, effects on cells and ability to bind to and detect prostate CTCs to determine their theranostic uses in PCa.

MATERIAL AND METHODS

Cell cultures

The human prostate cell lines PC-3, RWPE-1, DU-145 (ATCC, Manassas, VA, USA) and PNT-2 (ECACC, Wiltshire, UK) were cultured in monolayer, as recommended. These cells were successfully authenticated in 2018 by short tandem repeat (STR) analysis and evaluated for mycoplasma contamination monthly. PC-3 and DU-145 are tumor cells and RWPE-1 and PNT-2 are non-tumor cells.

Patient samples

This study was conducted according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and informed consent from all the participants was obtained. Research Ethics Committee of the Federal University of Uberlândia (CEP-UFU) approved this study (CAAE: 71108817.2.0000.5152).

Peripheral blood from eight PCa patients who were about to undergo radical prostatectomy for localized PCa treatment was collected in a second tube with EDTA to reduce contamination by epithelial cells from the skin. Samples were centrifuged at 200 g for 15 minutes at room temperature to isolate sera and mononuclear cells.

Design and synthesis of aptamers

Designs of R4 and D4 aptamers were based on the portion of the RNA aptamer A4 that contains its unique sequence¹⁰ –the variable portion without the flanked regions of the ends that allow the PCR amplification–: 5'rArGrCrCrGrArGrArGrGrUrArArGrCrArArArArCrCrArCrGrCrCrCrG3'. This sequence was attached to a biotin molecule at its 5' end and an inverted deoxythymidine by reverse linkage at its 3' end to synthesize the R4 aptamer. In addition to these modifications, the ribonucleotides (r) were changed to deoxyribonucleotides (d) to synthesize the DNA-like aptamer D4 (Figures 1A and 2A). Both aptamers were synthesized by Integrated DNA Technology (IDT, Coralville, IA, USA) and resuspended in nuclease-free water for injection to a 100 µM working solution.

Prediction of the aptamer structures

Top secondary structures of D4 and R4 were obtained through minimizing folding free energy by the Mfold web server¹¹. Corresponding tertiary structures were predicted by the RNA Composer web server¹² and pictured by the Discovery Studio Visualizer software (v19.1.0.18287, Dassault Systèmes BIOVIA, San Diego, CA, USA), as previously described.¹³

Nuclease stability assay

Sera from three participants were mixed and incubated independently with the aptamers D4 and R4 for different times, under conditions and with procedures previously described to establish aptamer half-lives.¹³

Enzyme linked aptasorbent assay (ELASA)

One day before the experiment, RWPE-1, PC-3, PNT-2 and DU-145 cells were plated in 96-well plates at a density of 1×10^4 cells per well. Then, wells were blocked by a phosphate-buffered saline (PBS) solution containing 5% bovine serum albumin (BSA) for 1 hour at 37 °C. Cells were incubated with D4 aptamer, R4 aptamer or none of them for 1 hour at 37 °C and in a concentration sufficient for binding (2 μ M, four times higher than the one used in the A4 binding assays¹⁰). Streptavidin (SAv)-conjugated horseradish peroxidase (HRP, 1:250, BioLegend, San Diego, CA, USA) was added to each well and incubated for 30 minutes at 37°C. After each step, cells were washed three times with PBS. The color-developing reaction was initiated by o-phenylenediamine (OPD) (Sigma Aldrich, Saint Louis, MO, USA) dissolved in 0.05 M phosphate-citrate buffer with 3% H₂O₂, according to manufacturer's instructions, and terminated upon addition of H₂SO₄ (4 N). The absorbance was measured at 492 nm using a microplate reader (Titertek Multiskan Plus, Flow Laboratories, Sunnyvale, CA, USA).

Aptacytofluorescence of prostate cell lines

RWPE-1, PC-3, PNT-2 and DU-145 cells were plated on round coverslips, in 12-well plates at a density of 1×10^5 cells per well. One day after plating, cells were washed twice with PBS 1% BSA 0.1% saponin, fixed and permeabilized by incubation with a commercial kit (Fix&Perm, Thermo Fisher, Waltham, MA, USA) for 20 minutes at room temperature. They were incubated with glycine 0.1 M in PBS 1% BSA 0.1% saponin for 10 minutes at room temperature and washed again in the same way. Then, cells were incubated with D4 aptamer (2 μ M), R4 aptamer (2 μ M) or none of them in PBS 1% BSA

0.1% saponin for 1 hour at 37 °C, washed accordingly and incubated with SAV-FITC conjugate in PBS 1% BSA 0.1% saponin (1:200, 405201, BioLegend) for 30 minutes at room temperature.

After the washing step, cells were incubated with TO-PRO-3 iodide (1:250, Thermo Fisher) in PBS 1% BSA 0.1% saponin for 30 minutes at room temperature and washed again. Then, the coverslips were placed cell-side down onto a mountant (ProLong Gold Antifade, Thermo Fisher) on microscope slides. Fluorescence was recorded by an inverted confocal microscopy (LSM 510 Meta, Zeiss, Oberkochen, Germany).

Cell viability and migration assays

Prostate cell lines used in the SELEX process of the original version of the aptamers were also used for the *in vitro* assays of D4 and R4 aptamers. Therefore, the non-tumoral RWPE-1 and tumoral PC-3 cells were treated separately with the aptamers at concentrations 0, 1 and 2 μ M in proper medium without FBS and analyzed at times 0, 24 and 48 hours after the treatments. For the viability assay, cells were plated in 96-well plates at a density of 2×10^3 cells per well and assessed by Neutral Red (Sigma Aldrich), according to the manufacturer's instructions. For the migration assay, cells were seeded in 12-well plates containing 2×10^5 cells per well. The Wound Healing test was performed from a scratch made with the tip of a pipette, as previously described.¹³

Affinity analysis

PC-3 and DU-145 cell lines, at a density of 2×10^5 cells, were incubated in blocking solution (PBS with 1% BSA) for 15 minutes at room temperature and in binding buffer (0.01 M PBS with 5 mM $MgCl_2$, 25 mM glucose and 15 mM BSA) with D4 and R4 aptamers, separately, for 1 hour at 37 °C. The concentrations of aptamers were 200, 100, 50, 25, 12.5, 6.25 and 0 nM. Then, cells were incubated with SAV-FITC conjugate in PBS

(1:500, 405201, BioLegend). After each step, cells were washed with PBS. A flow cytometer Accuri C6 (BD, Franklin Lakes, NJ, USA) was used to obtain the data by counting at least 5000 events.

Data were analyzed by FlowJo software (Version X 10.0.7r2 for Windows, FlowJo, Ashland, OR, USA). Equilibrium dissociation constants (K_d) of D4 and R4 aptamers were obtained by measuring the mean fluorescence intensity (MFI) of specific binding at the different aptamer concentrations, subtracting the MFI of nonspecific binding from cells incubated only with SAV-FITC conjugates. Afterwards, results were fit as $Y=B_{max} \cdot X/(K_d+X)$, using One site specific binding equation of PrismGraph program (v.7, GraphPad Software, La Jolla, CA, USA).

Characterization of CTCs

After erythrocyte lysis of the eight blood samples with Pharm Lyse (BD) according to manufacturer's instructions, the resulting mononuclear cell suspensions were incubated in blocking solution (PBS with 1% BSA) with 10% (v/v) inactivated human AB plasma for 15 minutes at room temperature. On one hand, they were incubated in PBS for 1 hour at 4 °C with a viability stain (FVS620, 1:1000, BD), unlabeled D4 or R4 aptamer (1:500) and labeled antibodies (anti-EpCAM-APC (324208) and anti-CD45-PE (368510), or anti-CD44-APC (338806) and anti-CD133-PE (372804), 1:400, BioLegend). Then, they were incubated at 4 °C for 30 minutes with a SAV-FITC conjugate (1:500, 405201, BioLegend) for indirect staining of aptamers.

On the other hand, cell suspensions were incubated in PBS at 4 °C for 30 minutes with the same viability stain, SAV-FITC conjugate and corresponding labeled isotype antibodies (400322 and 400114, or 338806 and 372804, 1:400, BioLegend). Incubations were also carried out without markers as a control.

After each staining step, cells were washed with washing buffer (PBS with 1% BSA and 0.1% sodium azide) and centrifuge for 5 minutes at 200 g and 4 °C. Cells resuspended in washing buffer were examined by a flow cytometer Accuri C6 (BD). The data from at least 10,000 viable cells from every sample were analyzed by a proper software (FlowJo, Version X 10.0.7r2 for Windows, BD, Ashland, OR, USA). Percentages of different stained populations of viable cells were considered for characterization of CTCs.

Target protein isolation

Suspension of 4×10^6 PC-3 cells in PBS were subjected to protein extraction by cytoplasmic extraction reagents of the NE-PER kit (Thermo Fisher) according to the manufacturer's instructions. Proteins from the resulting extracts were isolated from their binding to D4 or R4 aptamers immobilized on SAv-coupled magnetic beads (Dynabead MyOne Streptavidin T1, Invitrogen, Waltham, MA USA) following the manufacturer's directions. Aptamers were coupled onto the magnetic beads from a 2 μ M aptamer solution in DEPC-treated water, respectively. For binding of target proteins to the aptamers immobilized on the magnetic beads, a buffer solution of 0.01 M PBS with 5 mM $MgCl_2$, 25 mM glucose and 15 mM BSA was used for incubation at 37°C for 1 hour.

Target protein identification

Three target protein solutions for each aptamer (D4 and R4) were quantified by the Pierce BCA Protein assay Kit (Thermo Fisher Scientific) according to manufacturer's directions, mixed and treated with 0.2% surfactant Rapigest SF (Waters, Milford, MA, USA) and reduced with 100 mM dithiothreitol at 60°C for 45 minutes with continuous agitation. Then, protein solutions were alkylated with iodoacetamide 300 mM at room temperature for 30 minutes in the dark and digested by trypsin (1:100, Promega, Madison, WI, USA) at 37 °C overnight with continuous agitation. Digestion was stopped by

incubation with trifluoroacetic acid 0.5% (v/v), for 30 minutes at 37 °C. The peptides were purified and concentrated using ZipTip pipette tips (Millipore, Billerica, MA, USA) following the manufacturer's instructions.

Samples solubilized in trifluoroacetic acid 0.1% (v/v) were analyzed using a liquid chromatograph (1260 Infinity II, Agilent Technologies, Santa Clara, CA, USA) coupled to an electrospray ionization quadrupole time-of-flight mass spectrometer (6520, Agilent Technologies). The peptides were then separated using an AdvanceBio Peptide Mapping column (C18, 120 Å, 2.7 µm, 2.1 x 100 mm, Agilent) and eluted by a mobile phase consisting of solvent A: water with 0.1% formic acid (v/v), and organic solvent B: acetonitrile with 0.1% formic acid (v/v) with a flow rate of 400 µL/minutes. The gradient was as follows: 2% of B (0 minutes), 2% of B (10 minutes), 15% of B (40 minutes), 50% of B (150 minutes), 70% of B (200 minutes), 98% of B (220 minutes), 98% of B (300 minutes), 100% of B (301 minutes) and 100% of B (400 minutes). The capillary voltage was 4000 V, the nebulizer gas flow rate was 8 L/minutes, the pressure was maintained at 45 psi and the temperature, at 325 °C.

Data were analyzed by Spectrum Mill MS Proteomics Workbench program (Agilent Technologies) using the Human UniProt Complete Proteome database (<https://www.uniprot.org/>) to identify the proteins present in the samples. Only proteins from the plasma membrane or extracellular region, and with Distinct Summed MS/MS Search Score larger than two were considered as target protein candidates. The CRAPome repository was also used to rule out common contaminants, considering only proteins with a positive hit in less than 10% of the datasets.

***In silico* binding of aptamers and target proteins**

The molecular structures of the selected target protein candidates were prepared using AutoDock Tools (version 1.5.7) to delete water molecules and add polar hydrogens and Kollman charges,¹⁴ and UCSF Chimera (version 1.15) to rename chains.¹⁵

The blind docking simulations between the predicted most stable structures of D4 and R4 aptamers and their target proteins were performed through HDOCK online server from the Protein Data Bank (.pdb) files of all molecules¹⁶ and pictured by RasMol (Windows version 2.7.5.2).¹⁷ The interactions with the lowest energy score were used to identify the most stable complexes and therefore, the target proteins. The interacting residues between the target proteins and the aptamers were identified by the NUCPLOT program¹⁸ in the PDB Sum Generate web tool (<http://www.ebi.ac.uk/pdbsum>) and the DIMPLOT program in the LigPlot+ tool (version 2.2.5).¹⁹ The non-covalent interactions were also identified by the Protein-Ligand Interaction Profiler (PLIP) algorithm.²⁰

Statistical analyses

PrismGraph software (v.7, GraphPad, La Jolla, CA, USA) was used to perform Student's t-test or Mann-Whitney U test; Kruskal-Wallis test with Dunn's post-test or one-way ANOVA test with Tukey's post-test for comparing groups, as appropriate. All tests considered $p < 0.05$ as statistically significant and were performed at least three times and in triplicate, if not stated otherwise.

RESULTS

The top D4 and R4 aptamer structures

In silico characterization of D4 and R4 aptamer structures revealed five most energetically stable structures for D4 and four most energetically stable structures for R4 (Table 1). Four of the top structures for D4 and all the top structures for R4 shared a structural domain with potential binding functions (Figure 1).

D4 aptamer demonstrated more stability than R4 aptamer in human plasma

The contact of the aptamers with nucleases in human plasma for 24 hours displayed time-dependent degradation profiles. The half-life of D4 was 28.36 hours and the half-life of R4 was only 6.21 hours (Figure 2).

D4 and R4 aptamers bound selectively to tumor prostate cell lines in the ELASA

D4 and R4 aptamers in prostate cell lines indicated a similar selectivity of both aptamers for the tested conditions in the ELASA. Tumor prostate PC-3 cells (used as target cells for the positive selection of the original version of the aptamers) and DU-145 cells displayed an increase in absorbance when compared to non-tumor RWPE-1 cells (used as non-target cells for the negative selection of A4) and PNT-2 cells. Changes in absorbance indicated that both aptamers bind around five times more to DU-145 cells than to PC-3 cells (Figure 3).

Aptacytofluorescence of prostate cell lines backed ELASA results

The same prostate cell lines were subject of fluorescence staining in the same incubation conditions and aptamer concentrations as in the ELASA. Microscopy images support the previous observations about D4 and R4 selectivity (Figure 4).

D4 and R4 aptamers had no effect in PC-3 and RWPE-1 viability and migration

Viability and migration of RWPE-1 and PC-3 cell lines were unaffected by the presence of D4 and R4 aptamers at the tested conditions, as they showed statistically non-significant differences among the times and concentrations tested (Figure 5).

Affinity analysis

According to its K_d , R4 had a strength thirteen times higher to remain bound to PC-3 than to DU-145 cells, whereas the affinity of D4 was greater to DU-145 than to PC-3 cells with only 5nM of difference (Figure 6).

Characterization of D4⁺ and R4⁺ CTCs

D4⁺ and R4⁺ live cells from blood samples of PCa patients were separately stained with antibodies against epithelial (anti-EpCAM) and leukocyte (anti-CD45) markers or stem cell (anti-CD44 and anti-CD133) markers (Figure 7). On one hand, the phenotypes of D4⁺ and R4⁺ CTCs differed for epithelial and leukocyte markers. The largest population of D4⁺ CTCs was epithelial, with EpCAM⁺CD45⁻ cells. Most R4⁺ CTCs were non-epithelial, with a main population of EpCAM⁻CD45⁺ cells followed by a smaller one of EpCAM⁻CD45⁻ cells (Figure 7A). On the other hand, the phenotypes of D4⁺ and R4⁺ CTCs were similar for stem cell markers, with a predominant population of CD44⁺CD133⁻ cells (Figure 7B).

ADAM30, VNN2, DLGAP4, DNAH10OS, MUC6, TMPRSS13, WDR13, HLA-DMB and NXPE2 were identified as candidate target proteins

Around 203 µg/mL of protein were isolated in each of the three aptoprecipitates performed with D4 from PC-3 cells, and over 229 µg/mL of protein were isolated in each one of R4. The peptide spectra of protein solutions precipitated with D4 (Supplementary table 1) and R4 (Supplementary table 2) detected 51 proteins in total: 12 proteins found only in the D4's spectrum, 24 proteins found exclusively in the R4's spectrum and 15 proteins found in both spectra (Figure 8).

The proteins from the spectra that met the three selection criteria (from the plasma membrane or extracellular region, with a Distinct Summed MS/MS Search Score larger than two and with a positive hit in less than 10% of the datasets of the CRAPome repository) were considered as target protein candidates for further analyses were A

disintegrin and metalloproteinase domain 30 (ADAM30), Vanin 2 (VNN2) and Disks large-associated protein 4 (DLGAP4) from D4's spectrum; Dynein axonemal heavy chain 10 opposite strand (DNAH10OS), Mucin 6 (MUC6), transmembrane protease, serine 13 (TMPRSS13) and WD repeat domain 13 (WDR13) from R4's spectrum; and Major histocompatibility complex, class II, DM beta (HLA-DMB) and Neurexophilin and PC-esterase domain family member 2 (NXPE2) from both spectra.

Molecular docking revealed NXPE2-D4c, MUC6-R4i, ADAM30-D4e and NXPE2-R4f as the most stable complexes among the aptamers and candidate target proteins

Based on the HDock scores, the dockings between D4 and R4 aptamers and their target protein candidates predicted the most energetically stable complexes in this order: NXPE2-D4c, MUC6-R4i, ADAM30-D4e and NXPE2-R4f (Table 2).

The *in silico* interface between the amino acids and nucleotides of the four best scoring models formed several kinds of interactions (Figure 9). According to PLIP, there were 20 hydrogen bonds and two salt bridges between the protein and aptamer of the complex ADAM30-D4e (Supplementary table 3). NXPE2-D4c complex included 27 hydrogen bonds, one π -stacking interaction, two π -cation interactions and seven salt bridges (Supplementary table 4), whereas the same protein with R4f aptamer displayed only 16 hydrogen bonds (Supplementary table 5). MUC6-R4i complex showed 25 hydrogen bonds and seven salt bridges (Supplementary table 6).

DISCUSSION

Selecting the single-stranded DNA aptamer D4 or the RNA aptamer R4 for further research on their applications for PCa it is a choice that depends on the results presented in this paper. Both kinds of aptamers have proved their effectiveness in different

settings.²¹ However, their properties and functionalities may be different in specific conditions and they can determine the best aptamer in every case.

The lowest free energy and highest melting temperature (T_m) for D4 and R4 were found in the D4a and R4f structures, respectively. Consequently, these structures may be the most thermodynamically stable and feasible ones for every aptamer.

In silico predicted R4 structures were considered superior in terms of thermodynamics. RNA 3D-structures are considered more intricate than ssDNA 3D-structures, mainly due to the presence of the functional group 2'OH.²² The fact that the original aptamer was selected from an RNA library¹⁰ may also explain it. However, *in silico* strategies to study aptamers are limited for aptamers of ssDNA because algorithms are mainly based on RNA aptamers and databases for DNA aptamers are rare.²³ Consequently, our study is limited by the *in silico* resources available at this moment.

The structural domain repeated in D4 and R4 aptamers is a conserved hairpin tetraloop closed by two guanosine-cytosine base pairs that was observed in previous versions of these aptamers,¹³ and it may function as a binding site for their common target protein. This domain is not found in D4c aptamer structure, and it is not the richest region of interactions in the complex ADAM30-D4e. However, it is the second most important interfacing region for NXPE2-R4a and the main one for MUC6-R4d.

The fact that the only D4 conformation without the potential binding region (D4c) showed the best docking result with the complex formed with NXPE2 may be partially explained because a conformation with a higher affinity to the target is not necessarily the most stable in terms of free energy.²⁴ Moreover, within aptamer-protein complexes, aptamers tend to selectively bind to positively charge areas that usually attract nucleic acids simply due to their opposite charge.²⁵

As it was expected, degradation for the RNA aptamer was faster than that for the DNA aptamer certainly because of its highly reactive hydroxyl groups.²⁶ Conjugation with inverted thymidine and biotin used to stabilize these aptamers from nuclease activity was insufficient. However, the fact that D4 had a prolonged presence supports the rationale for the synthesis of this DNA homolog: to reduce the cost of production, to ease the handling, and to extend the degradation time in the presence of nucleases in clinical applications such as diagnosis by flow cytometry from blood samples.⁴ For theranostic applications, protection against nucleases is generally gained after polyethylene glycol (PEG) coupling.⁶ Thus, further modifications may enhance the performance of D4 and R4 aptamers in biological samples.

In the ELASA, D4 and R4 aptamers demonstrated a selective binding to tumor prostate cell lines, specially to DU-145 cells, in comparison to non-tumor prostate cell lines. These results were further corroborated by the aptacytofluorescence assay. The microscopy showed both aptamers bound to tumor prostate cells. Aptamers are anionic molecules that are unable to cross the plasma cell membrane by themselves; but can be internalized by endocytosis, micropinocytosis or phagocytosis mechanisms depending on the function of their target molecules.²⁷ Further investigation of the aptamer subcellular location with 3D microscopy techniques or co-localization assays without permeabilized cells are needed.

The use of a hormone-dependent cell line such as LNCaP could have been used to test the selectivity of the aptamers to prostate cells other than the classical hormone-independent ones (DU-145 and PC-3) and to better assess the ability to detect tumor progression and metastasis. In this way, we could have compared with the aptacytofluorescence results from A4 when incubated with LNCaP cells. However, all the aptamers (A4, D4 and R4) showed similar fluorescence patterns when incubated with

PC-3 and RWPE-1 cells in this assay.¹⁰ Therefore, they seem to have a similar cellular selectivity.

The binding of aptamers to their targets may trigger a cellular response, such as inhibition or promotion of cell death or migration. Considering that we used standard concentrations and times for the aptamer tests, the lack of a significant reaction in the viability and migration of non-target RWPE-1 cells may indicate that the aptamers do not bind to these cells. The absence of a significant response in target PC-3 cells may mean that despite binding to them, the target molecule does not have sufficient expression to trigger a response in these cells, it is involved in constitutively active downstream signaling pathways²⁸ related to cell viability and migration or it is involved in signaling pathways unrelated to cell viability and migration.

Few reported aptamers lack effects in the function of their target proteins or cells because researchers prioritize the study of inhibitory aptamers.²⁵ Therefore, D4 and R4 aptamers may be used directly for diagnostic approaches, but they need to be modified with therapeutic agents for PCa treatment proposals.

Taking into consideration the conditions for the selection of the original aptamer,¹⁰ predictably the RNA aptamer R4 showed the highest affinity to PC-3 cells. However, R4 displayed the largest K_d to DU-145 cells, so that it is not able to bound so tightly to these tumor cells. On the other side, D4 showed a similar affinity to both tumor prostate cells. Since both R4 and D4 bound with more selectivity to DU-145 cells in the ELISA, it may evidence that they bind weakly to more than one motif on their surface. The higher expression of the target molecule on DU-145 cells may be another plausible explanation. The K_d between PC-3 cells and both D4 and R4 improved when compared to the one between these cells and the original aptamer, as it was around 55 and 65 nM higher, respectively.¹⁰

In the present study, we used a dual immune approach to identify stem cell and epithelial cell characteristics on CTCs from blood samples of PCa patients that bind to D4 and R4 aptamers. Biomarkers related to the EMT and its reverse process –the mesenchymal-epithelial transition (MET)– may become evident on CTCs during metastasis. These CTCs go through hybrid transition states, with intermediate phenotypes that show different levels of epithelial and mesenchymal features.^{29,30}

Target CTCs of D4 and R4 aptamers showed mesenchymal stem cell characteristics due to their binding to CD44⁺ cells. As CD44 is an independent predictor of shorter survival in PCa patients treated with docetaxel chemotherapy,³¹ these aptamers could be used as molecular tools for therapy personalization. Pre-cancerous lesions of prostate cancer also express the cell adhesion molecule CD44. In this case, CD44 acts as a receptor of the osteopontin secreted by tumor-associated macrophages that activates cell proliferation via Akt and JNK pathways.³² D4 and R4 aptamers could be used to identify PCa patients in an early phase –when the tumor is localized, as in the case of the volunteer patients in this study– to prevent the spreading of metastatic cells. Their use in liquid biopsies could assist in both cancer diagnosis and interception. Thus, PCa interception would contribute to strategies for the long-awaited cancer prevention.³³

While D4 aptamers bind to CTCs with a more epithelial profile, R4 aptamers bind to non-epithelial cells. Due to the dynamic expression of markers in CTCs undergoing EMT and MET,³⁴ D4 and R4 aptamers may detect CTCs in different stages of tumor progression. Therefore, the identification of the surface biomarkers CD44 and EpCAM on CTC ligands of D4 and R4 aptamers has promising clinical applications in patient stratification. The detection of different profiles of CTCs is historically relevant since most of the CTC detection strategies are based only on the selection of EpCAM⁺ cells and they have ignored other types of CTCs.^{35,36}

The spectra of the aptoprecipitates of D4 and R4 aptamers showed that they have both unique and shared target protein candidates. The different proteins found in the aptoprecipitates may be because the aptamers have different sugars, so they are different molecules despite having the same sequence of nitrogenous bases. The presence of several proteins in the spectra may indicate non-specific binding of both aptamers that requires to be reduced by further aptamer optimization to enhance and validate their use as theranostic tools in complex samples such as blood.³⁷ This may also suggest that the modifications to the original aptamer A4 impaired the specificity of D4 and R4 aptamers, or even that the SELEX procedure had a low efficiency and selected not specific enough aptamers.³⁸

According to the most stable molecular dockings, we can consider NXPE2 as the most probable target protein for both aptamers; MUC6, for R4 aptamer; and ADAM30, for D4 aptamer. Nevertheless, these targets were selected from a blind SELEX technique and they require additional confirmation,³⁹ for instance with aptamer-antibody competition assays by confocal microscopy and flow cytometry.

The protein NXPE2 has no identified function.⁴⁰ However, future research may uncover an interesting biomarker. In the human male reproduction tissues, NXPE2 is expressed, particularly in the testis.⁴¹ NXPE2 was associated to inflammatory diseases of the digestive system and lately it was defined as a new predictive indicator for diffuse-type gastric cancer involved in tumor cell migration and proliferation.⁴² At the level of its mRNA, alternative splicing events were considered as prognostic markers for colorectal cancer.⁴³

Mucins are glycoproteins highly coated with O-glycans that constitute the main component of mucus secretions establishing the protective barriers of internal surfaces. Recently, the gene expression of *MUC6* was reported to be associated with a higher risk

of aggressive PCa. However, further research is needed to determine its theranostic value.⁴⁴ Intriguingly, other mucins such as MUC1 have already been studied in the field of cancer theranostics as a target for aptamer-based approaches.⁴⁵

MUC6 aberrant expression may lead to EMT and is known to play an important role in tumorigenesis, cancer progression, metastasis, and recurrence. In spite of that, its role remains uncertain in several types of cancers.^{46,47} For example, promotor methylation downregulates *MUC6* expression leading to cancer progression in gastric cancer.⁴⁸ Overexpression of *MUC6* was associated with favorable survival outcomes in intermediate stages of colorectal cancer.⁴⁹ *MUC6* expression is downregulated in invasive stomach, pancreas, and bile duct cancers and it is a positive prognostic marker of disease-free survival in lung cancer.⁵⁰

ADAM30 is able to promote the spread of tumor cells to adjacent and distant normal tissues with its proteolytic activity. ADAMs degrade the extracellular matrix and shed the ectodomain of transmembrane proteins such as growth factors, cytokines, receptors and adhesion molecules participating in different phases of the metastatic process.^{51,52} Depending on the substrate, ADAMs act as signaling scissors⁵³ promoting uncharacterized downstream effects that need further analyses.⁵⁴ Overexpression of *ADAM30* has been reported in endometrial tumors and it is associated to endometrial cancer diagnosis and immunotherapy decision making.⁵⁵

The analysis of the structures of D4 and R4 together with our experimental and modelling data shed some light upon the future performance of these aptamers as theranostic tools for PCa. From the parent aptamer A4, the truncated aptamers D4 and R4 have proved to be more suitable tools in the context of clinical applications. Additional chemical modifications may help to improve their stability and binding in clinical settings and the physiological conditions found in body fluids such as blood. Meanwhile, the

identified interacting proteins NXPE2, MUC6 and ADAM30 require further validation as PCa biomarkers. Currently, we need more data from both aptamers to weigh up their pros and cons and determine the best choice for every clinical scenario.

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FIGURE LEGENDS

Figure 1. *In silico* aptamer structures. **A.** D4 aptamer sequence. **B.** (a, b, c, d, and e) Most stable 2D structures of D4 aptamer. **C.** Corresponding 3D structures of D4 aptamer. **D.** R4 aptamer sequence. **E.** (f, g, h, and i) Most stable 2D structures of R4 aptamer. **F.** Corresponding 3D structures of R4 aptamer. Deoxyribonucleotides are represented as 'd_' and ribonucleotides, as 'r_'. Green dots indicate a common base pair between the minimum free energy (MFE) and centroid structure models for each aptamer, red dots indicate base pairs only in the centroid model, and blue dots indicate base pairs only in the MFE model. Balls in blue represent nitrogen; balls in red, oxygen; balls in grey, hydrogen; balls in orange, phosphorus; and balls in green, carbon. The putative binding domain is shown in yellow. 2D structures were obtained by the Mfold server. 3D structures were obtained by the RNA Composer server and pictured by the Discovery Studio Visualizer software.

Figure 2. Degradation profile of D4 and R4 aptamers incubated in human plasma up to 24 hours. Non-linear one-phase decay model was used to calculate the half-lives. Values represent the mean of triplicate incubations of three independent experiments, where plasma from three patients were pooled and incubated with 33.34 μ M aptamer solutions at 37 °C for different times. $t_{1/2}$, half-life.

Figure 3. Selectivity of D4 and R4 aptamers to prostate cell lines. **A.** PC-3 cells incubated with no aptamer (control), D4 or R4 aptamers at 2 μ M for 1 hour at 37 °C. **B.** DU-145 cells. **C.** RWPE-1 cells. **D.** PNT-2 cells. Values represent the mean of triplicates + standard deviation (SD) of the absorbance of the cells at 492 nm, from three independent ELISA experiments. * $p < 0.05$.

Figure 4. Binding of D4 and R4 aptamers in representative fluorescence microscopy images of fixed prostate cells. **A.** PC-3 cells. **B.** DU-145 cells. **C.** RWPE-

1 cells. **D.** PNT-2 cells. Cells were incubated with SAv-FITC (1:200), (control, first row); D4 aptamer at 2 μ M and SAv-FITC (1:200), (D4, second row); or R4 aptamer at 2 μ M and SAv-FITC (1:200), (R4, third row). First column: overlay of bright field, nuclei (blue) and aptamer (green) images. Second column: nuclei images (TO-PRO3 iodide, 633 nm, blue). Third column: aptamer images (green, 488 nm, FITC). Scale bar 50 μ m. FITC, fluorescein; SAv, streptavidin.

Figure 5. Effects of D4 and R4 aptamers in cellular parameters of prostate cell lines. **A.** Viability of PC-3 cells incubated with D4 and R4 at 0, 1 and 2 μ M for 24 and 48 hours and analyzed by the Neutral Red assay. **B.** Migration of PC-3 cells incubated with D4 at 0, 1 and 2 μ M for 24 and 48 hours and analyzed by the Wound Healing assay. **C.** Migration of PC-3 cells incubated with R4 in the same conditions. **D.** Viability of RWPE-1 cells incubated with D4 and R4 at 0, 1 and 2 μ M for 24 and 48 hours and analyzed by the Neutral Red assay. **E.** Migration of RWPE-1 cells incubated with D4 at 0, 1 and 2 μ M for 24 and 48 hours and analyzed by the Wound Healing assay. **F.** Migration of RWPE-1 cells incubated with R4 in the same conditions. Values represent the mean percentage + standard deviation (SD) of live cells and wound closure of triplicates of three independent experiments, respectively.

Figure 6. Equilibrium dissociation constants (Kd) of D4 and R4 in tumor prostate cell lines from the One site specific binding equation: $Y=B_{max} \cdot X/(K_d+X)$. **A.** Mean fluorescence intensity (MFI) of PC-3 cells at concentrations of aptamers between 200 and 0 nM. **B.** MFI of DU-145 cells at concentrations of aptamers between 200 and 0 nM. a. u.: arbitrary units.

Figure 7. Immunophenotyping of D4⁺ and R4⁺ CTCs from prostate cancer patients.

Data represent the percentage of aptamer D4 (D4⁺, left) or R4 (R4⁺, right) positive live cells stained with corresponding antibodies from blood samples of eight prostate cancer

patients diagnosed with localized PCa. **A.** Distribution of the four possible phenotypes of live cells stained with EpCAM and CD45 antibodies in the blood samples. **B.** Distribution of the four possible phenotypes of live cells stained with CD44 and CD133 antibodies in the blood samples. Boxes represent the median values $\pm$ standard deviation (SD), whiskers represent minimum and maximum values and '+' symbols into the boxes represent the mean values. CTCs, circulating tumor cells.

Figure 8. Heatmap analysis of mass spectrometry-based proteomics. List of proteins found in solutions precipitated with D4 and R4 aptamers. Rows display the proteins and columns represent the solutions. Color of the squares indicate the abundance of the proteins in the spectra: green palette, from dark green (high) to light green (low), and red (absence).

Figure 9. *In silico* binding of top complexes between D4 and R4 aptamers and target protein candidates. **A.** ADAM30-D4e, **B.** NXPE2-D4c, **C.** NXPE2-R4f and **D.** MUC6-R4i complex images by RasMol and schematic diagram of their interaction interface by NUCPLOT. Complexes: ADAM30 in purple, NXPE2 in light blue and MUC6 in red. D4 in dark blue and R4 in light green. Diagram: hydrogen bonds and nonbonded contacts are represented as blue and red dotted lines, respectively.

BACKGROUND

Aptamers still have a long way to go before they are used in the clinical setting. Herein we show the preclinical study of two aptamers applied in the context of prostate cancer, a type of cancer that has evidenced the need for alternative diagnostic and therapeutic strategies for a long time.

TRANSLATIONAL SIGNIFICANCE

This article reports the characteristics of two aptamer candidates for prostate cancer theranostics. Although not all the features described are desirable outcomes for clinical uses, the significance of this study lies in the applicability of the methods displayed to bring aptamers closer to clinical practice. In addition, new potential biomarkers for prostate cancer have been revealed.

FIGURES AND TABLES

Table 1. Thermodynamic parameters in a 50 mM Na⁺ solution at 25 °C (298 K) and melting temperatures (T_m) of aptamer structures.

Structure	ΔG (kJ/mol)	T _m (°C)	ΔH (kJ/mol)	ΔS (J/K·mol)	
D4	a	-7.15	48.2	-99.16	-308.57
	b	-5.86	35.3	-174.89	-566.93
	c	-4.48	36.5	-120.08	-387.77
	d	-4.35	33.8	-151.88	-494.76
	e	-4.02	43.2	-69.45	-219.53
R4	f	-39.16	70.6	-294.97	-857.97
	g	-29.58	66	-244.76	-721.74
	h	-27.5	62.1	-245.60	-732.53
	i	-25.27	62.4	-226.77	-675.84

Table 2. Energy scores of the best molecular docking results between each of the top aptamer structures and the target protein candidates, based on the HDock algorithm.

	D4					R4			
	a	b	c	d	e	f	g	h	i
ADAM30	-334.06	-327.46	-330.29	-332.16	-346.68	-	-	-	-
VNN2	-333.21	-264.38	-319.56	-269.54	-290.27	-	-	-	-
DLGAP4	-239.45	-208.34	-242.67	-210.07	-229.56	-	-	-	-

HLA-DMB	-295.99	-281.88	-348.44	-310.39	-335.94	-313.18	-313.18	292.81	-299.01
NXPE2	-322.31	-292.23	-386.9	-321.99	-320.55	-320.72	-320.72	-286.01	-306.06
DNAH10OS	-	-	-	-	-	-301.9	-301.9	-299.61	-290.14
MUC6	-	-	-	-	-	-329.75	-329.75	-333.4	-358.67
TMPRSS13	-	-	-	-	-	-319.63	-319.63	-313.15	-335.54
WDR13	-	-	-	-	-	-318.08	-318.08	-309.18	-295

Bold scores are the best ones.

Figure 1

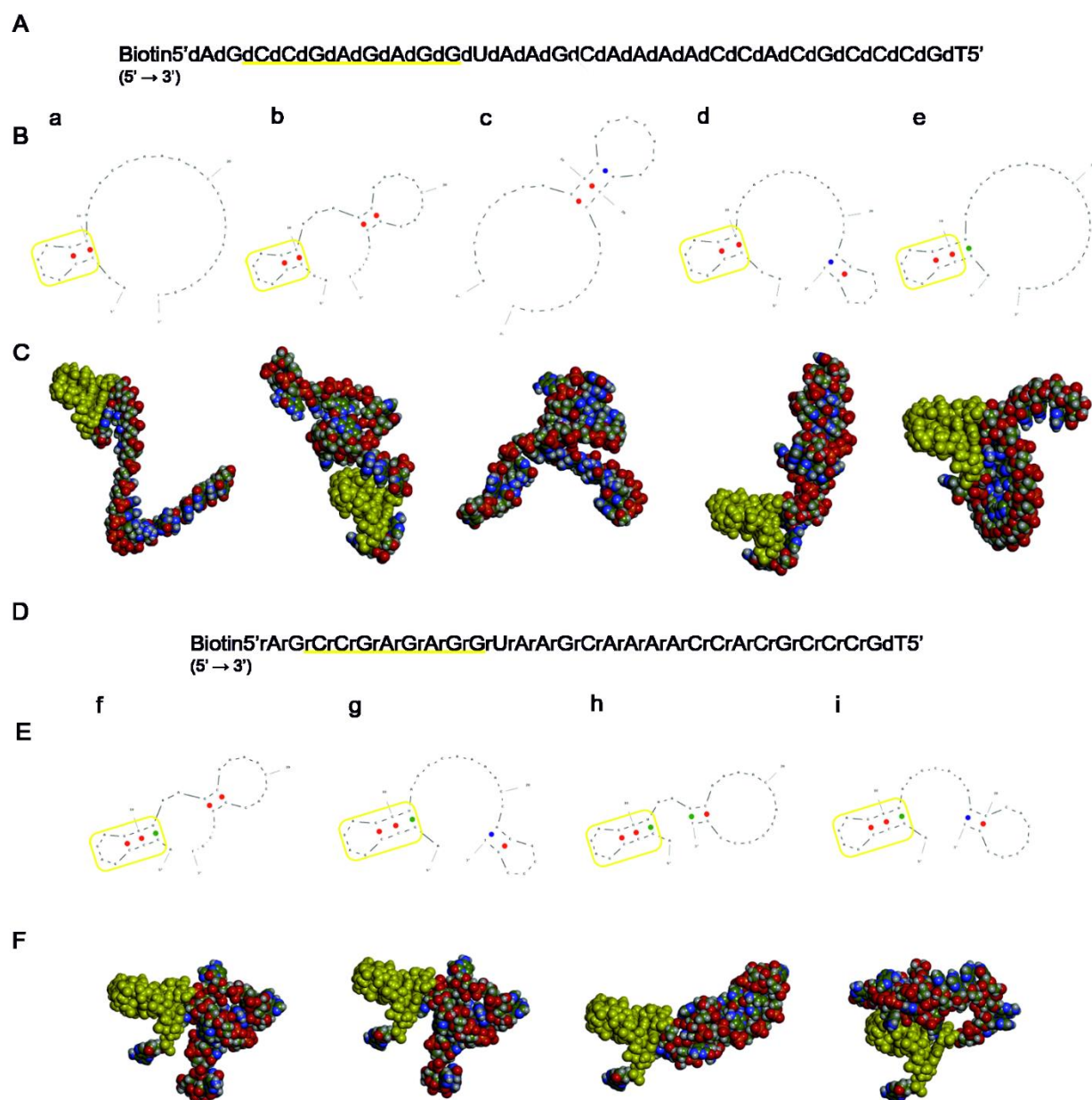


Figure 2

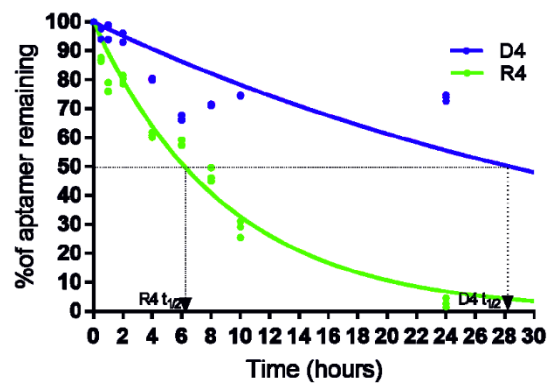


Figure 3

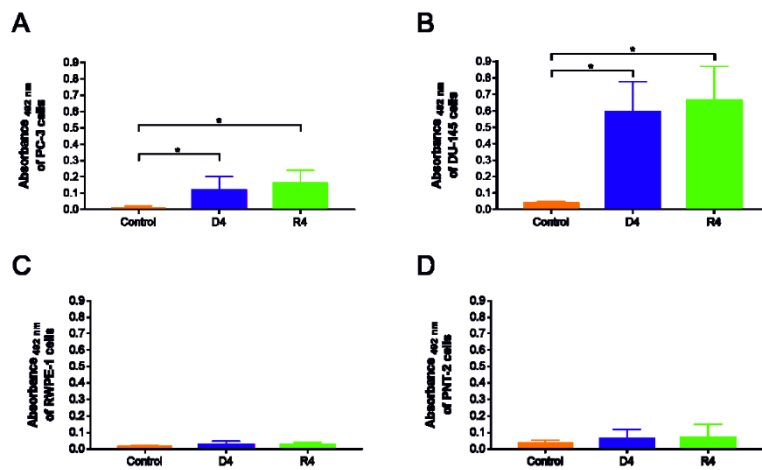


Figure 4

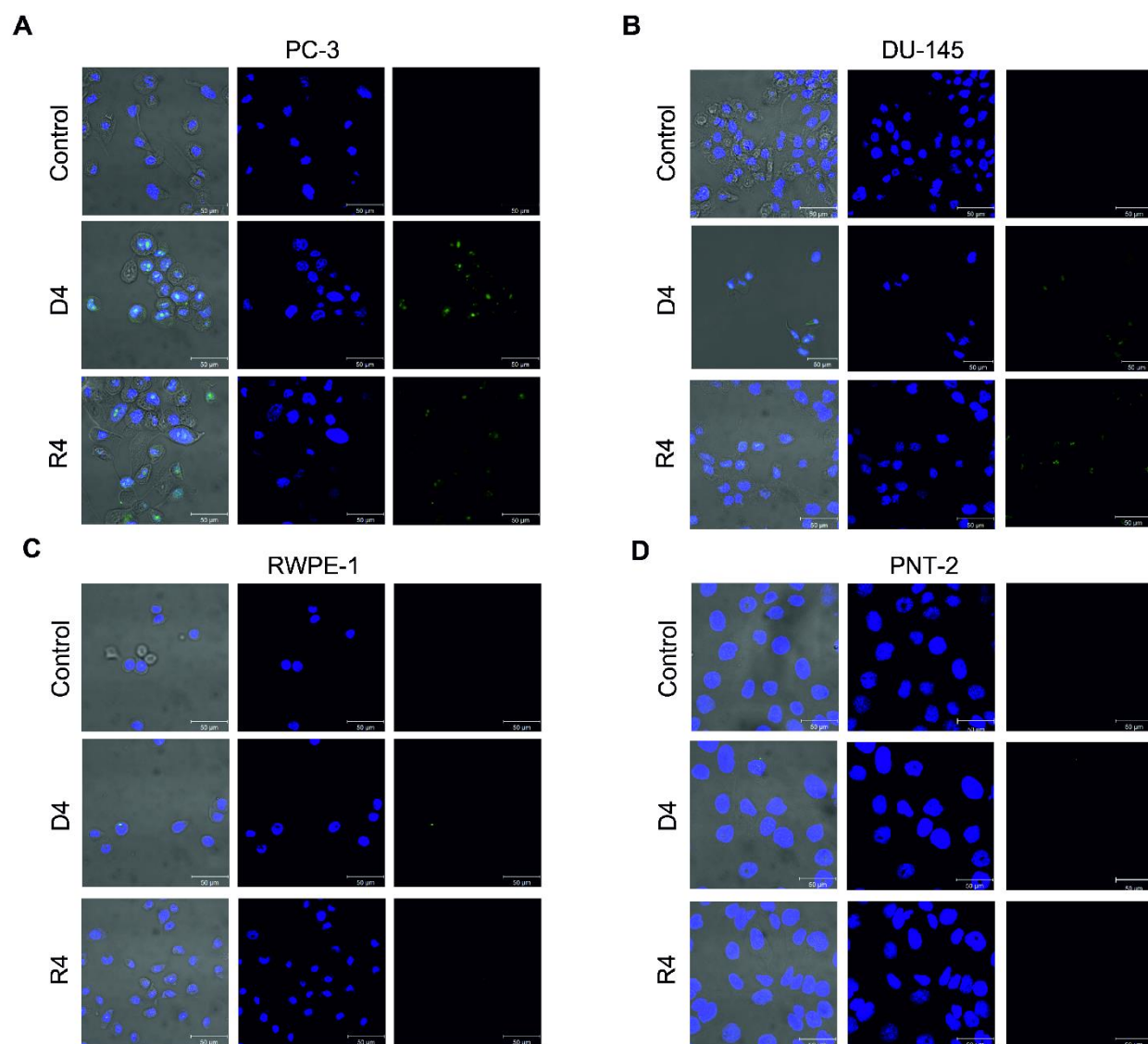


Figure 5

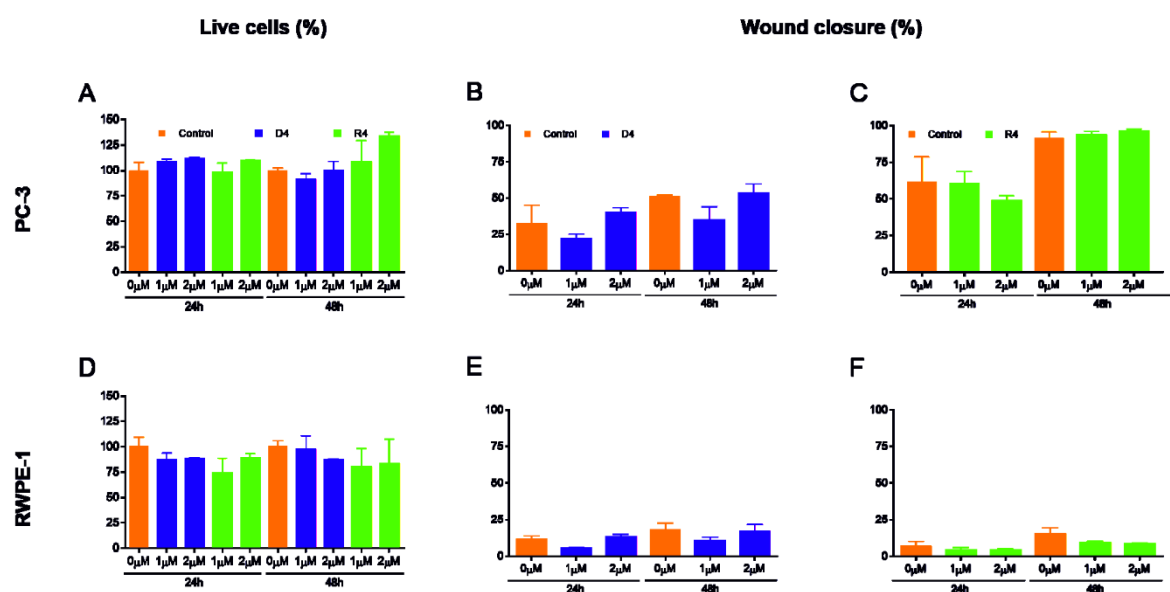


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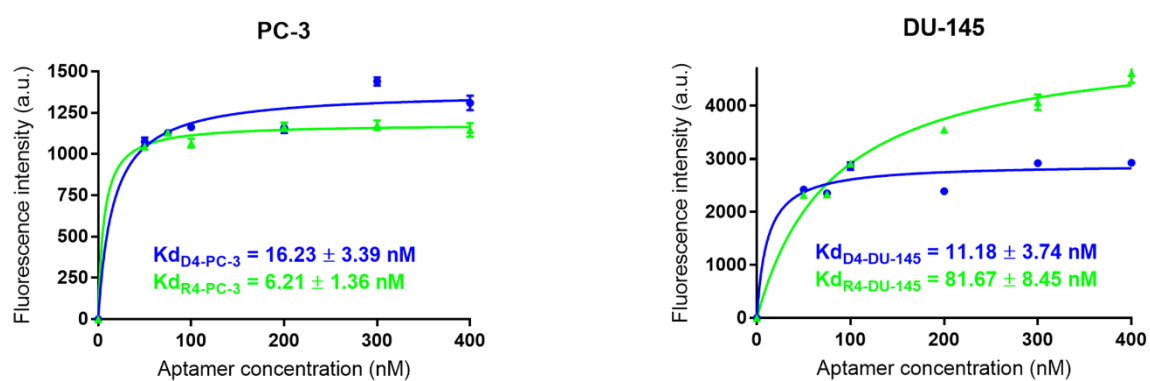


Figure 7

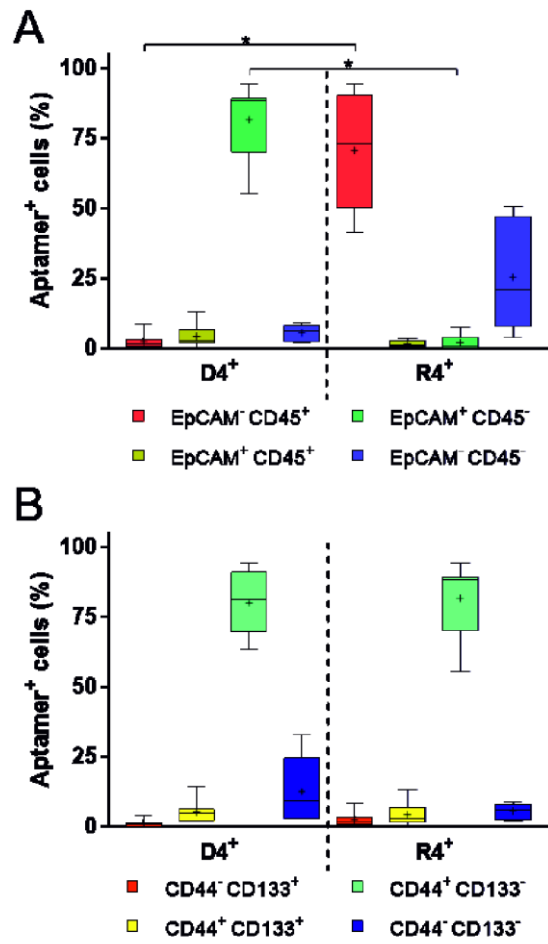


Figure 8

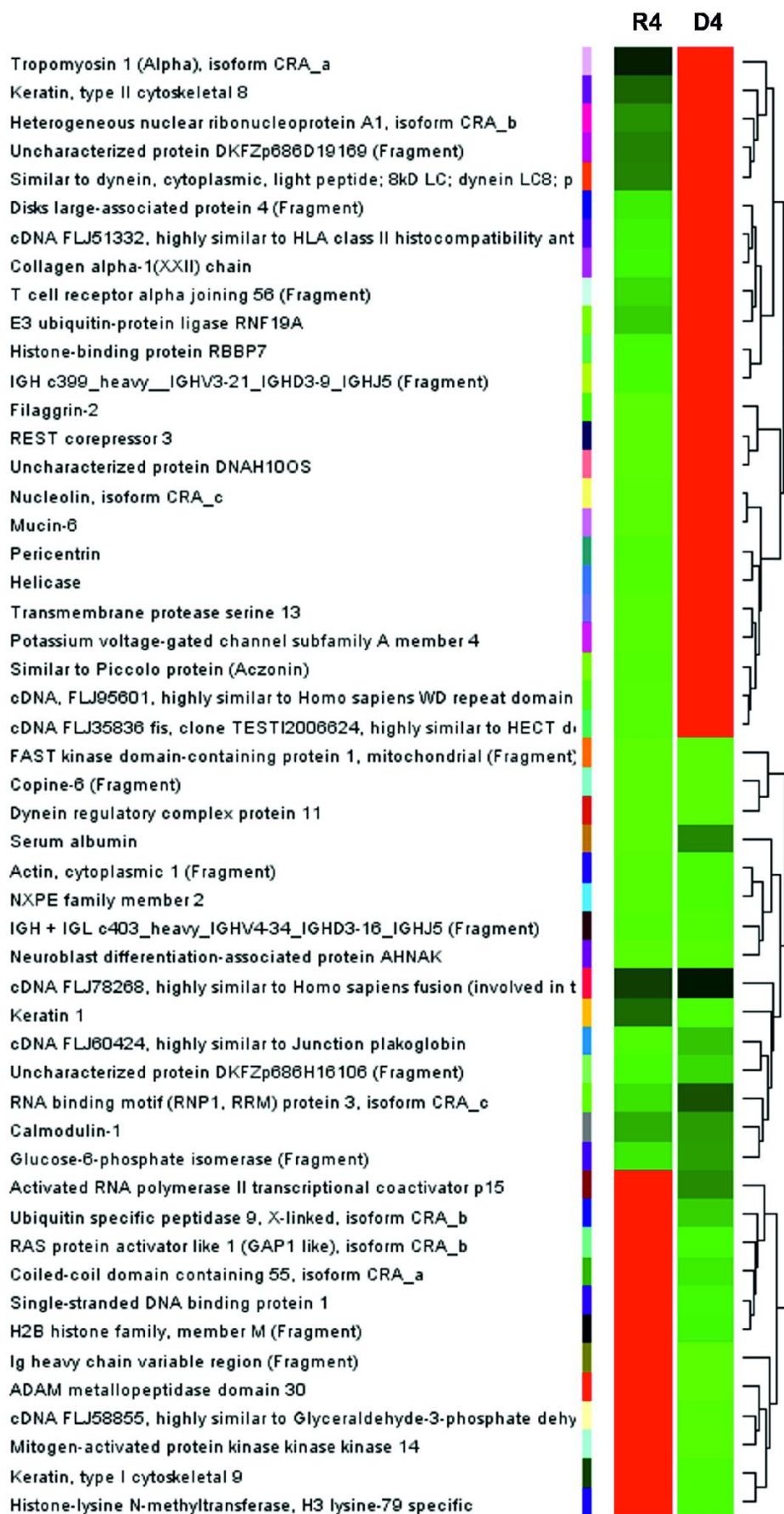
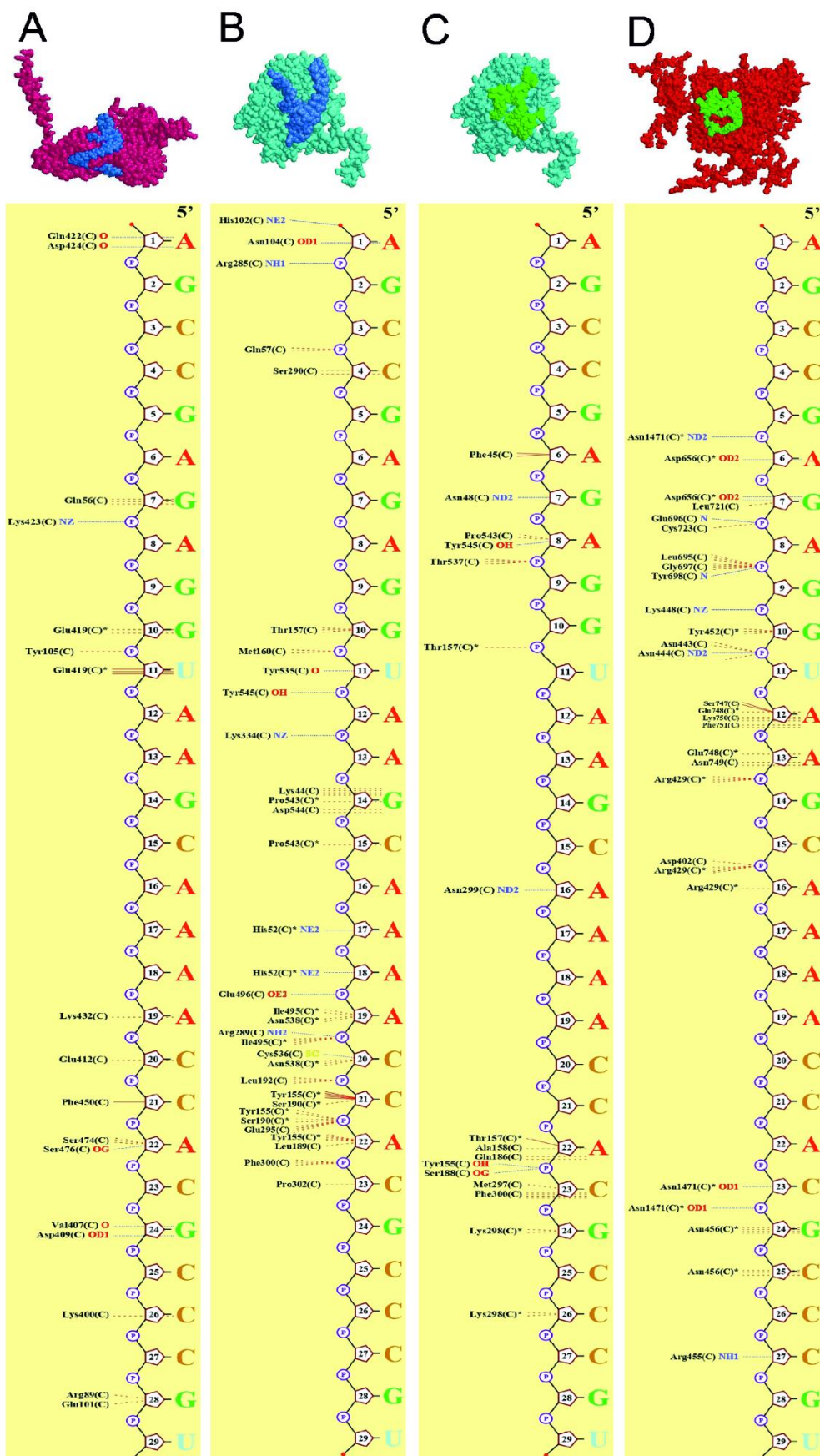


Figure 9



SUPPLEMENTARY FIGURES AND TABLES

Supplementary table 1. Hits of the mass spectrometry analysis for the protein solution precipitated with D4 aptamer.

Group	Subgroup	Spectra	Distinct peptides	Distinct summed MS/MS search score	% Amino acid coverage	Total protein spectral intensity	Protein molecular weight (Da)	Database accession
1	1.1	29	14	133.98	27.6	9.77E+05	66222.7	H6VRF8
2	1.2	5	2	15.68	4.7	1.63E+05	53703.3	P05787
3	2.1	24	12	133.4	32.5	9.58E+05	62291.5	P35527
4	3.1	18	8	79.57	24	4.98E+05	44105.2	P08727
5	4.1	12	8	47.86	52.7	2.02E+05	17259.4	A4D1U3
6	5.1	14	5	44.64	8.2	1.41E+06	71222.2	A0A0C4DGB6
7	6.1	10	4	30.93	17.7	3.30E+05	34426.5	A0A2R8Y793
8	7.1	2	2	12.68	3.6	2.34E+04	70531.2	B3KXU8
9	8.1	3	2	12.16	21	2.49E+04	17170.1	A0A024QYX3
10	9.1	2	2	11.97	22	3.04E+04	16965.4	A0A590UK56
11	10.1	2	2	10.97	22.5	1.64E+04	16077.2	B4E2A3
12	11.1	3	2	9.68	4.1	2.92E+04	53638.2	A8K4H1
13	12.1	1	1	5.53	14.1	1.13E+04	12110.7	B4DVC2
14	13.1	1	1	5.52	1.5	8.92E+03	59054.3	P13645

15	14.1	1	1	5.17	2.6	4.22E+03	58610.5	B3KM80
16	15.1	2	1	5.02	0.5	2.43E+05	629603.7	Q09666
17	16.1	1	1	4.74	4.8	6.23E+04	21429.7	J3QRE9
18	17.1	1	1	4.32	7.9	3.19E+03	30659.7	A0A384NL53
19	18.1	1	1	4.02	13.2	1.38E+04	25080.9	J3KQ55
20	19.1	1	1	4.01	0.7	1.54E+06	249437.6	Q5D862
21	20.1	2	1	3.78	16.6	5.69E+05	11006.1	A0A2Y9CYF9
22	21.1	1	1	3.7	4.7	9.97E+03	14395.1	P53999
23	22.1	1	1	3.54	8	1.10E+06	16755.8	H0YNM7
24	23.1	1	1	3.29	5.1	1.11E+05	23539.6	B4DRV9
25	24.1	1	1	3.21	0.6	8.57E+05	96565.7	A0A0A0MSY6
26	25.1	1	1	3.03	2	2.01E+05	91449.8	Q8TBZ7
27	26.1	1	1	2.99	1	5.23E+04	165665	A0A1C9J732
28	27.1	1	1	2.69	0.2	2.13E+04	321432.1	Q6AI22
29	28.1	1	1	2.53	1.4	5.26E+04	65641.8	Q96DL1
30	29.1	1	1	2.49	0.7	1.96E+04	274501.4	D3DWB6
31	30.1	1	1	2.27	16.3	2.01E+05	12009.6	C9JMM4
32	31.1	1	1	2.16	16.3	8.73E+04	13690.1	A0A5C2G9M0
33	32.1	1	1	1.79	2.1	1.22E+04	31661.6	A0A0J9YX90
34	33.1	1	1	1.71	19.2	2.75E+04	5866.9	A9UJN3

35	34.1	1	1	1.44	2.6	3.45E+04	91037.6	A0A024RBK8
36	35.1	1	1	0.78	1.9	2.45E+04	60668.6	A0A024QZ33
37	36.1	1	1	0.53	2.5	1.44E+05	105351.9	Q99558

Supplementary table 2. Hits of the mass spectrometry analysis for the protein solution precipitated with R4 aptamer.

Group	Subgroup	Spectra	Distinct peptides	Distinct summed MS/MS search score	% Amino acid coverage	Total protein spectral intensity	Protein molecular weight (Da)	Database accession
1	1.1	25	14	130.31	34.7	1.08E+06	44105.2	P08727
2	2.1	27	12	105.22	24.4	9.21E+05	58610.5	B3KM80
3	3.1	19	6	55.8	8.2	3.24E+06	71222.2	A0A0C4DGB6
4	4.1	9	3	34.91	17.6	3.03E+05	42360.1	H0Y449
5	5.1	12	4	33.31	17.7	5.19E+05	34426.5	A0A2R8Y793
6	6.1	8	4	33.27	6.2	1.52E+05	66222.7	H6VRF8
7	7.1	4	4	24.09	9.3	3.67E+04	53638.2	A8K4H1
8	8.1	4	3	20.91	26.6	1.12E+05	16965.4	A0A590UK56
9	9.1	4	2	17.37	21	6.53E+04	17170.1	A0A024QYX3
10	10.1	3	2	15.91	4.7	2.36E+04	53703.3	P05787
11	11.1	4	2	13.75	3.6	4.43E+04	70531.2	B3KXU8

12	12.1	2	2	13.72	3.5	1.46E+04	59054.3	P13645
13	13.1	3	2	11.31	4	2.69E+04	62291.5	P35527
14	14.1	1	1	8.06	13.4	4.91E+03	11504.1	K7EJ44
15	15.1	1	1	7.76	7.7	8.23E+03	16077.2	B4E2A3
16	16.1	2	1	6.54	4.3	1.34E+04	35122	B4DMY3
17	17.1	1	1	6.34	13.4	8.91E+03	11232.5	Q6MZP9
18	18.1	2	1	5.97	16.3	9.95E+05	12009.6	C9JMM4
19	19.1	2	1	5.06	0.5	3.47E+05	629603.7	Q09666
20	20.1	1	1	4.83	2.6	2.36E+06	42144.3	B4DV59
21	21.1	1	1	4.8	8	2.36E+06	16755.8	H0YNM7
22	22.1	1	1	4.73	4.3	1.05E+04	38860.1	A0A024RAZ7
23	23.1	1	1	4.56	4.9	2.85E+03	32734.1	A0A024R5W6
24	24.1	1	1	4.33	0.6	1.42E+06	96565.7	A0A0A0MSY6
25	25.1	1	1	4.14	3.2	1.18E+05	54262.7	B2RBN3
26	26.1	1	1	4	18.4	2.41E+04	14055.1	A0A087X246
27	27.1	1	1	3.87	1.8	1.46E+05	64250	Q9BYE2
28	28.1	1	1	3.74	16.3	9.70E+04	13690.1	A0A5C2G9M0
29	29.1	1	1	3.71	38	2.17E+04	2223.6	A0A075B6Z2
30	30.1	1	1	3.61	0.2	3.76E+04	321432.1	Q6AI22
31	31.1	1	1	3.58	1	1.15E+05	55414.7	B3KSA4

32	32.1	1	1	3.54	14.1	2.57E+04	12110.7	B4DVC2
33	33.1	1	1	3.51	11	2.29E+06	17993	P0CZ25
34	34.1	1	1	3.45	0.2	1.10E+05	411895.6	A4D1A8
35	35.1	1	1	3.17	0.7	3.41E+06	249437.6	Q5D862
36	36.1	1	1	3.1	2.1	2.39E+04	31661.6	A0A0J9YX90
37	37.1	1	1	2.74	1.4	1.60E+05	65641.8	Q96DL1
38	38.1	1	1	2.72	1.3	1.77E+05	263306.4	A0A0G2JN42
39	39.1	1	1	2.7	7.4	3.61E+04	47279.6	E9PC52
40	40.1	1	1	1.51	1.9	1.31E+05	74111.8	P22459
41	41.1	1	1	1.48	0.4	8.39E+04	380826.2	O95613
42	42.1	1	1	1.44	32.2	3.92E+04	13597	A0A5C2G615
43	43.1	1	1	1.18	1.4	2.67E+04	162169.4	Q8NFW1
44	44.1	1	1	1.13	4.7	9.32E+03	33054.5	A4D1D1
45	45.1	1	1	0.81	1.9	9.56E+04	94099.8	A0A126LB09
46	46.1	1	1	0.07	3.1	1.93E+04	92691.7	Q9NV58

Supplementary table 3. Interactions in ADAM30-D4e complex interface based on PLIP.

Hydrogen bond	Residue	Amino acid	Distance H-A	Distance D-A	Donor angle
1	56C	GLN	3.00	3.57	118.43

2	56C	GLN	2.64	3.59	163.67
3	89C	ARG	2.61	3.46	143.74
4	105C	TYR	2.19	3.13	162.33
5	105C	TYR	3.49	3.92	110.84
6	287C	ARG	3.06	4.03	167.36
7	400C	LYS	2.45	3.40	154.67
8	400C	LYS	3.26	4.06	138.69
9	409C	ASP	2.40	2.84	108.31
10	419C	GLU	2.63	3.53	161.76
11	419C	GLU	2.80	3.53	131.12
12	419C	GLU	2.79	3.49	128.24
13	422C	GLN	2.78	3.47	127.48
14	424C	ASP	2.02	2.71	125.41
15	432C	LYS	2.78	3.65	142.71
16	432C	LYS	2.21	3.11	150.25
17	432C	LYS	2.64	3.46	141.29
18	474C	SER	3.00	3.57	118.86
19	476C	SER	2.36	3.23	148.78
20	476C	SER	3.69	4.02	102.67

Salt bridge	Residue	Amino acid	Distance	Ligand group
1	409C	ASP	4.57	Guanidine
2	423C	LYS	3.30	Phosphate

H, hydrogen atom; A, acceptor atom; D, donor atom.

Supplementary table 4. Interactions in NXPE2-D4c complex interface based on PLIP.

Hydrogen bond	Residue	Amino acid	Distance H-A	Distance D-A	Donor angle
1	41C	ASP	2.92	3.87	167.37
2	44C	LYS	2.66	3.65	163.37
3	48C	ASN	3.46	3.85	106.31
4	57C	GLN	2.73	3.20	109.66
5	104C	ASN	1.68	2.50	137.70
6	104C	ASN	2.95	3.60	125.03
7	188C	SER	2.52	3.37	145.18
8	190C	SER	3.55	3.90	104.00
9	285C	ARG	3.37	3.89	114.71
10	285C	ARG	3.50	3.98	113.44
11	288C	HIS	3.35	3.98	125.24
12	290C	SER	3.22	3.99	136.80
13	290C	SER	2.64	3.10	108.74
14	290C	SER	3.37	4.10	133.87
15	291C	ASN	2.98	3.70	131.58
16	295C	GLU	1.40	2.36	169.83
17	300C	PHE	3.39	3.74	103.19
18	334C	LYS	3.05	3.45	106.24
19	495C	ILE	2.26	3.23	167.75

20	496C	GLU	2.98	3.39	105.93
21	496C	GLU	3.54	4.04	114.77
22	535C	TYR	1.79	2.23	104.04
23	537C	THR	2.74	3.41	125.60
24	538C	ASN	2.82	3.74	155.62
25	539C	ASN	3.51	3.95	109.79
26	544C	ASP	2.18	3.09	152.91
27	545C	TYR	2.56	3.34	140.15

π-Stacking interaction	Residue	Amino acid	Distance	Angle	Offset	Stacking type
1	288C	HIS	3.98	21.66	1.05	P

π-Cation interaction	Residue	Amino acid	Distance	Offset	Ligand group
1	44C	LYS	3.05	0.73	Aromatic
2	378C	LYS	5.16	1.62	Aromatic

Salt Bridge	Residue	Amino acid	Distance	Ligand group
1	41C	ASP	4.44	Guanidine
2	285C	ARG	2.84	Phosphate
3	289C	ARG	3.19	Phosphate
4	334C	LYS	3.74	Phosphate
5	334C	LYS	4.63	Phosphate
6	334C	LYS	5.30	Phosphate
7	544C	ASP	5.23	Guanidine

H, hydrogen atom; A, acceptor atom; D, donor atom; P, parallel.

Supplementary table 5. Interactions in NXPE2-R4f complex interface based on PLIP.

Hydrogen Bond	Residue	Amino acid	Distance H-A	Distance D-A	Donor angle
1	104C	ASN	3.38	3.87	113.30
2	104C	ASN	2.80	3.52	131.42
3	155C	TYR	2.08	2.88	141.62
4	157C	THR	2.48	3.16	126.67
5	157C	THR	2.59	3.16	117.15
6	158C	ALA	3.14	3.95	139.86
7	188C	SER	1.56	2.51	164.16
8	291C	ASN	2.83	3.25	107.04
9	291C	ASN	1.81	2.27	105.39
10	297C	MET	2.81	3.26	109.54
11	298C	LYS	3.32	3.87	115.52
12	299C	ASN	2.45	2.89	107.01
13	299C	ASN	2.24	3.18	158.00
14	300C	PHE	2.80	3.72	155.92
15	545C	TYR	2.18	2.92	134.58
16	545C	TYR	2.35	2.92	116.86

H, hydrogen atom; A, acceptor atom; D, donor atom.

Supplementary table 6. Interactions in MUC6-R4i complex interface based on PLIP.

Hydrogen bond	Residue	Amino acid	Distance H-A	Distance D-A	Donor angle
1	429C	ARG	3.42	3.92	113.46
2	431C	ASP	3.33	3.94	124.56
3	443C	ASN	2.87	3.43	117.40
4	444C	ASN	2.12	2.97	142.93
5	446C	GLU	3.30	3.63	102.15
6	453C	LYS	2.72	3.37	125.66
7	454C	THR	2.91	3.59	128.45
8	455C	ARG	2.77	3.74	169.72
9	455C	ARG	1.43	2.33	150.13
10	456C	ASN	2.60	3.44	144.16
11	472C	SER	1.95	2.75	138.50
12	472C	SER	1.96	2.75	135.76
13	656C	ASP	2.60	3.55	161.63
14	695C	LEU	3.22	3.67	110.36
15	696C	GLU	2.21	2.87	122.67
16	697C	GLY	2.53	3.44	152.86
17	698C	TYR	1.25	2.24	177.59
18	720C	ARG	3.04	3.94	153.24
19	723C	CYS	2.81	3.50	127.69
20	748C	GLU	2.85	3.44	119.44
21	749C	ASN	2.74	3.28	114.63

22	750C	LYS	2.76	3.62	147.15
23	1471C	ASN	2.83	3.41	118.27
24	1471C	ASN	2.42	3.31	149.76
25	1472C	GLN	3.19	3.97	137.55

Salt Bridge	Residue	Amino acid	Distance	Ligand group
1	429C	ARG	5.37	Phosphate
2	429C	ARG	3.78	Phosphate
3	448C	LYS	3.56	Phosphate
4	448C	LYS	4.50	Phosphate
5	656C	ASP	3.91	Guanidine
6	696C	GLU	4.77	Guanidine
7	750C	LYS	5.26	Phosphate

H, hydrogen atom; A, acceptor atom; D, donor atom.

CAPÍTULO III – PATENTE

Patente intitulada “Aptâmero modificado, sistema aptaimunológico, painel aptaimunológico, kit, método e uso no diagnóstico de câncer de próstata” (depósito nacional BR102020013625-9 A2 e internacional PTC/BR2021/050290).

Esse capítulo inclui a primeira página da patente depositada no Instituto Nacional de Propriedade Industrial (INPI), a listagem de sequências, o relatório descritivo, a reivindicação, os desenhos e o resumo.



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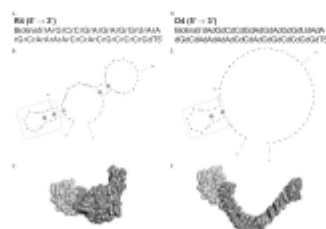
(54) Título: APTÂMERO MODIFICADO, SISTEMA APTAIMUNOLÓGICO, PAINEL APTAIMUNOLÓGICO, KIT, MÉTODO E USO NO DIAGNÓSTICO DE CÂNCER DE PRÓSTATA

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(57) Resumo: APTÂMERO MODIFICADO, SISTEMA APTAIMUNOLÓGICO, PAINEL APTAIMUNOLÓGICO, KIT, MÉTODO E USO NO DIAGNÓSTICO DE CÂNCER DE PRÓSTATA. A presente invenção descreve novos aptâmeros modificados e um sistema aptaimunológico compreendendo ao menos um dos referidos aptâmeros modificados R4 e/ou D4 combinado com ao menos um anticorpo específico, painéis aptaimunológicos compreendendo o referido sistema, kit; método de diagnóstico ou prognóstico e seus usos para identificar biomarcadores do câncer de próstata em células tumorais circulantes de amostras biológicas. A partir da análise destes biomarcadores por citometria de fluxo, é possível discriminar pacientes com câncer de próstata de uma maneira superior ao antígeno prostático específico, que atualmente é o biomarcador padrão-ouro no câncer de próstata. Assim, o sistema aptaimunológico da presente invenção apresenta um menor índice de falsos positivos e falsos negativos, o que é útil na triagem, diagnóstico, prognóstico e monitoramento terapêutico do câncer de próstata para aprimorar seu manejo clínico.



LISTAGEM DE SEQUÊNCIAS

APTÂMERO MODIFICADO, SISTEMA APTAIMUNOLÓGICO, PAINEL APTAIMUNOLÓGICO, KIT, MÉTODO E USO NO DIAGNÓSTICO DE CÂNCER DE PRÓSTATA

Sequências de aptâmero formadas por ribonucleotídeos (r) e deoxirribonucleotídeos (d) modificadas no extremo 5' por uma biotina e no extremo 3' por uma timina invertida.

Sequência do aptâmero de RNA R4 (5' → 3')

Biotina5'rArGrCrCrGrArGrArGrGrUrArArGrCrArArArArCrCrArCrGrCrCrCrGdT5'

Sequência do aptâmero de DNA D4 (5' → 3')

Biotina5'dAdGdCdCdGdAdGdAdGdGdUdAdAdGdCdAdAdAdAdCdCdAdCdGdCdCdCdGdT5'

APTÂMERO MODIFICADO, SISTEMA APTAIMUNOLÓGICO, PAINEL APTAIMUNOLÓGICO, KIT, MÉTODO E USO NO DIAGNÓSTICO DE CÂNCER DE PRÓSTATA

Campo da invenção

[001] A presente invenção refere-se a uma ferramenta teranóstica que auxilia nos procedimentos clínicos de pacientes com câncer de próstata através de um sistema baseado em anticorpos e aptâmeros modificados que identificam células tumorais da próstata utilizando a técnica de citometria de fluxo para análise de amostras biológicas. Em particular, a ferramenta serve para auxiliar no diagnóstico de câncer de próstata, mas também na triagem, prognóstico, monitoramento de respostas a tratamentos e inclusive no desenvolvimento de tratamentos quando acoplada a agentes terapêuticos.

Fundamentos da invenção

[002] O câncer de próstata é bastante frequente, representou 10% dos novos casos de câncer nos Estados Unidos no ano de 2019 ⁵⁶. No Brasil, a incidência prevista para 2020 é de 66.000 novos casos, sendo o segundo tipo de câncer mais comum nos homens ⁵⁷.

[003] Atualmente, o câncer de próstata é rastreado pelo exame do antígeno prostático específico (PSA, do inglês *Prostate-Specific Antigen*) no soro sanguíneo, complementado pelo exame de toque retal. O resultado positivo no PSA, precisa ser confirmado pelo exame histopatológico a partir de uma biópsia invasiva do tecido prostático, guiada por ultrassom. Após um resultado negativo na biópsia, pode ser feita adicionalmente uma ressonância magnética multiparamétrica ⁵⁸. Assim, o PSA é hoje a técnica padrão ouro na prática clínica do câncer de próstata. Entretanto, não é um teste conclusivo, pois precisa de técnicas complementares que validem seu resultado ⁵⁹.

[004] Além disso, valores de PSA elevados, ou seja, maiores que 4,0 ng/mL, podem levar a resultados falsos positivos que indicam presença de câncer quando não há. Ao mesmo tempo, resultados abaixo do valor de referência podem apresentar falsos negativos, não detectando casos reais de câncer ⁶⁰. Ainda, no período entre 2009 e 2015 nos Estados Unidos, todos os pacientes diagnosticados com câncer de próstata em estadiamento local ou loco-regional mostraram sobrevivência relativa de cinco anos, enquanto que apenas cerca de 30% dos pacientes diagnosticados em estadiamento avançado apresentaram uma sobrevivência relativa a cinco anos ⁵⁶. Com isso, é

necessária a detecção precoce de câncer de próstata, com testes mais específicos e sensíveis do que o PSA.

[005] Novas abordagens chamadas de biópsias líquidas analisam os componentes do tumor liberados nos fluidos corporais, como sangue, urina, sêmen, saliva e outras secreções. As biópsias líquidas são capazes de representar o estado atual do câncer em um indivíduo ⁶¹ e sua amostragem é menos invasiva que as biópsias teciduais, que são cirúrgicas. Entre outros constituintes, os tumores liberam células que podem ser detectadas por exemplo, no sangue do indivíduo acometido ⁶². Essas células são conhecidas como células tumorais circulantes (CTCs). A expressão de marcadores (também chamados de antígenos) de superfície em CTCs e sua quantificação já foram estudadas como fatores para o monitoramento clínico de vários tipos de câncer, principalmente a partir da marcação imunológica, usando anticorpos.

[006] A presença de CTCs em amostras de sangue é muito rara, na ordem de várias CTCs em dezenas de bilhões de células sanguíneas. Para detectar as CTCs, muitas abordagens enriquecem primeiramente as amostras com a população de CTCs para depois analisá-las. O enriquecimento de CTCs é feito atualmente com base à diferença nas características físicas (tamanho, morfologia, densidade, carga, etc.) ou na expressão de antígenos de superfície presentes na membrana plasmática. Assim, um dos problemas que as tecnologias de enriquecimento enfrenta é a alta heterogeneidade destas células, pois podem apresentar variabilidade tanto nas características físicas, quanto na expressão destes antígenos.

[007] Por exemplo, a patente CN110004062A apresenta uma interessante estratégia de enriquecimento negativo de CTCs em sangue por eliminação de outros tipos celulares. Diante disso, as hemácias são lisadas e um anel magnético giratório com anticorpos anti-CD45 captura os leucócitos para seu descarte, desta forma, concentrando com um alto rendimento todos os tipos de CTCs.

[008] As CTCs expressam na sua membrana plasmática diversos antígenos de diferenciação ou desdiferenciação celular e em diferentes graus, segundo a etapa de transição epitélio-mesenquimal (EMT, do inglês *epithelial-mesenchymal transition*) na qual se encontram. Estes antígenos proporcionam às células um fenótipo híbrido entre as epiteliais (com características diferenciadas) e as mesenquimais (com características desdiferenciadas).

[009] Durante a metástase, que acontece nas fases mais avançadas do câncer, a EMT permite que as CTCs do tumor primário extravasem para a corrente sanguínea e invadam outros tecidos, formando nichos celulares que darão lugar a tumores secundários após um processo de transição inverso (MET, de inglês *mesenchymal-epithelial transition*). À medida que a doença progride e os nichos metastáticos proliferam, são liberadas cada vez mais e mais CTCs, tanto do tumor primário quanto dos secundários. Estas CTCs podem ser capturadas na corrente sanguínea e fornecer informações relevantes acerca do quadro clínico atual dos pacientes.

[0010] A presente invenção abrange a utilização tanto de antígenos epiteliais, como EpCAM, quanto de antígenos mesenquimais, como CD44 e CD133 que são indicativos de células tronco tumorais. EpCAM é uma glicoproteína de transmembrana expressa em células epiteliais normais e de forma exacerbada em carcinomas, com um papel relevante na formação de estruturas adesivas e na polaridade celular, sendo correlacionada com pior sobrevida global, mau prognóstico e maior risco de recorrência ⁶³. CD133 é também conhecido como proeminina-1, uma glicoproteína de transmembrana presente em células tronco tumorais e não tumorais. Sua alta expressão em tumores é considerada como fator de prognóstico ruim e é responsável pela inibição de apoptose, a angiogênese e a proliferação celular nos processos de tumorigênese, metástase e quimioresistência ⁶⁴. CD44 é um receptor de adesão que se encontra na superfície das células e é altamente expresso em vários tipos de tumores, onde regula a migração e invasão celular durante o processo de metástase ⁶⁵. Além destes antígenos, CD45 também faz parte desta invenção excluindo da análise as células de origem leucocitária através da ausência da sua expressão. Na presente invenção, esses antígenos são identificados por anticorpos.

[0011] Por outra parte, a presente invenção abrange os aptâmeros D4 e R4 que foram modificados a partir do aptâmero original A4 ¹⁰ e ligam especificamente a antígenos ainda não identificados na membrana plasmática de células tumorais da próstata. Os aptâmeros são biomoléculas de tipo oligonucleotídeos de fita simples ou peptídeos, formadas pela ligação de vários nucleotídeos ou aminoácidos, respectivamente, e com a capacidade de se ligar de maneira específica a moléculas alvo de maneira similar aos anticorpos monoclonais. A técnica de evolução sistemática de ligantes por enriquecimento exponencial (SELEX, do inglês *systematic evolution of ligands by*

exponential enrichment) é usada para selecionar aptâmeros a partir de um conjunto de sequências aleatórias de oligonucleotídeos ou aminoácidos.

[0012] Comparado aos anticorpos, os aptâmeros de oligonucleotídeos apresentam melhor penetração tecidual por seu menor tamanho, maior especificidade, maior reprodutibilidade, versatilidade e durabilidade devido a sua síntese química, que ainda facilita sua produção, modificação, manipulação e estocagem, além da baixa imunogenicidade que aumenta o sucesso em terapias, entre outras vantagens ⁶⁶.

[0013] Diante da problemática do câncer de próstata, a presente invenção propõe o uso conjunto dos aptâmeros de natureza oligonucleotídica D4 e/ou R4 com os anticorpos anti-EpCAM e anti-CD45 ou anti-CD44 e anti-CD133, como marcadores para triagem, diagnóstico, prognóstico e monitoramento terapêutico do câncer de próstata a partir da identificação desses biomarcadores por citometria de fluxo convencional em amostras biológicas, como fluídos corporais ou biópsias teciduais.

[0014] Em um contexto mais amplo, a patente US2008206757A1 refere-se, entre outros, a um método de enriquecimento de CTCs a partir de amostras de sangue de indivíduos com qualquer tipo de câncer para sua posterior análise clínica. Este método, igual ao nosso, compreende etapas de centrifugação, lavagem em tampões, lise ou depleção de eritrócitos e incubação com anticorpos ou outras moléculas ligantes para separar as células de interesse ou descartar aquelas indesejáveis. Entre essas moléculas ligantes não mencionam os aptâmeros, nem os anticorpos anti-CD133 ou anti-EpCAM. Entretanto, propõem o uso dos anticorpos anti-CD44 e anti-CD45 e anticorpos contra moléculas de adesão diferentes a EpCAM de forma combinada com muitos outros anticorpos. Diferentemente da nossa, esta proposta usa anticorpos acoplados a *beads* magnéticas para facilitar a separação das células na amostra enriquecida, inclui uma etapa de filtração com uma prensa de tipo francesa e utiliza uma amostra de sangue armazenada a temperatura ambiente a qual pode ser analisada em até 4 dias.

[0015] A principal desvantagem desse modelo, frente ao apresentado neste pedido, é o comprometimento da integridade das células devido ao tempo de armazenamento das amostras e às etapas de enriquecimento por métodos físico. O custo elevado das *beads* magnéticas acopladas a anticorpos e da prensa francesa são outras das desvantagens do modelo apresentado na patente US2008206757A1.

[0016] Outro método e dispositivo para o enriquecimento e análise de CTCs em amostras de sangue de pacientes com câncer é o proposto na patente CN107449713A, que também limita-se apenas ao uso de anticorpos: anti-CD45 para a remoção de leucócitos, anti-EpCAM para a concentração de CTCs com perfil epitelial, anti-vimentina para as CTCs com perfil mesenquimal e anti-pancitoqueratina para a confirmação do enriquecimento de CTCs.

[0017] O sistema de detecção de CTCs apresentado pela patente CN106645726A compreende a lise dos eritrócitos de amostras de sangue, o uso de um suporte adsorvente de CTCs a partir da ligação destas ao anticorpo monoclonal anti-EpCAM ou anti-EGFR, a filtração da solução resultante, a fixação das células, a marcação fluorescente nuclear das células com o corante DAPI ou Hoechst 33258, a marcação das células tumorais com anticorpos anti-CK8/CK18/CK19, anti-PanCK, anti-vimentina ou anti-plastina-3 acoplados a *quantum dots*, a marcação de leucócitos com anticorpos anti-CD45, anti-CD15 ou anti-CD33 acoplados a *quantum dots* e a avaliação da marcação por um operador através de um microscópio de fluorescência.

[0018] Este sistema alega uma menor taxa de perda de CTCs devido à combinação do suporte adsorvente e a filtração e uma maior qualidade da marcação pelo uso de *quantum dots*, cuja fluorescência é mais brilhante e estável que a dos fluorocromos comumente usados. Entretanto, o uso de sistemas físicos de separação compromete a integridade das células e a avaliação das marcações por um técnico pode induzir a resultados variáveis devido à subjetividade da análise.

[0019] A patente US8329422B2 refere-se a métodos e reagentes para a análise de CTCs, aglomerados ou fragmentos de células por citometria de fluxo ou microscopia de fluorescência, para a triagem, monitoramento e diagnóstico de tumores. Esta patente já rendeu a plataforma comercial CellSearch, que quantifica as CTCs provenientes de sangue periférico para o prognóstico de câncer de próstata metastático resistente a castração, dentre outros tumores metastáticos e é o único sistema aprovado pela *Food and Drug Administration* para esta finalidade ⁶⁷.

[0020] No CellSearch é necessário dispor de vários dispositivos e softwares para o processamento das amostras e sua posterior análise. Estes dispositivos usam anticorpos anti-EpCAM e anti-CD45 de maneira similar à presente invenção, mas adicionam os anticorpos citoplasmáticos (marcadores internos, não de membrana plasmática) anti-

citoquinas 8, 18 e 19 e o corante nuclear DAPI para a identificação das CTCs prostáticas por microscópio de fluorescência. A partir da identificação de cinco CTCs nas amostras destes pacientes, considera-se um prognóstico desfavorável, com baixa sobrevida global. Mas um ponto importante a considerar é que o CellSearch não detecta CTCs em pacientes com câncer de próstata localizado, seu uso apenas foi aprovado para pacientes com metástase (MEYER et al., 2016).

[0021] Entretanto, o maior erro do sistema é a identificação de fragmentos de CTCs como células inteiras, resultando em uma grande variabilidade na contagem de CTCs ⁶⁸. Ainda, o CellSearch precisa de caros dispositivos exclusivos do fabricante para o processamento e análise das amostras. Isso cria uma total dependência do fabricante e faz com que nem todos os laboratórios clínicos possam instalar este sistema, sendo necessário o transporte de amostras até laboratórios de referência para o seu processamento. Assim, este transporte pode agravar o dano celular e interferir na análise e resultado da amostra.

[0022] Portanto, nenhuma patente combina de fato o uso de anticorpos e aptâmeros. Aptâmeros de superfície celular já tem sido propostos para a detecção de células tumorais: de forma individual como o aptâmero ligante de CD133 para carcinoma indiferenciado da tireoide descrito na patente CN109536503A ou de forma conjunta como na patente US2011124015A1 e nos sistemas multiplex das patentes WO2013185078A1 ou JP2017079634A. Esta última propõe uma interessante estratégia de detecção de CTCs a partir da amplificação de sinais luminescentes baseada na hibridação de aptâmeros complexados nos quais participa um aptâmero de captura de CTCs ligante de EpCAM.

[0023] Na presente abordagem, apenas é necessário uma ultracentrífuga refrigerada e um citômetro de fluxo com no mínimo quatro filtros ópticos para o processamento e análise das amostras. Este fato evita a dependência de centros de referência, o que reduz o tempo desde a coleta até a análise e assim a degradação e variabilidade das amostras. Além disso, comparado com a baixa especificidade do teste do PSA e sua moderada sensibilidade ⁶⁹, o presente teste de biopsia líquida tem uma maior acurácia, apresentando um melhor desempenho diagnóstico.

[0024] Diante disso, atendendo às deficiências da tecnologia atual, combinamos anticorpos bem estabelecidos com aptâmeros de superfície celular isolados por nosso

grupo e posteriormente modificados, para desenvolver um sistema de marcação de CTCs da próstata confiável, rápido, de baixo custo, fácil de popularizar entre os pacientes e de operação e equipamentos simples.

Breve descrição dos desenhos

A Figura 1 apresenta as sequências e as conformações bi- e tridimensionais mais prováveis dos aptâmeros R4 e D4 com seu correspondente potencial domínio de ligação.

A Figura 2 ilustra o diagrama de fluxo do método para o processamento da amostra.

A Figura 3 mostra a estratégia de análise dos dados por citometria de fluxo.

A Figura 4 apresenta os grupos de indivíduos saudáveis e de pacientes com câncer de próstata segundo as variáveis analisadas no painel 1 com o aptâmero R4, o *cutoff* que melhor os diferencia e sua correspondente curva ROC.

A Figura 5 apresenta os grupos de indivíduos saudáveis e de pacientes com câncer de próstata segundo as variáveis analisadas no painel 1 com o aptâmero D4, o *cutoff* que melhor os diferencia e sua correspondente curva ROC.

A Figura 6 apresenta os grupos de indivíduos saudáveis e de pacientes com câncer de próstata segundo as variáveis analisadas no painel 2 com o aptâmero R4, o *cutoff* que melhor os diferencia e sua correspondente curva ROC.

A Figura 7 apresenta os grupos de indivíduos saudáveis e de pacientes com câncer de próstata segundo as variáveis analisadas no painel 2 com o aptâmero D4, o *cutoff* que melhor os diferencia e sua correspondente curva ROC.

Descrição da invenção

[0025] O presente pedido de patente descreve um aptâmero modificado em sua forma de RNA ou de DNA que combinado com anticorpos específicos identifica células tumorais circulantes de câncer de próstata em amostras biológicas. O aptâmero foi selecionado na sua forma de RNA, a partir de uma biblioteca randômica de oligonucleotídeos de setenta a quatrocentos pares de bases, pela técnica de SELEX usando células prostáticas cultivadas em cultura tridimensional (*3D Cell-SELEX*) e chamado de A4 ¹⁰.

[0026] Os aptâmeros modificados contém as 28 bases nitrogenadas originais do aptâmero A4 ligadas a um radical fosfato e a uma pentose, ribose no caso do aptâmero de RNA e 2' desoxirribose no caso do aptâmero de DNA. Adicionalmente, foram modificados com uma molécula de biotina acoplada no extremidade 5' para permitir sua ligação a moléculas de estreptavidina acopladas a um fluorocromo e um nucleotídeo monofosfato 2'desoxitimidina invertido, unido por uma ligação 3'-3', na extremidade 3'

para evitar a degradação pelas exonucleases 3' e a extensão pelas polimerases de DNA. Assim, os aptâmeros usados na presente invenção apresentam um total de 29 nucleotídeos e são denominados de R4 e D4, o aptâmero modificado de RNA e de DNA, respectivamente (Figura 1, A e D). Estas modificações são particularmente preferíveis mas também podem ser suscetíveis de outras, como por exemplo a marcação direta com fluorescência a partir da adição na extremidade 5' de um fluorocromo.

[0027] Na figura 1 aparece destacado em amarelo o potencial domínio de ligação à proteína alvo dos aptâmeros R4 e D4 (PBD, do inglês *protein binding domain*). Estes PBDs compartilham uma estrutura de alça em forma de grampo na conformação secundária mais provável de ambos aptâmeros R4 e D4 (Figura 1, B e E). De esta maneira, os aptâmeros apresentam uma conformação tridimensional (Figura 1, C e F) que permite o reconhecimento do seu alvo.

[0028] Os aptâmeros D4 e R4 são combinados com dois painéis de anticorpos para analisar as marcações e assim identificar células tumorais de amostras de indivíduos por citometria de fluxo. O painel aptaimunológico 1 está formado por um dos aptâmeros combinado com os anticorpos anti-EpCAM e anti-CD45 e o painel aptaimunológico 2, por um dos aptâmeros combinado com os anticorpos anti-CD44 e anti-CD133. No painel 1 é usada a marcação positiva do anticorpo anti-EpCAM que liga em moléculas de adesão de células epiteliais e a marcação negativa do anticorpo pan-leucocitário CD45 para analisar a marcação positiva de D4 ou R4 em uma população de células epiteliais não leucocitárias. No painel 2 é usada a marcação positiva dos anticorpos anti-CD44 e anti-CD133 que ligam a células tronco tumorais para analisar a marcação positiva de D4 ou R4 em esta população celular.

[0029] A presente invenção proporciona ainda um kit compreendendo os materiais necessários para a detecção de células de câncer de próstata em amostras de pacientes, preferencialmente de sangue periférico. Os materiais incluem ao menos um dos dois painéis aptaimunológicos com seus respectivos controles e as marcações personalizadas para o citômetro de fluxo de cada usuário, e adicionalmente os tubos de coleta, os tampões de lise de hemácias, bloqueio e lavagem, sem estarem limitados aos mesmos. Estes materiais são caracterizados nos próximos parágrafos, sem estarem restritos às especificações apresentadas.

[0030] As seguintes etapas (1, 2, 3, 4, 5, 6, 7 e 8) explicam o processamento das amostras biológicas dos pacientes, de tal maneira que permitem a um técnico no assunto desenvolver a presente invenção proporcionando um método para detectar e quantificar CTCs de câncer de próstata atribuindo-lhes um valor clínico que permite um melhor gerenciamento médico desta doença:

[0031] (1) A primeira etapa é a coleta da amostra, podendo ser esta uma biópsia tecidual ou líquida (de um fluido corporal), mas preferivelmente a amostra trata-se de sangue periférico por ser uma amostra pouco invasiva, de fácil acesso e operação e amplamente aceita entre os pacientes. Para a coleta da amostra de sangue periférico a punção venosa deve ser realizada na fossa cubital do braço, usando tubos para coleta a vácuo com EDTA de 4 mL de preferência. O primeiro volume coletado, aproximadamente 1 mL, deve ser descartado em um tubo, pois pode conter células epiteliais da pele que interferem na análise da amostra. Assim, um segundo tubo deve ser usado e este tubo será processado nas seguintes etapas.

[0032] (2) A segunda etapa consiste no isolamento da camada de células mononucleares do sangue periférico. O sangue do segundo tubo deve ser homogeneizado invertendo várias vezes o tubo suavemente e em seguida colocado em uma ultracentrífuga a temperatura ambiente durante 15 minutos a 200 g. Após a centrifugação, a camada de células mononucleares do sangue periférico também chamada de anel leucocitário deve ser transferida a um tubo de citometria. O método de separação do anel leucocitário não é limitado a este e podem ser usadas outras técnicas que combinam por exemplo a centrifugação por gradiente de densidade ou a filtração.

[0033] (3) A terceira etapa envolve a lise das hemácias. Esta lise pode ser realizada com tampões desenvolvidos *in-house* ou disponíveis comercialmente, com cloreto de amônia como componente principal e sem fixadores como o formaldeído para manter as células vivas na hora da análise. De preferência, a suspensão celular resultante deve ser lavada duas vezes e estas lavagens devem ser feitas com solução de lavagem (composta por albumina sérica bovina (BSA, do inglês *bovine serum albumin*) 1% e azida básica 0,1% (m/v) em tampão fosfato (PBS, do inglês *phosphate buffer solution*) com pH 7,4 e filtrada por membrana de 0,22 µm) após centrifugação de 5 minutos a 200g e temperatura ambiente.

[0034] (4) A quarta etapa é o bloqueio de ligações inespecíficas da porção Fc dos anticorpos usados nos painéis com os receptores de superfície das células da amostra. Neste passo a suspensão celular deve ser incubada por 15 minutos com uma solução de bloqueio com plasma humano inativado de sangue tipo AB 10% (v/v) na solução de lavagem ou uma solução de bloqueio comercial equivalente.

[0035] (5) A quinta etapa é a primeira incubação por 30 minutos a 4 °C da suspensão celular na solução de bloqueio com os painéis de aptâmeros e anticorpos que devem ser adicionados em diferentes tubos, segundo as seguintes combinações:

[0036] Tubo sem marcação: não adicionar nada.

[0037] Tubo painel 1: aptâmero (R4 ou D4), anti-EpCAM e anti-CD45.

[0038] Tubo controles painel 1: estreptavidina, isotipo de anti-EpCAM e isotipo de anti-CD45.

[0039] Tubo painel 2: aptâmero (R4 ou D4), anti-CD44 e anti-CD133.

[0040] Tubo controles painel 2: estreptavidina, isotipo de anti-CD44 e isotipo de anti-CD133.

[0041] Os anticorpos, seus correspondentes isotipos e a estreptavidina estão marcados com diferentes fluorocromos, de acordo com os filtros do citômetro de fluxo. Exceto no tubo sem marcação, deve ser adicionado um corante de viabilidade com pico de emissão diferente aos fluorocromos já utilizados. Desta maneira, o citômetro de fluxo usado deve ser capaz de ler pelo menos quatro fluorocromos diferentes. Depois da incubação, a suspensão celular deve ser lavada duas vezes com a solução de lavagem após centrifugação de 5 minutos a 200g e 4 °C.

[0042] (6) A sexta etapa é a adição de estreptavidina marcada aos tubos com aptâmeros (Tubo aptâmero + painel 1 e Tubo aptâmero + painel 2) e sua incubação por 30 minutos a 4 °C. A marcação indireta dos aptâmeros é preferida na presente invenção, mas no caso de marcação direta dos aptâmeros com fluorocromos esta etapa deve ser omitida. Após a incubação, a suspensão celular deve ser lavada duas vezes com a solução de lavagem após centrifugação de 5 minutos a 200g, à 4 °C.

[0043] (7) A sétima etapa corresponde à leitura da fluorescência no citômetro de fluxo. Uma vez ressuspensas as células, as amostras são lidas no citômetro de fluxo a partir da determinação de uma janela que deve considerar uma população de até 500.000 leucócitos identificados segundo seu tamanho e granulosidade, desprezando os *debris* celulares.

[0044] (8) A oitava etapa é a análise dos dados obtidos na citometria de fluxo. Dentro desses 500.000 leucócitos, são selecionadas as células únicas (*singlets*), com relação de área e altura do seu tamanho próxima a 1, excluindo os *doublents*. Dentro dos *singlets*, são selecionadas as células vivas pela fluorescência do corante de viabilidade. Dentro das células vivas, são selecionadas individualmente por histograma as marcações específicas de cada painel, segundo os correspondentes controles (estreptavidina e isotipos).

[0045] No painel 1, são selecionadas as células com marcação negativa para CD45, dentro desta população são selecionadas as células com marcação positiva para EpCAM e dentro desta subpopulação são selecionadas as células com marcação positiva para os aptâmeros (R4 ou D4). No painel 2, são selecionadas as células com marcação positiva para CD133, dentro desta população são selecionadas as células com marcação positiva para CD44 e dentro desta subpopulação são selecionadas as células com marcação positiva para os aptâmeros (R4 ou D4).

[0046] De preferência, o resultado é a percentagem de células selecionadas em cada painel. O resultado também pode ser o grau de expressão do alvo dos aptâmeros nas mesmas células, calculado a partir da intensidade média de fluorescência. Portanto, a presente invenção também inclui como resultado um índice calculado a partir de expressões matemáticas que usam esses dois parâmetros. O resultado indica a presença de CTCs de origem prostática, servindo como diagnóstico para o câncer de próstata.

[0047] Durante o processamento das amostras na presente invenção, as CTCs podem ser submetidas a outros meios de detecção, tais como exame microscópico de fluorescência. Além disso, as CTCs podem ser separadas, por exemplo, por citômetros de fluxo com *sorting* celular ou por aplicação de *beads* magnéticas acopladas a estreptavidina, e obtidas como amostras para pesquisa básica para a análise de marcadores adicionais ou outras aplicações clínicas, tais como triagem, análise de

disposição à doença, prognóstico, predição do melhor tratamento (tratamento personalizado), farmacogenômica, monitoramento do tratamento, e inclusive desenvolvimento de tratamentos com direcionamento de drogas ou outros agentes terapêuticos.

[0048] Dado que a presente invenção é apresentada com certo grau de detalhamento, esta é suscetível de quaisquer modificações, adaptações e aplicações adicionais por um técnico no assunto de acordo com os princípios dos métodos, materiais e usos expostos neste documento. Assim, as reivindicações anexas e o próprio relatório cobrem quaisquer modificações, adaptações e aplicações adicionais abrangidas no escopo da presente invenção.

[0049] Daqui em diante, iremos ressaltar apenas a título de exemplo os resultados com os procedimentos que permitem uma melhor compreensão dos materiais, métodos e usos apresentados. Contudo, a presente invenção não é limitada aos seguintes exemplos de concretizações.

Exemplos de concretizações da invenção

Exemplo 1: PREPARAÇÃO DE AMOSTRAS DE SANGUE PERIFÉRICO PARA A ANÁLISE DOS PAINÉIS 1 E 2 POR CITOMETRIA DE FLUXO

[0050] O protocolo de pesquisa com Certificado de Apresentação para Apreciação Ética (CAAE) 71108817.2.0000.5152 foi aprovado pelo Comitê de Ética em Pesquisas com Seres Humanos da Universidade Federal de Uberlândia (UFU). Todos os pacientes convidados a participar do estudo assinaram o correspondente termo de consentimento livre e esclarecido (TCLE) antes de doar as amostras de sangue no Serviço de Urologia do Hospital de Clínicas da UFU para serem encaminhadas ao Laboratório de Nanobiotecnologia do Instituto de Biotecnologia da UFU, onde foram processadas e analisadas.

[0051] O critério de inclusão para os pacientes com câncer de próstata foi o diagnóstico de câncer de próstata e para os controles, ausência do diagnóstico de câncer de próstata o que incluiu indivíduos saudáveis ou com outras doenças da próstata como hiperplasia benigna ou prostatites. Diagnóstico anterior de qualquer tipo de câncer foi o critério de exclusão para todos os participantes da pesquisa.

[0052] Amostras de sangue periférico de 34 indivíduos foram coletadas em tubos a vácuo com EDTA para 4 mL (Vacuette Greiner Bio-One, Americana, SP, Brasil) no Hospital de

Clínicas da UFU, segundo o protocolo de pesquisa com Certificado de Apresentação para Apreciação Ética (CAAE) 71108817.2.0000.5152, aprovado pelo Comitê de Ética em Pesquisas com Seres Humanos da UFU. Dessas amostras, 20 eram de controles e 14 de pacientes com câncer de próstata.

[0053] Após homogeneizar e centrifugar os tubos com as amostras a 200 g por 15 minutos a temperatura ambiente, o anel leucocitário de cada amostra foi coletado e dividido em sete tubos para citometria de 5 mL (Sarstedt, Nümbrecht, Alemanha), colocando 90 µL em cada um. A solução de lise de hemácias BD Pharm Lyse (Franklin Lakes, NJ, EUA) foi adicionada (2 mL por tubo) e vortexada para sua incubação por 15 minutos no escuro a temperatura ambiente. Após a incubação os tubos foram centrifugados a 200 g por 5 minutos a temperatura ambiente e o sobrenadante foi descartado por inversão. O *pellet* de cada tubo foi ressuspensionado duas vezes com 2 mL de solução de lavagem *in-house* (tampão PBS 1x, pH 7,4, 1% BSA e 0,1% azida básica), homogeneizado e centrifugado a 200 g por 5 minutos a temperatura ambiente.

[0054] O *pellet* foi então ressuspensionado em cada tubo teste com 90 µL da solução de lavagem e 10 µL de plasma humano inativado de sangue tipo AB, como solução de bloqueio, e incubado por 15 minutos a temperatura ambiente. Após o bloqueio, foram adicionados os marcadores: o corante de viabilidade (Fixable Viability Stain 620 (FVS620), BD, Franklin Lakes, NJ, EUA), os aptâmeros e os anticorpos nos tubos correspondentes. Os aptâmeros R4 e D4 foram sintetizados (IDT, Coralville, IA, EUA) com suas respectivas sequências e modificações (Figura 1 A e D) e liofilizados. Para seu uso, os aptâmeros foram ressuspensionados em água de injeção até uma solução estoque com uma concentração de 100 µM. Para a marcação dos aptâmeros foi utilizada estreptavidina ligada ao fluorocromo FITC (SAv-FITC, 405201, BioLegend, San Diego, CA, EUA). Os anticorpos também foram todos adquiridos da empresa BioLegend.

[0055] Após homogeneizar os tubos, os marcadores foram incubados a 4 °C por 30 minutos no escuro da seguinte maneira. O primeiro tubo (Tubo 1) foi incubado sem marcadores servindo como controle. O segundo tubo (Tubo 2) foi incubado com 0,1 µL de FVS620, 0,2 µL de R4, 0,25 µL de EpCAM-APC (324208) e 0,25 µL de CD45-PE (368510). O terceiro tubo (Tubo 3) foi incubado com 0,1 µL de FVS620, 0,2 µL de D4, 0,25 µL de EpCAM-APC (324208) e 0,25 µL de CD45-PE (368510). O quarto tubo (Tubo 4) foi incubado com 0,1 µL de FVS620; 0,2 µL de SAv-FITC, 0,25 µL do isotipo de EpCAM IgG2bκ-APC (400322) e 0,25 µL do isotipo de CD45 IgG1κ-PE (400114). O quinto tubo

(Tubo 5) foi incubado com 0,1 µL de FVS620, 0,2 µL de R4, 0,25 µL de CD44-APC (338806) e 0,25 µL de CD133-PE (372804). O sexto tubo (Tubo 6) foi incubado com 0,1 µL de FVS620, 0,2 µL de D4, 0,25 µL de CD44-APC (338806) e 0,25 µL de CD133-PE (372804). O sétimo tubo (Tubo 7) foi incubado com 0,1 µL de FVS620; 0,2 µL de SAv-FITC, 0,25 µL do isotipo de CD44 IgG1κ-APC, (400120) e 0,25 µL do isotipo de CD133 IgG1κ-PE (400114). Após a incubação, os tubos foram centrifugados a 200 g por 5 minutos a 4 °C e o sobrenadante descartado por inversão. O pellet de cada tubo foi ressuspensionado duas vezes com 2 mL da solução de lavagem, homogeneizado e centrifugado a 200 g por 5 minutos a 4 °C. Todos os tubos foram ressuspensionados em 100 µL de solução de lavagem.

[0056] O primeiro, quarto e sétimo tubo foram mantidos no escuro. O segundo, terceiro, quinto e sexto tubo foram homogeneizados e incubados a 4 °C por 30 minutos no escuro com 0,2 µL de SAv-FITC. Após a incubação, estes tubos foram centrifugados a 200 g por 5 minutos a 4 °C e o sobrenadante descartado por inversão. O *pellet* de cada tubo foi ressuspensionado duas vezes com 2 mL da solução de lavagem, homogeneizado e centrifugado a 200 g por 5 minutos a 4 °C. Estes tubos foram ressuspensionados em 100 µL da solução de lavagem e mantidos no escuro. Assim, as amostras estão prontas para sua análise. A figura 2 representa o diagrama de fluxo para o processamento destas amostras.

Exemplo 2: ANÁLISE DOS DADOS CITOMÉTRICOS DOS PAINÉIS 1 E 2

[0057] Para a análise da marcação por citometria de fluxo foi usado o citômetro Accuri C6 (BD, Franklin Lakes, NJ, EUA) com o laser azul de 488 nm e os filtros 533/30, 585/40 e 670 LP para ler fluorescência de FITC (FL-1), PE (FL-2) e FVS620 (FL-3) respectivamente, e com o laser vermelho de 640 nm e o filtro 670 LP para ler a fluorescência de APC (FL-4).

[0058] A figura 3 ilustra de maneira geral como foi feita esta análise no programa FlowJo, versão X 10.0.7r2 para Windows. Primeiramente, foi selecionada uma população de 500 mil leucócitos a partir dos dados citométricos do primeiro tubo, identificada segundo seu tamanho e granulosidade, desprezando os *debris* celulares. Desta população foram selecionados os *singlets*, como aqueles leucócitos com relação entre a área e altura do seu tamanho próxima a 1, excluindo os *doublets*. Dentro dos *singlets*, foram selecionadas as células vivas pela fluorescência do FVS620, a partir da ausência de marcação nos

dados do primeiro tubo. Estas populações foram aplicadas nos dados citométricos dos outros tubos, do segundo ao sétimo (Figura 3A).

[0059] Dentro das células vivas do segundo ao sétimo tubo, foram determinadas individualmente por histograma as marcações específicas de cada painel. A partir das marcações dos isotipos dos anticorpos e da SAV-FITC do quarto tubo, foram estabelecidas as marcações de CD45 e EpCAM e dos alvos dos aptâmeros no segundo e no terceiro tubo (Painel 1): foram identificadas as células negativas para CD45, dentro destas células foram identificadas as células positivas para EpCAM e dentro desta subpopulação, foram identificadas as células com marcação positiva para os aptâmeros (no segundo tubo para R4 e no terceiro para D4) (Figura 3B).

[0060] A partir das marcações dos isotipos dos anticorpos e da SAV-FITC do sétimo tubo, foram estabelecidas as marcações de CD133 e CD44 e dos alvos dos aptâmeros no quinto e no sexto tubo (Painel 2): foram identificadas as células positivas para CD133, dentro destas células foram identificadas as células positivas para CD44 e dentro desta subpopulação, foram identificadas as células com marcação positiva para os aptâmeros (no quinto tubo para R4 e no sexto para D4) (Figura 3C).

[0061] A partir desta análise foram obtidos os dados da percentagem de células marcadas com cada um dos aptâmeros em cada painel (do painel 1, células CD45⁻ EpCAM⁺ R4/D4⁺ e do painel 2, células CD133⁺ CD44⁺ R4/D4⁺) e dessas células, os dados da intensidade média da fluorescência (MFI, do inglês *Mean Fluorescence Intensity*) e da intensidade mediana da fluorescência (MDFI, do inglês *Median Fluorescence Intensity*) de cada aptâmero.

[0062] A comparação entre os tipos de amostra foi feita pelo teste de Mann-Whitney para variáveis não paramétricas e pelo teste *t* não pareado para variáveis paramétricas. O melhor ponto de corte (*cutoff*) das variáveis foi calculado em base à análise da curva ROC (do inglês *Receiver Operating Characteristic*). O desempenho diagnóstico das variáveis foi determinado pelo teste exato de Fisher com o método de Wilson-Brown para calcular os intervalos de confiança de 95% (IC de 95%). Todas as análises estatísticas foram desenvolvidas no programa Graph Pad Prism, versão 7.0 para Windows, considerando o valor de *p* menor que 0,05 como significativo.

Exemplo 3: RESULTADOS DA MARCAÇÃO DO PAINEL 1

[0063] A informação obtida das células marcadas com o painel 1 foi a porcentagem das células negativas para CD45 e positivas para EpCAM marcadas com a fluorescência dos aptâmeros R4 e D4, separadamente, e suas respectivas intensidades em termos de média (MFI) e mediana (MDFI) expressas em unidades arbitrárias (u. a.) (Tabela 1).

[0064] A diferença das variáveis entre os grupos de indivíduos analisados, controles e pacientes, está representada na figura 4 A, C e E para o aptâmero R4 e na figura 5 A, C e E para o aptâmero D4 no painel 1. As barras representam a média e o desvio padrão em cada grupo, sendo a linha vermelha o valor do *cutoff* (plotado à direita do eixo Y), usado para a curva ROC representada à direita (Figura 4 B, D e F e Figura 5 B, D e F). As variáveis que apresentaram diferença estatística entre os grupos foram aquelas relacionadas ao aptâmero R4: % de células R4⁺ (0,0033), MFI de R4 (0,0249) e MDFI de R4 (0,0164).

[0065] Tabela 1. Porcentagem de células positivas para o alvo do aptâmero R4 ou D4 e seu grau de expressão na população de células CD45⁻ EpCAM⁺.

Amostras		Painel 1: Células CD45 ⁻ EpCAM ⁺					
Nº	Tipo	% R4 ⁺	MFI de R4 (u. a.)	MDFI de R4 (u. a.)	% D4 ⁺	MFI de D4 (u. a.)	MDFI de D4 (u. a.)
1	Controle	6	5562	3981	2.38	15150	7594
2	Controle	0	0	0	0	0	0
3	Controle	0	0	0	0	0	0
4	Controle	0	0	0	0	0	0
5	Controle	0	0	0	0	0	0
6	Controle	2.72	2489	2150	2.88	5529	2420
7	Controle	0.6	9249	5465	2.2	4762	4627
8	Controle	0	0	0	0.23	5333	5333
9	Controle	0	0	0	0.83	2678	2536
10	Controle	0	0	0	0.3	4353	4353
11	Controle	0.82	2255	2245	0	0	0
12	Controle	0.28	2619	2465	0.71	6723	6723
13	Controle	0	0	0	0	0	0
14	Controle	0.17	2536	2536	0.9	4120	2740

Amostras		Painel 1: Células CD45 ⁻ EpCAM ⁺					
Nº	Tipo	% R4 ⁺	MFI de R4 (u. a.)	MDFI de R4 (u. a.)	% D4 ⁺	MFI de D4 (u. a.)	MDFI de D4 (u. a.)
15	Controle	4.96	2346	2236	1.27	1873	1760
16	Controle	0.081	3440	3440	0.77	3397	2696
17	Controle	0.22	2139	2004	0.33	2020	2020
18	Controle	0.92	2682	2516	1.18	2650	2377
19	Controle	4.26	3679	3384	0.56	5646	5646
20	Controle	4.91	7108	6274	4.59	9518	7625
21	Paciente	5.88	4935	4780	5.1	10095	6049
22	Paciente	2.04	11261	11261	7.04	24927	24962
23	Paciente	66.7	7641	6945	11.1	3854	3854
24	Paciente	0.98	8405	8405	0	0	0
25	Paciente	7.92	4636	3656	13.5	4251	3807
26	Paciente	0	0	0	0	0	0
27	Paciente	0.74	2496	2496	1.29	3219	2599
28	Paciente	60	2433	2264	22.7	8178	3701
29	Paciente	7.08	6628	5623	11.2	7796	5248
30	Paciente	0.97	3905	3184	0.5	2037	2037
31	Paciente	6.52	3276	3146	3.62	3398	3371
32	Paciente	19.5	3390	3133	0	0	0
33	Paciente	2.75	2778	2785	1.1	3626	3626
34	Paciente	0	0	0	0	0	0

[0066] As melhores curvas ROC dos *cutoffs* definidos para as variáveis analisadas no painel 1, com área sob a curva ROC (AUC, do inglês *area under the curve ROC*) maior que 0,7, foram na seguinte ordem as de % R4⁺, MDFI de R4 e MFI de R4 (Figura 4 B, F e D). Portanto, o teste diagnóstico para câncer de próstata resultante da análise da variável % R4⁺ no painel 1 seria o mais preciso.

[0067] O teste exato de Fisher mostrou que os parâmetros diagnósticos para o câncer de próstata % R4⁺, MFI e MDFI de R4 em células CD45⁻ EpCAM⁺ foram estatisticamente

significantes (destacados em negrito na Tabela 2). Estes parâmetros apresentaram a mesma sensibilidade, sendo a percentagem de células R4⁺ o parâmetro mais específico.

[0068] Tabela 2. Desempenho diagnóstico dos aptâmeros R4 e D4 em células CD45⁻ EpCAM⁺ para o câncer de próstata.

Variável (<i>cutoff</i>)	Tamanho do efeito (IC de 95%)					Acurácia
	Sensibilid ade	Especificid ade	Valor Preditivo Positivo	Valor Preditivo Negativo	Razão de Verossimil hança	
% R4⁺ (>0,945)	0,75 (0,5313 - 0,8881)	0,7857 (0,5241 - 0,9243)	0,8333 (0,6078 - 0,9416)	0,6875 (0,444 - 0,8584)	3,5	76,4706
MFI de R4 (u. a.) (>2730)	0,75 (0,5313 - 0,8881)	0,7143 (0,4535 - 0,8828)	0,7895 (0,5667 - 0,9149)	0,6667 (0,4171 - 0,8482)	2,625	73,5294
MDFI de R4 (u. a.) (>2661)	0,75 (0,5313 - 0,8881)	0,7143 (0,4535 - 0,8828)	0,7895 (0,5667 - 0,9149)	0,6667 (0,4171 - 0,8482)	2,625	73,5294
% D4⁺ (>1)	0,7 (0,481 - 0,8545)	0,6429 (0,3876 - 0,8366)	0,7368 (0,5121 - 0,8819)	0,6 (0,3575 - 0,8018)	1,96	67,6471
MFI de D4 (u. a.) (>3398)	0,55 (0,3421 - 0,7418)	0,5714 (0,3259 - 0,7862)	0,6471 (0,413 - 0,8269)	0,4706 (0,2617 - 0,6904)	1,283	55,8824
MDFI de D4 (u. a.) (>3056)	0,65 (0,4329 - 0,8188)	0,5714 (0,3259 - 0,7862)	0,6842 (0,4601 - 0,8464)	0,5333 (0,3012 - 0,7519)	1,517	61,7647

[0069] A performance das variáveis relacionadas ao aptâmero R4 no painel 1 em testes diagnósticos em um contexto de rastreamento (ou *screening*) de câncer de próstata seria a mesma para os *cutoffs* avaliados se apenas considerarmos a sensibilidade. Entretanto, para a variável % R4⁺ a razão de verossimilhança é maior e consequentemente a acurácia do teste diagnóstico também. Já para o contexto onde é preciso um teste

confirmatório para decidir a realização de um procedimento invasivo, por exemplo uma prostatectomia radical, o resultado da variável % R4⁺ seria o mais indicado para tomar a decisão. Assim, a chance de falsos positivos seria menor evitando sobretratamentos lesivos ou biópsias desnecessárias.

Exemplo 4: RESULTADOS DA MARCAÇÃO DO PAINEL 2

[0070] Os dados extraídos das células incubadas com o painel 2 consistiram na percentagem da população de células positivas simultaneamente para CD133 e CD44 e que foram marcadas com a fluorescência de cada aptâmero e suas respectivas intensidades média e mediana em u. a. (Tabela 3).

[0071] Tabela 3. Percentagem de células positivas para R4 ou D4 e o grau de expressão do alvo de cada aptâmeros na população de células CD133⁺ CD44⁺.

Amostras		Painel 2: células CD133 ⁺ CD44 ⁺					
Nº	Tipo	% R4 ⁺	MFI de R4 (u. a.)	MDFI de R4 (u. a.)	% D4 ⁺	MFI de D4 (u. a.)	MDFI de D4 (u. a.)
1	Controle	36.2	19476	10173	69.2	29888	7321
2	Controle	23.2	22003	6274	25	7571	3197
3	Controle	20.1	3414	2912	23.8	6084	2853
4	Controle	14.5	4422	2387	27.9	6648	2588
5	Controle	26.1	3348	2455	34.2	4589	2674
6	Controle	33.3	3331	2391	22.2	3478	2439
7	Controle	60.2	7965	7145	60.4	17202	7411
8	Controle	12.5	4389	4389	100	2503	2426
9	Controle	23.1	2890	2853	26.1	7013	2888
10	Controle	11.6	2734	2406	22.1	4191	2578
11	Controle	5.69	9598	2227	21.7	3535	2729
12	Controle	17.4	6383	2485	17	8203	5465
13	Controle	21.1	7396	2547	22.9	8262	3289
14	Controle	17.2	6795	2853	7.69	2636	2445
15	Controle	10.8	2365	2227	11.4	2658	2445
16	Controle	25	3290	2948	37.6	3008	2936
17	Controle	8.45	3022	2455	10.1	3825	2358

Amostras		Painel 2: células CD133 ⁺ CD44 ⁺					
Nº	Tipo	% R4 ⁺	MFI de R4 (u. a.)	MDFI de R4 (u. a.)	% D4 ⁺	MFI de D4 (u. a.)	MDFI de D4 (u. a.)
18	Controle	4.49	4041	3158	19.6	8257	3045
19	Controle	19.1	4623	3303	21.9	3949	3412
20	Controle	31.9	13270	7718	20.5	16617	9495
21	Paciente	81.8	22365	8861	40	17728	6074
22	Paciente	49.6	60569	39339	42.6	129401	93438
23	Paciente	36.9	8555	3997	40.4	18871	4283
24	Paciente	20.3	12043	6300	20.5	21440	6377
25	Paciente	48.1	8227	4515	30.7	9468	5577
26	Paciente	41.9	9316	6074	37.6	8921	4683
27	Paciente	14.7	3595	2406	45.1	26279	2936
28	Paciente	41.7	18270	5532	21.7	17357	4479
29	Paciente	34.8	9098	6588	54.2	9829	6588
30	Paciente	15.1	5756	2445	27.2	17183	3303
31	Paciente	20.7	8266	2568	13.5	9880	2426
32	Paciente	22.3	3766	2762	27.5	5026	2842
33	Paciente	11.8	2751	2387	12.2	2457	2485
34	Paciente	28	5365	2674	29.5	7556	2865

[0072] As figuras 6 e 7 (A, C e E) representam a diferença das variáveis entre os indivíduos controles e pacientes do painel 2, para o aptâmero R4 e D4, respectivamente. As barras representam a média e o desvio padrão em cada grupo, sendo a linha vermelha o valor do *cutoff* (plotado à direita do eixo Y) da curva ROC representada à direita (Figuras 6 e 7, B, D e F). A diferença entre os grupos foi estatisticamente significativa para a percentagem de células R4⁺ (0,03), MFI de R4 (0,047) e MFI de D4 (0,0026). Assim, os valores da percentagem de células R4⁺ e da MFI de R4 foram estatisticamente significantes nos dois painéis apresentados.

[0073] A melhor curva ROC do painel 2 foi a da variável MFI de D4, com AUC 0,8 (Figura 7 D), superando às variáveis do painel 1. Também mostraram uma boa curva ROC as

variáveis % R4+, e MFI de R4 para o painel 2 (Figura 6 B e D), as quais também mostram um bom desempenho no painel 1.

[0074] Os parâmetros diagnósticos para o câncer de próstata MFI de R4, % D4+, MFI e MDFI de D4 em células CD133⁺ CD44⁺ foram estatisticamente significantes segundo o teste exato de Fisher (destacados em negrito na Tabela 4). A sensibilidade da variável MFI de D4 em células CD133⁺ CD44⁺ foi a maior dos dois painéis seguida pela MFI de R4 em células CD133⁺ CD44⁺. A especificidade da MFI de D4 em células CD133⁺ CD44⁺ também foi superior à das outras variáveis do mesmo painel e igual à da % de células R4⁺ em células CD45⁻ EpCAM⁺.

[0075] Tabela 4. Desempenho diagnóstico dos aptâmeros R4 e D4 em células CD133⁺ CD44⁺ para o câncer de próstata.

Variável (<i>cutoff</i>)	Tamanho do efeito (IC de 95%)					Acurácia
	Sensibilid ade	Especificid ade	Valor Preditivo Positivo	Valor Preditivo Negativo	Razão de Verossimil hança	
% R4 ⁺ (>21,7)	0,6 (0,3866 - 0,7812)	0,6429 (0,3876 - 0,8366)	0,7059 (0,4687 - 0,8672)	0,5294 (0,3096 - 0,7383)	1,68	61,7647
MFI de R4 (u. a.) (>8096)	0,8 (0,584 - 0,9193)	0,6429 (0,3876 - 0,8366)	0,7619 (0,5491 - 0,8937)	0,6923 (0,4237 - 0,8732)	2,24	73,5294
MDFI de R4 (u. a.) (>3650)	0,75 (0,5313 - 0,8881)	0,5714 (0,3259 - 0,7862)	0,7143 (0,5004 - 0,8619)	0,6154 (0,3552 - 0,8229)	1,75	67,6471
% D4 ⁺ (>26,65)	0,7 (0,481 - 0,8545)	0,7143 (0,4535 - 0,8828)	0,7778 (0,5479 - 0,91)	0,625 (0,3864 - 0,8152)	2,45	70,5882
MFI de D4 (u. a.) (>8592)	0,85 (0,6396 - 0,9476)	0,7857 (0,5241 - 0,9243)	0,85 (0,6396 - 0,9476)	0,7857 (0,5241 - 0,9243)	3,967	82,3529

Variável (<i>cutoff</i>)	Tamanho do efeito (IC de 95%)					Acurácia
	Sensibilid ade	Especificid ade	Valor Preditivo Positivo	Valor Preditivo Negativo	Razão de Verossimil hança	
MDFI de D4 (u. a.) (>3296)	0,75 (0,5313 - 0,8881)	0,6429 (0,3876 - 0,8366)	0,75 (0,5313 - 0,8881)	0,6429 (0,3876 - 0,8366)	2,1	70,5882

[0076] Nas amostras analisadas, a variável MFI de D4 no painel 2 revelou-se como o melhor parâmetro diagnóstico para o câncer de próstata, inclusive com a maior razão de verossimilhança e acurácia. Esta variável poderia ser usada tanto para o rastreamento de câncer de próstata, quanto para a diferenciação de tumores indolentes, com baixo potencial de disseminação, e tumores com comportamentos mais agressivos.

[0077] O presente sistema aptaimunológico mostrou potencial diagnóstico superior ao PSA, o biomarcador de referência para o câncer de próstata. As variáveis analisadas neste sistema poderiam ser aplicadas para diminuir o sobrediagnóstico no rastreamento do câncer de próstata que chega até 67% de falsos positivos em casos de pacientes com hiperplasia benigna da próstata ou prostatites e PSA maiores que 4,0 ng/mL ⁷⁰ e até cerca de 30% de falsos negativos em pacientes com câncer de próstata que não são diagnosticados pelo PSA ⁷¹.

[0078] Comparando o PSA com a MFI de D4 em células CD133⁺ CD44⁺ D4⁺ que apresentou apenas 21,43% de falsos positivos e 15% de falsos negativos, esta variável demonstrou ser um biomarcador mais efetivo para a detecção de câncer de próstata. Deste modo, o uso clínico do presente sistema aptaimunológico otimizaria os recursos financeiros destinados ao câncer de próstata nos sistemas de saúde, tanto públicos quanto privados, diminuiria a mortalidade desta doença e melhoraria a qualidade de vida dos pacientes.

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REIVINDICAÇÕES

1. Sistema aptaimunológico **caracterizado por** uma composição teranóstica que combina anticorpos e aptâmeros modificados em um processo que compreende as etapas de aquisição, processamento e análise da amostra por citometria de fluxo para a determinação do uso clínico de células de câncer de próstata detectadas em pacientes.
2. Composição teranóstica, de acordo com a reivindicação 1, **caracterizada por** compreender dois painéis aptaimunológicos (painel 1 e painel 2) que incluem uma combinação dos aptâmeros R4 e D4 e dos anticorpos anti-EpCAM, anti-CD45, anti-CD44 e anti-CD133 com seus respectivos controles.
3. Painel 1, de acordo com a reivindicação 2, **caracterizado por** consistir em um aptâmero (R4 ou D4) com seu ligante estreptavidina marcado com um fluorocromo e os anticorpos anti-EpCAM e anti-CD45 também marcados com outros fluorocromos diferentes entre si; e seus respectivos controles (estreptavidina, isotipo de anti-EpCAM e isotipo de anti-CD45) com a mesma marcação.
4. Painel 2, de acordo com a reivindicação 2, **caracterizado por** consistir em um aptâmero (R4 ou D4) com seu ligante estreptavidina marcado com um fluorocromo e os anticorpos anti-CD44 e anti-CD133 também marcados com outros fluorocromos diferentes entre si; e seus respectivos controles (estreptavidina, isotipo de anti-CD44 e isotipo de anti-CD133) com a mesma marcação.
5. Dois aptâmeros modificados denominados R4 e D4, **caracterizados por** serem oligonucleotídeos de fita simples que contêm a sequência de 28 pares de bases do aptâmero A4 com o açúcar ribose (no caso de R4) ou desoxirribose (no caso de D4) modificados com uma biotina acoplada no extremidade 5' e uma 2'desoxitimidina monofosfato unida por uma ligação 3'-3' na extremidade 3'.
6. Um kit, **caracterizado por** conter ao menos um dos dois painéis aptaimunológicos, tubos de coleta a vácuo com EDTA, tampão de lise de hemácias com cloreto de amônia e sem fixador celular, tampão de bloqueio com plasma humano inativado de sangue tipo AB, tampão fosfato com albumina sérica bovina e azida básica como solução de lavagem e corante de viabilidade com fluorescência diferente às usadas nos painéis.

7. Processo, de acordo com a reivindicação 1, **caracterizado por** compreender os métodos utilizados nas diferentes etapas que envolvem a aquisição, o processamento e a análise de amostras de sangue venoso periférico de indivíduos humanos.
8. Primeira etapa do processo, de acordo com a reivindicação 7, **caracterizada por** coletar as amostras a partir da punção venosa na fossa cubital do braço do indivíduo, usando os tubos proporcionados pelo kit, de acordo com a reivindicação 6, e descartar o primeiro volume de sangue (1 mL) para a seguinte etapa.
9. Segunda etapa do processo, de acordo com a reivindicação 7, **caracterizada por** isolar a camada de células mononucleares do sangue não descartado após sua centrifugação.
10. Terceira etapa do processo, de acordo com a reivindicação 7, **caracterizada por** lisar as hemácias e em seguida lavar a suspensão celular resultante usando o tampão de lise e a solução de lavagem respectivamente, proporcionado pelo kit, de acordo com a reivindicação 6.
11. Quarta etapa do processo, de acordo com a reivindicação 7, **caracterizada por** bloquear as ligações inespecíficas da porção Fc dos anticorpos usados nos painéis com a solução de bloqueio proporcionado pelo kit, de acordo com a reivindicação 6.
12. Quinta etapa do processo, de acordo com a reivindicação 7, **caracterizada por** incubar separadamente a suspensão celular no tampão de bloqueio com os painéis aptaimunológicos e o corante de viabilidade e em seguida lavar com solução de lavagem, itens proporcionados todos eles pelo kit, de acordo com as reivindicações 2 a 6.
13. Sexta etapa do processo, de acordo com a reivindicação 7, **caracterizada por** marcar indiretamente os aptâmeros com seu ligante estreptavidina e em seguida lavar com solução de lavagem, proporcionados ambos pelo kit, de acordo com as reivindicações 2 a 6.
14. Sétima etapa do processo, de acordo com a reivindicação 7, **caracterizada por** ler a fluorescência de 500 mil leucócitos das amostras processadas, a partir da identificação dos mesmos por seu tamanho e granulosidade em um citometro de fluxo.

15.Oitava etapa do processo, de acordo com a reivindicação 7, **caracterizada por** analisar os dados da citometria obtidos na etapa anterior para identificar células tumorais circulantes (CTCs) da próstata, de acordo com a reivindicação 14.

16. Identificação de CTCs da próstata, de acordo com a reivindicação 15, **caracterizada por** selecionar os *singlets*, e de entre os *singlets* selecionar as células vivas, e de entre as células vivas selecionar individualmente por histograma as CTCs de cada painel, segundo seus controles.

17. Identificação de CTCs da próstata no painel 1, de acordo com a reivindicação 16, **caracterizada por** selecionar as células com marcação negativa para CD45 e de entre elas, selecionar as células com marcação positiva para EpCAM e de entre estas selecionar as células com marcação positiva para os aptâmeros (R4 ou D4).

18. Identificação de CTCs da próstata no painel 2, de acordo com a reivindicação 16, **caracterizada por** selecionar as células com marcação positiva para CD133 e de entre elas, selecionar as células com marcação positiva para CD44 e de entre estas selecionar as células com marcação positiva para os aptâmeros (R4 ou D4).

19. Uso clínico do sistema aptaimunológico, de acordo com todas as reivindicações anteriores, **caracterizado por** estar baseado na quantificação de CTCs da próstata identificadas por ao menos um dos painéis e no grau de expressão dos alvos dos aptâmeros, a partir da intensidade média de fluorescência nas mesmas células.

20. Uso clínico do sistema aptaimunológico, de acordo com a reivindicação 19, **caracterizado por** indicar a presença de células tumorais circulantes da próstata no indivíduo.

21. Uso clínico do sistema aptaimunológico, de acordo com a reivindicação 20, **caracterizado por** incluir as aplicações de diagnóstico, triagem, análise de disposição à doença, prognóstico, predição do melhor tratamento, farmacogenômica, monitoramento do tratamento, e desenvolvimento de tratamentos com direcionamento de qualquer agente terapêutico acoplado ao aptâmero D4 ou R4, de acordo com a reivindicação 5.

DESENHOS

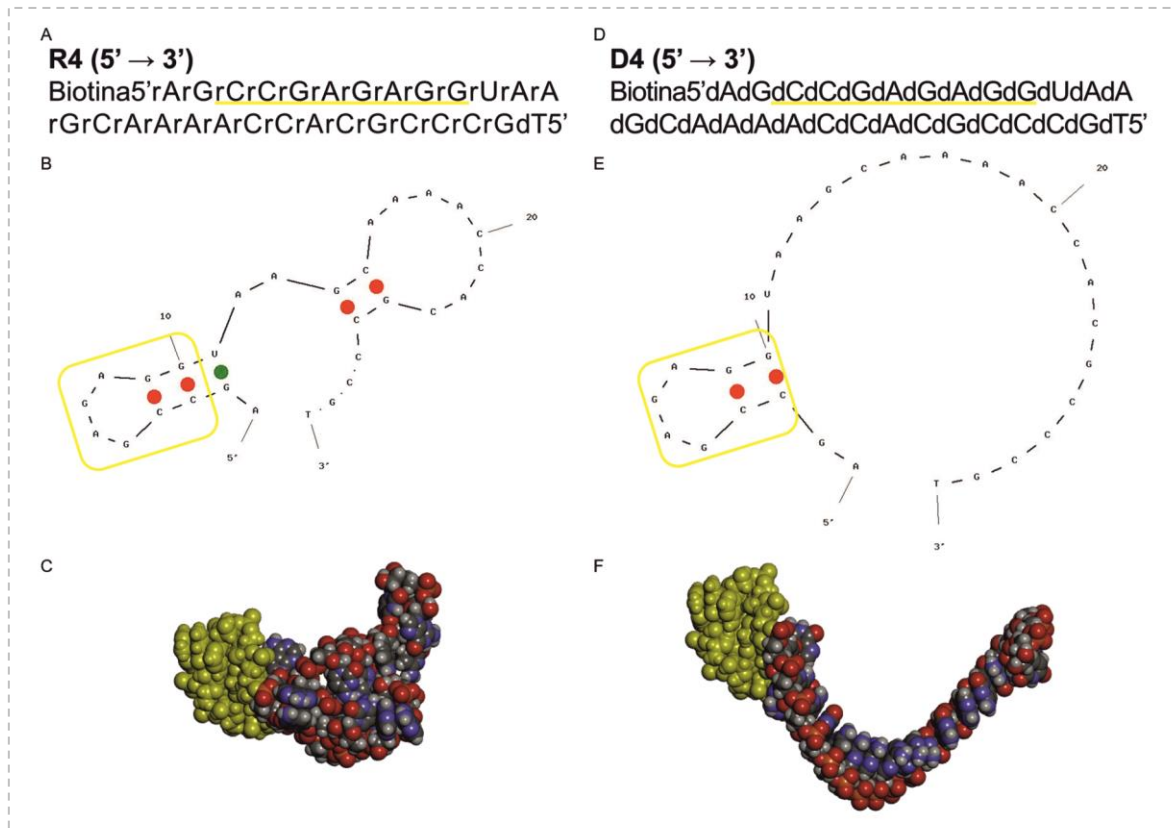


Figura 1

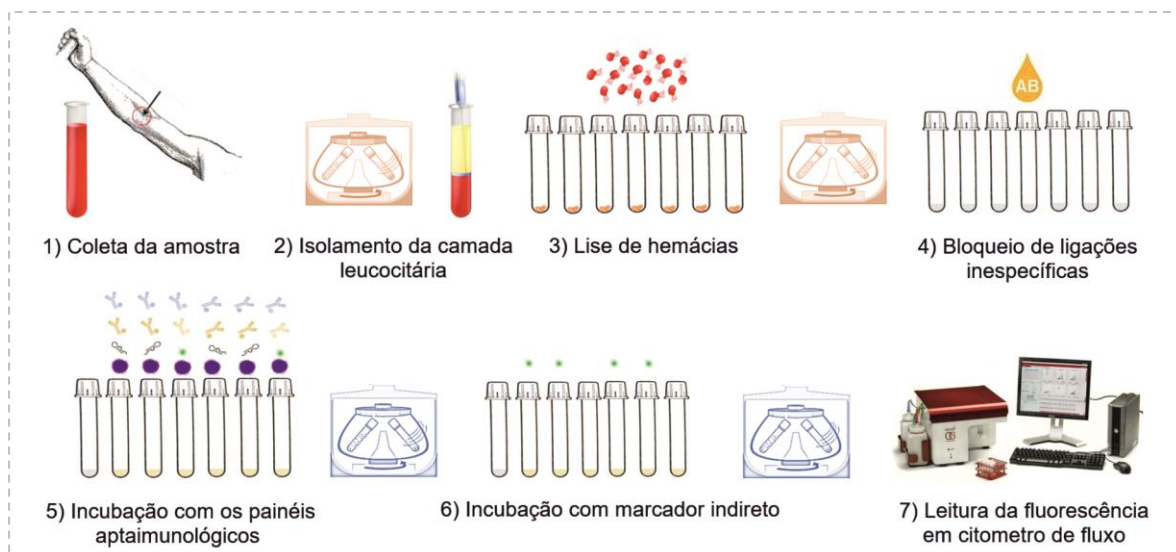


Figura 2

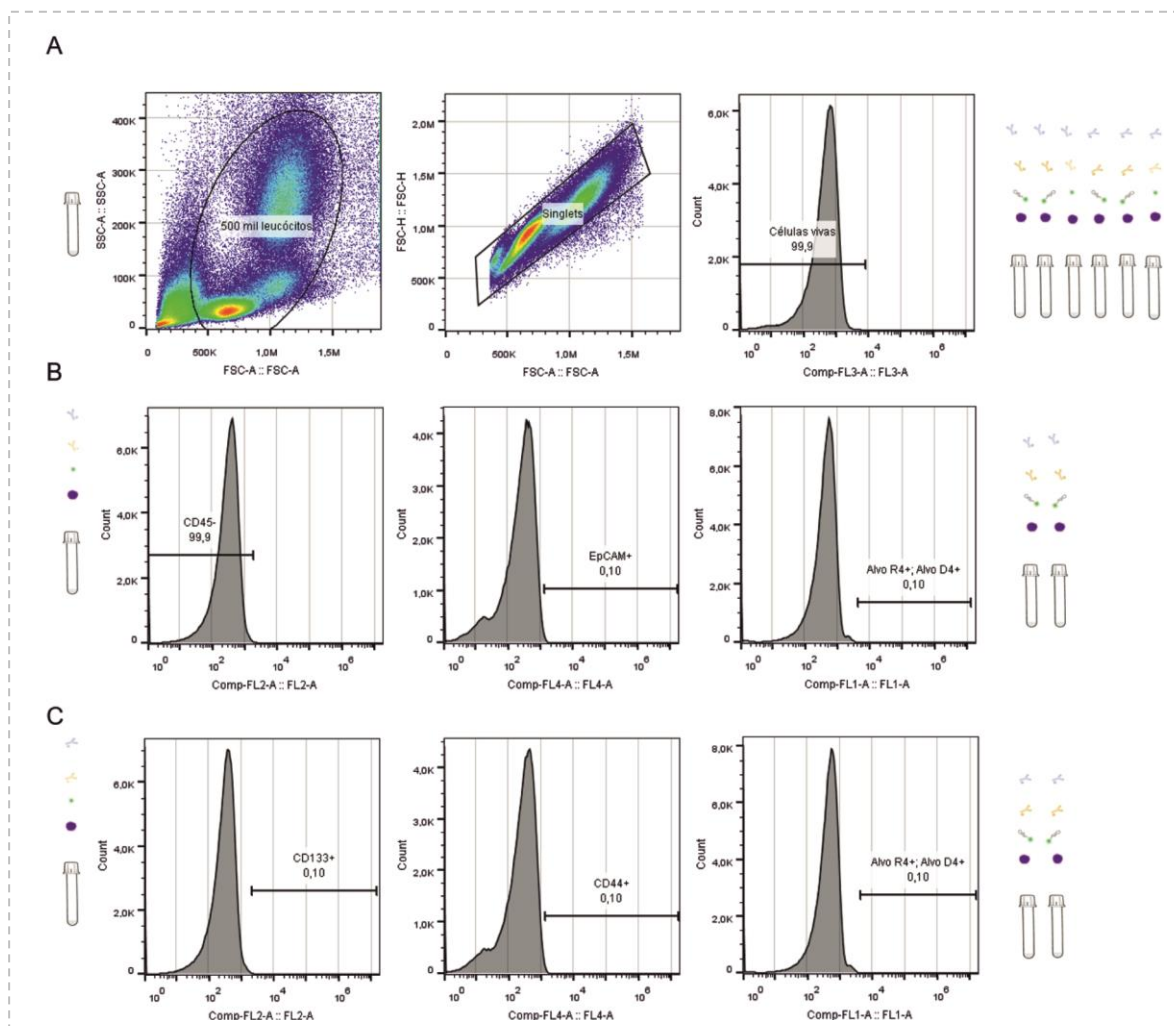


Figura 3

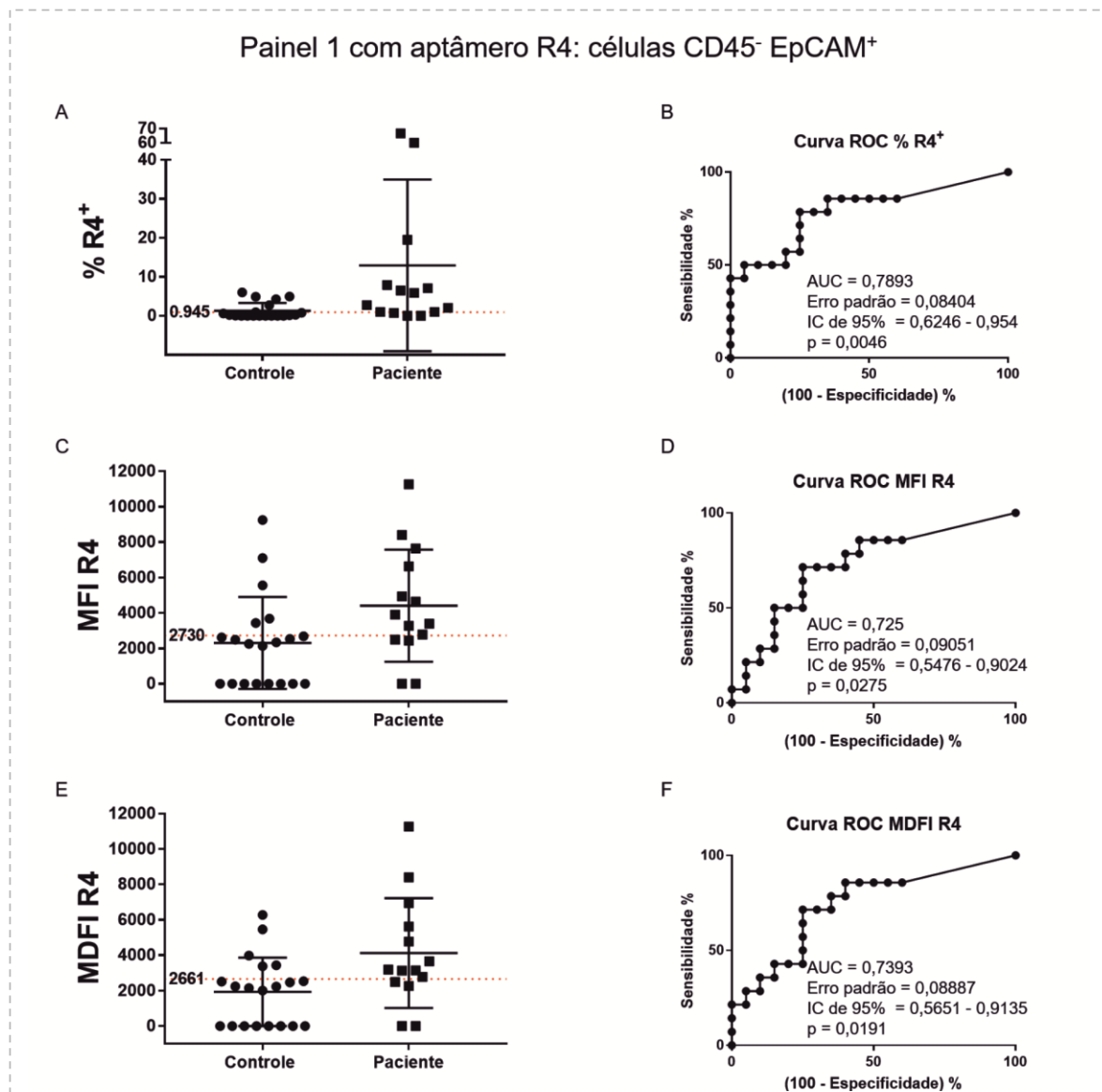


Figura 4

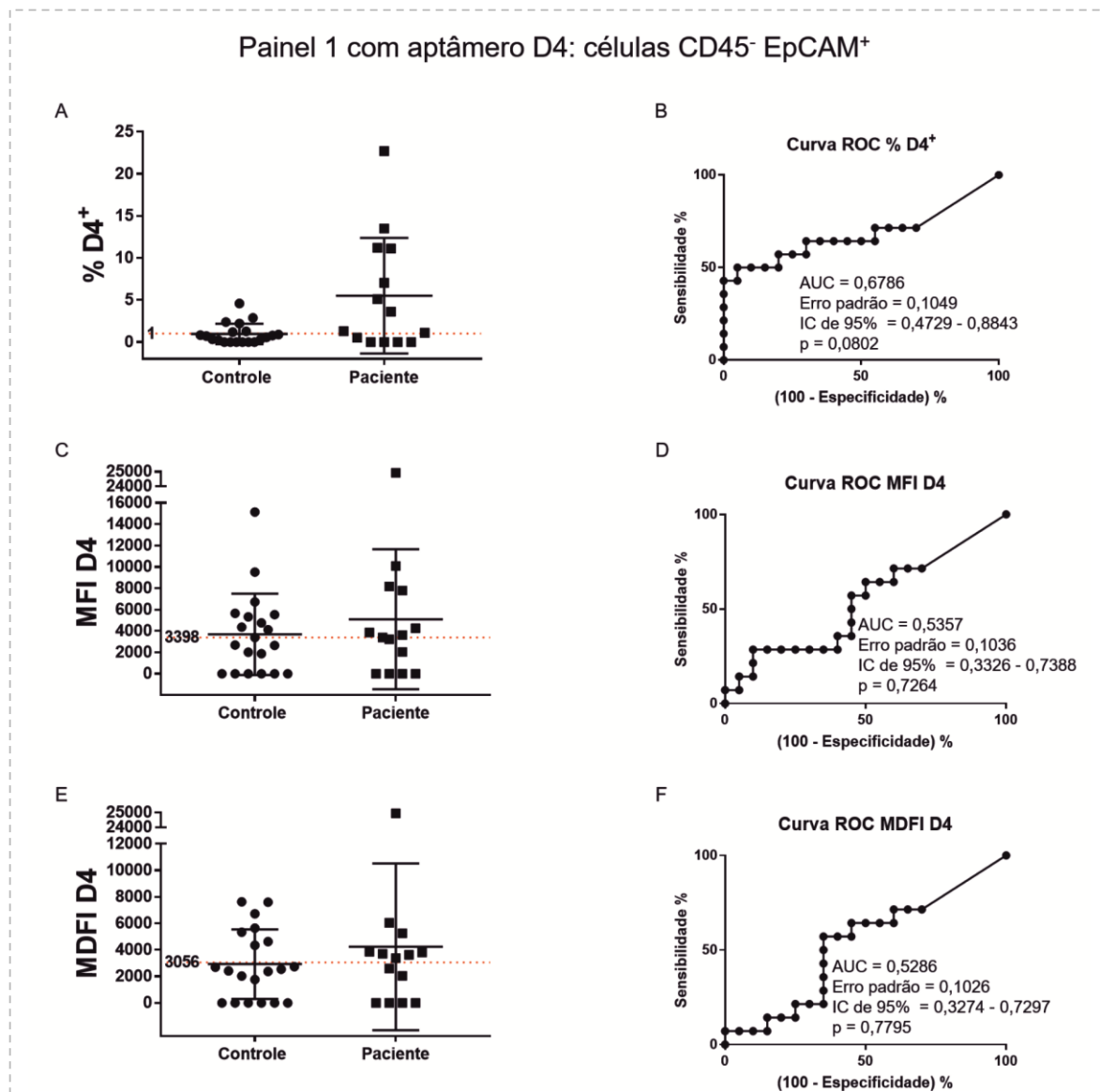


Figura 5

Painel 2 com aptâmero R4: células CD133⁺ CD44⁺

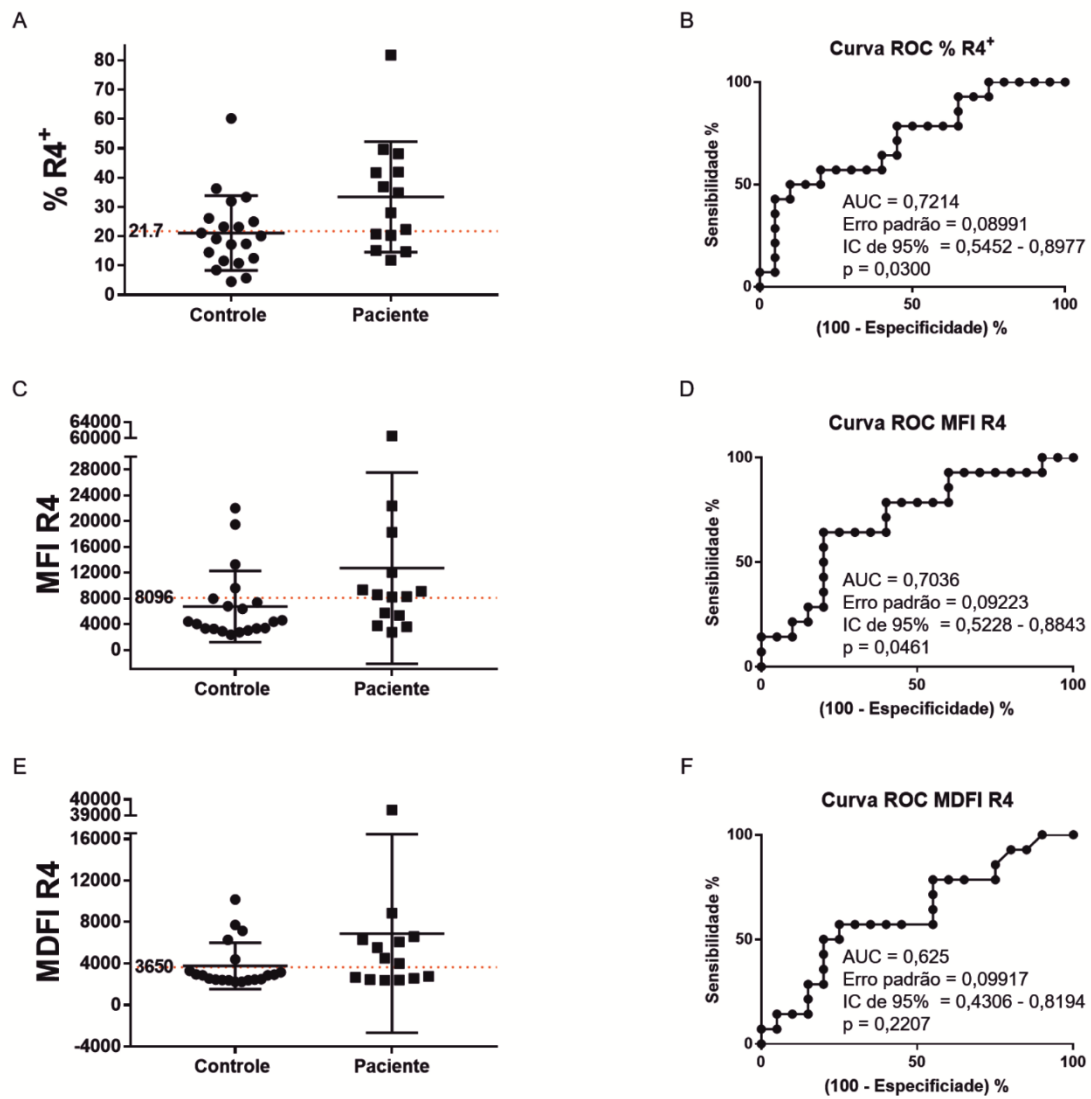


Figura 6

Painel 2 com aptâmero D4: células CD133⁺ CD44⁺

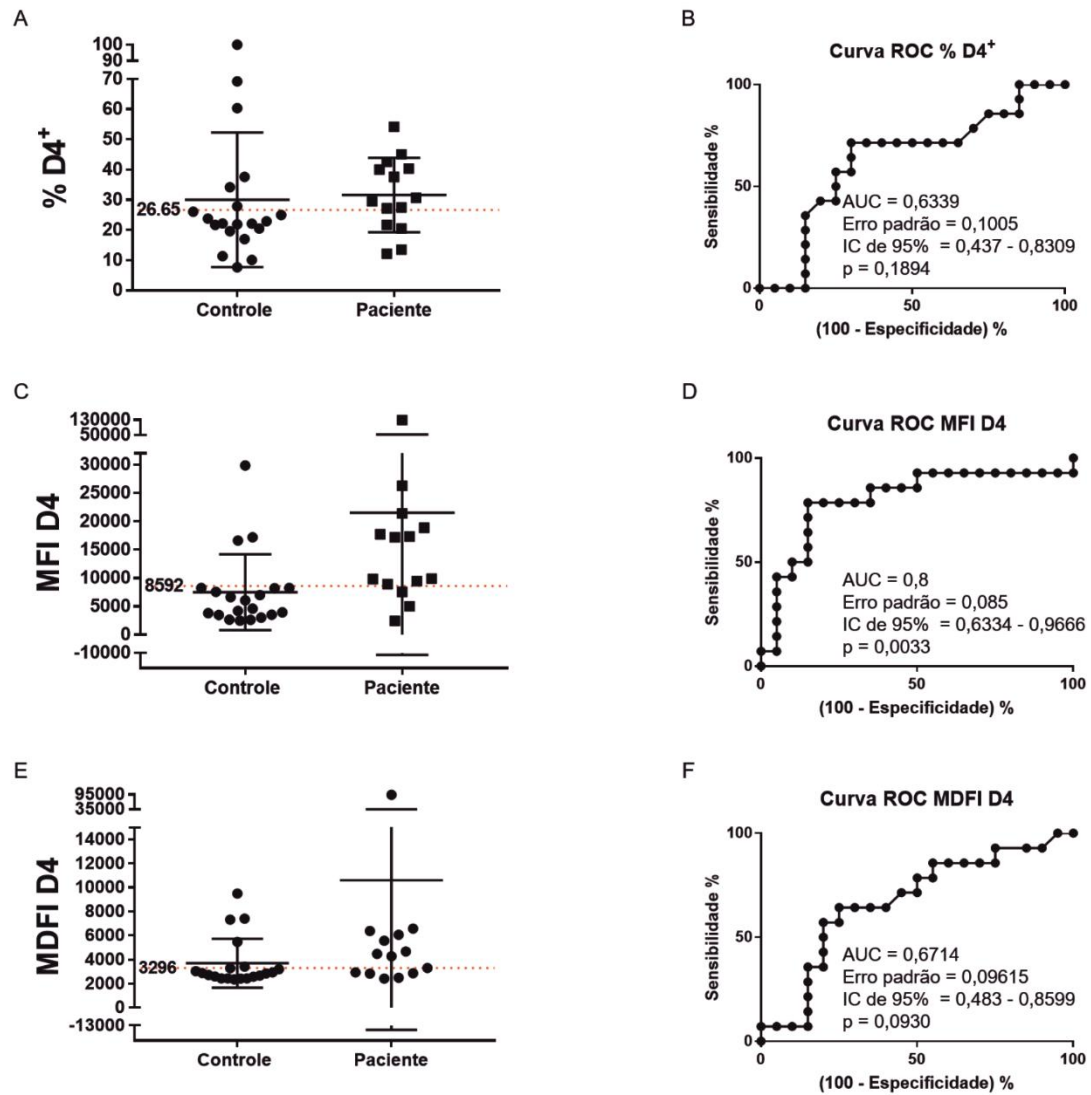


Figura 7

RESUMO

SISTEMA APTAIMUNOLÓGICO PARA A DETECÇÃO DE CÂNCER DE PRÓSTATA

[0079] A presente invenção refere-se a um conjunto de anticorpos e aptâmeros que identificam biomarcadores do câncer de próstata em células tumorais circulantes de amostras biológicas. A partir da análise destes biomarcadores por citometria de fluxo é possível discriminar pacientes com câncer de próstata de uma maneira superior ao antígeno prostático específico que é o biomarcador padrão-ouro no câncer de próstata. Assim, este sistema aptaimunológico apresenta um menor índice de falsos positivos e falsos negativos, o que seria útil na triagem, diagnóstico, prognóstico e monitoramento terapêutico do câncer de próstata para aprimorar seu manejo clínico.