MONYSE DE NÓBREGA

“MICRORNAS LIVRES DE CÉLULAS E MICRORNAS ASSOCIADOS A VESÍCULAS EXTRACELULARES COMO BIOMARCADORES EM BIÓPSIA LÍQUIDA PARA O CÂNCER DE PRÓSTATA”

Londrina
2023

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Tese apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Estadual de Londrina, área de concentração Genômica e Biologia Molecular, como requisito parcial para obtenção do título de doutor.

Orientador: Profa. Dra. Ilce Mara de Syllos Cólus.
Coorientador: Profa. Dra. Mariana Bisarro dos Reis.

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MONYSE DE NÓBREGA

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Londrina, 28 de fevereiro de 2023.

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À minha família, especialmente aos meus pais por terem fornecido lápis e borracha para que eu pudesse escrever nas linhas da vida.

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“There's always gonna be another mountain
I'm always gonna wanna make it move
Always gonna be an uphill battle
Sometimes I'm gonna have to lose
Ain't about how fast I get there
Ain't about what's waiting on the other side
It's the climb”.

Miley Cyrus

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RESUMO

MicroRNAs (miRNAs) são moléculas que têm importante papel como reguladores da expressão gênica, e desregulações na expressão de miRNAs têm sido associadas com a progressão de muitos tipos de tumores humanos. Devido ao baixo custo, ser uma molécula instável e minimamente invasiva, o miRNA apresenta um importante potencial como biomarcador. Por este motivo, este estudo teve como objetivo avaliar miRNAs circulantes (cell-free e derivados de vesículas extracelulares) para serem usados como biomarcadores minimamente invasivos, capazes de promover diagnóstico precoce, discriminar diferentes subgrupos do câncer de próstata (CaP) e predizer progressão tumoral. MiR-25-3p, miR-92a-1-5p, miR-92a-2-5p e miR-148a-3p foram selecionados dos dados do *The Cancer Genome Atlas* para serem avaliados em amostras de plasma total de pacientes com CaP e de indivíduos controles. O miR-25-3p em pacientes com CaP mostrou expressão aumentada e foi associado a pior prognóstico. Este miRNA aumentou a proliferação, migração e invasão celular em estudo funcional em cultura de células LNCaP. A análise de expressão gênica indicou que a expressão dos genes *BCL2L11, CDH1, CDKN1C, EZH2* e *TP53,* alvos diretos do miR-25-3p, estava diminuída após a transfecção. Posteriormente, com o objetivo de comparar a relação de expressão de EV-miRNAs e miRNAs livres de células, escolhemos investigar miR-21-5p, miR-200c-3p, miR-375-3p e miR-1290-3p. Os EV-miRNAs miR-21-5p, miR-200c-3p, miR-375-3p e miR-1290-3p estavam super expressos em amostras de pacientes. Estes resultados demonstram que apesar de ambas as formas de miRNAs serem relevantes para a patogênese do CaP, EV-miRNAs mostraram um maior potencial como biomarcadores quando comparados com cell-free miRNAs.

Palavras-chave: miRNAs circulantes; *cell-free* miRNAs; miRNAs exossomais; biópsia líquida; câncer de próstata; vesículas extracelulares (VEs).

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ABSTRACT

MicroRNAs (miRNAs) play an important role as gene expression regulators, and their dysregulation has been associated with the progression of many types of human tumours, including prostate cancer (PCa). Due to their low cost, minimal invasiveness, and stability, miRNAs have significant potential as biomarkers. This study aimed to assess circulating miRNAs, including cell-free and those derived from extracellular vesicles (EVs), as minimally invasive biomarkers for early PCa diagnosis, subtyping, and progression prediction. MiR-25-3p, miR-92a-1-5p, miR-92a-2-5p, and miR-148a-3p were selected from The Cancer Genome Atlas data for assessing total plasma samples from PCa patients and control individuals. MiR-25-3p exhibited increased expression in PCa patients and was associated with a worse prognosis. Functional studies demonstrated that miR-25-3p increased LNCaP cell culture proliferation, migration, and invasion. Gene expression analysis indicated that miR-25-3p directly targets and downregulates the expression of *BCL2L11, CDH1, CDKN1C, EZH2*, and *TP53* genes. To compare the expression ratio of EV-miRNAs versus cell-free miRNAs, miR-21-5p, miR-200c-3p, miR-375-3p, and miR-1290-3p were investigated. The EV-miRNAs miR-21-5p, miR-200c-3p, miR-375-3p, and miR-1290-3p were overexpressed in patient samples. These results demonstrate that although both forms of miRNAs are relevant to PCa pathogenesis, EV-miRNAs showed greater potential as biomarkers when compared with cell-free miRNAs.

Key-words: circulating miRNAs; cell-free miRNAs; exosomal miRNAs; liquid biopsy; prostate cancer; extracellular vesicles.

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LISTA DE ABREVIATURAS E SIGLAS

3' UTR	região 3' não traduzida
5' UTR	região 5' não traduzida
AGO	Argonauta
BCL2L11	B-cell lymphoma 2 Like 11
CaP	Câncer de Próstata
CDH1	do inglês, Cadherin 1
CDKN1C	Cyclin Dependent Kinase Inhibitor 1C
cf-miRNA	do inglês, Cell-free microRNA
cfNAs	do inglês, *cell free Nucleic Acid*
CMV	Corpo Multivesicular
CTCs	Células Tumorais Circulantes
ctDNA	do inglês, *circulating tumoral DNA*
ctRNA	do inglês, *circulating tumoral RNA*
DHT	di-hidrotestosterona
DNA	do inglês, *Deoxyribonucleic acid*
DRE	do inglês, Digital Rectal Examination
EDTA	do inglês, Ethylenediaminetetraacetic Acid
EMT	do inglês, Epithelial Mesenchymal Transition;
EUA	Estados Unidos da América
EV-miRNA	do inglês, extracellular vesicles microRNAs
EZH2	do inglês, Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit
FC	do inglês, Fold-Change
FDA	do inglês, *Food and Drug Administration*
FDR	do inglês, False Discovery Rate
GS	do inglês, Gleason Score
HPB	Hiperplasia Prostática Benigna
HPV	do inglês, *Human Papillomavirus*
HRP	Peroxidase de Rábano
INCA	Instituto Nacional de Câncer José Alencar Gomes da Silva
ISEV	do inglês, *International Society of Extracellular Vesicles*
ISUP	do inglês, International Society of Urological Pathology
LB	do inglês, Liquid Biopsy

LIN-4	do inglês, *lineage-deficient-4*
LNCaP	do inglês, Lymph Node Carcinoma Prostatic
MCM7	do inglês Minichromosome Maintenance Complex Component 7
MDM2	MDM2 Proto-Oncogene
MiRNAs	microRNAs
mRNA	do inglês, Messenger RNA
MYC	do inglês, Avian myelocytomatosis viral oncogene homolog
ncRNAs	do inglês, Non-coding RNAs
NSCLC	do inglês, *Non Small-Cell Lung Carcinoma*
NTA	do inglês, *Nanoparticle Tracking Analysis*
oncomiR	do inglês, Oncogenic microRNA
PCa	do inglês, Prostate cancer
PIN	do inglês, *Prostatic Intraepithelial Neoplasia*
pré-miRNAs	Precursores de MicroRNAs
pri-miRNA	do inglês, *Primary transcripts microRNAs*
PSA	do inglês, *Prostatic antigen specific*
RISC	do inglês, *RNA-induced silencing complex*
RNA pol II	RNA polimerase II
RNA	do inglês*, Ribonucleic acid*
ROC	do inglês, Receiver Operating Characteristic
RT-qPCR	do inglês, Quantitative reverse transcription PCR
TBST	Solução Salina Tris-Tamponada -Tween
TCGA	do inglês, *The Cancer Genome Atlas*
TEM	do inglês, *Transmission Electron Microscopy*
TP53	do inglês, Tumor Protein P53 (human)
TRBP	do inglês, *Transactivation Responsive RNA-Binding Protein*
UICC	do inglês, *Union for International Cancer Control*
UTR	do inglês, Untranslated Regions
VEs	Vesículas Extracelulares
WB	do inglês: *Western Blotting*
WDR4	do inglês, WD repeat domain 4

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1. INTRODUÇÃO

O câncer de próstata (CaP) é um problema de saúde pública e a segunda neoplasia maligna mais frequente no mundo, sendo a quinta causa de mortalidade devido a câncer em homens. De acordo com a última estimativa do GLOBOCAN, o CaP teve 1.414.259 de novos casos e 375.304 mortes no ano de 2020 (SUNG et al., 2021). No Brasil, segundo o Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA), órgão do Ministério da Saúde, o CaP é o câncer mais frequente na população masculina com idade superior a 65 anos e a estimativa para cada ano do triênio 2023-2025 é de aproximadamente 72 mil novos casos (INCA, 2022). Apesar da alta incidência do CaP, sabe-se relativamente pouco sobre sua etiologia, mecanismos moleculares envolvidos na carcinogênese e sua progressão. Entretanto, é consenso que se trata de uma doença heterogênea, com comportamentos biológicos distintos (FAHMY et al., 2021).

A triagem para este tipo tumoral é tradicionalmente realizada por dois exames, o toque retal e o PSA (do inglês, *prostatic antigen specific*) (DUFFY, 2020). Contudo, a especificidade do PSA é limitada, sendo a biópsia positiva em somente 25% dos pacientes com concentrações séricas entre 2 e 10 ng/mL (SRIVASTAVA et al., 2019; WELCH; ALBERTSEN, 2020). O padrão ouro para diagnóstico de tumores é feito por biópsias de tecido, que devido à sua natureza invasividade e incompatibilidade, estão associadas a muitas limitações, incluindo risco para o paciente, preparação da amostra, sensibilidade e precisão, custos de procedimentos e invasividade, como também é incompatível com o acompanhamento clínico longitudinal (CORCORAN, 2020; QI et al., 2018). Para superar essas limitações, há uma busca por novos métodos capazes de predizer com precisão o comportamento biológico do tumor e acelerar o diagnóstico, prognóstico e a resposta terapêutica para estratificações de indivíduos.

As biópsias líquidas têm um grande potencial para superar essas limitações. Normalmente obtida de sangue, pode ser utilizada para fins de triagem, diagnóstico, prognóstico e acompanhamento de terapias em tempo real. O “circuloma tumoral”, definido como o subconjunto de componentes circulantes, é derivado do tecido tumoral e pode ser usado direta ou indiretamente como fonte de biomarcadores de câncer em biópsias líquidas. Esses componentes incluem proteínas tumorais circulantes, ácidos nucleicos tumorais circulantes (ctDNA e ctRNA), células tumorais circulantes e plaquetas “educadas” pelo tumor. Sendo assim, as biópsias líquidas apresentam vantagens importantes sobre as biópsias de tecidos convencionais, de tal modo que avanços tecnológicos referentes ao isolamento de amostras (por exemplo, o desenvolvimento de *chips* de extração de ácidos nucleicos para minimizar a manipulação de amostras) e plataformas de detecção, (como PCR digital ou plataformas de Western Blotting de célula única), têm evoluído

rapidamente e dado suporte a essa abordagem (DE RUBIS; RAJEEV KRISHNAN; BEBAWY, 2019).

O uso de microRNAs (miRNAs) como marcadores diagnósticos, prognósticos e preditivos para pacientes com CaP tem sido reportado na literatura (DE NÓBREGA et al., 2022). Essas moléculas têm papel fundamental na regulação da expressão gênica e, consequentemente, sua desregulação tem sido associada a diversas condições patológicas, entre elas o câncer (CONDRAT et al., 2020; WINKLE et al., 2021).

Os miRNAs podem ser liberados por células apoptóticas e necróticas, como também podem ser secretados ativamente para a circulação sanguínea na forma de cfNAs (do inglês*, cell free nucleic acid*s) ou em VEs (vesículas extracelulares). Um tipo de VE de origem endossomal são os denominados exossomos, nanovesículas que abrigam miRNAs, mRNAs e proteínas. Proteínas e miRNAs exossomais desempenham um papel importante na comunicação célula-a-célula. Além disso, a quantidade e composição de miRNAs derivados dessas nanovesículas difere entre pacientes com câncer e indivíduos livres de câncer, tornando os miRNAs potenciais biomarcadores de diagnóstico (PARDINI et al., 2019; RZHEVSKIY et al., 2022).

Os miRNAs são classificados em miRNAs oncogênicos (oncomiRs) e miRNAs supressores de tumor. Normalmente os oncomiRs são miRNAs super expressos e têm como alvos os genes supressores tumorais, e os miRNAs supressores de tumor são miRNAs pouco expressos ou deletados, o que permite o aumento da expressão de seus alvos, normalmente proto-oncogenes, levando à progressão do tumor (BARBATO; SOLAINI; FABBRI, 2017).

Alterações na expressão ou nos níveis de miRNAs dentro das células são comuns em cânceres humanos. Atualmente é possível mimetizar ou inibir a função dos miRNAs em ambientes monitorados utilizando metodologias específicas em cultura de células tumorais e, assim, inferir o impacto dessas alterações no câncer, como também seu possível potencial para o tratamento e cura dessa doença (RIOLO et al., 2020).

No presente estudo investigamos o perfil de expressão de oito diferentes miRNAs circulantes, 4 livres de células – cf-miRNAs e outros 4 associados a vesículas extracelulares - EV-miRNAs), comparando os resultados obtidos para os dois grupos. Um dos cf-miRNAs se apresentou diferencialmente expresso e sua funcionalidade foi investigada em linhagem celular humana de carcinoma prostático, identificando sua capacidade oncogênica e seu potencial como biomarcador no CaP. Os resultados obtidos no presente estudo contribuirão para a compreensão de como alterações nos padrões de expressão de miRNAs circulantes podem afetar o desenvolvimento tumoral, pois trazem novos conhecimentos sobre biomarcadores epigenéticos para o CaP.

2. OBJETIVOS

2. 1. Objetivo Geral

Avaliar miRNAs circulantes (cf-miRNAs e EV-miRNAs) como possíveis biomarcadores minimamente invasivos, capazes de promover diagnóstico precoce, discriminar diferentes subgrupos do CaP e predizer progressão tumoral e desenvolvimento de metástases em portadores da doença.

2.2. Objetivos Específicos

a) Selecionar cf-miRNAs candidatos para posterior validação, a partir de tecido tumoral e não tumoral adjacente de pacientes diagnosticados com CaP, oriundos de banco de dados público (TCGA).

b) Selecionar EVs-miRNAs obtidos de plasma, como candidatos para posterior validação, a partir de dados da literatura de pacientes diagnosticados com CaP.

c) Validar como biomarcadores de diagnóstico/prognóstico, um conjunto de cf-miRNAs e EV-miRNAs (observados diferencialmente expressos) obtidos de plasma de pacientes com CaP, local e metastático, e em indivíduos livres de neoplasias;

d) Associar os parâmetros clínicos e histopatológicos do tumor dos pacientes com os dados de expressão gênica dos cf-miRNAs e EV-miRNAs, a fim de relacioná-los com agressividade e progressão da doença.

e) Determinar a consequência da modulação de um cf-miRNA selecionado em relação à proliferação, migração e invasão celulares observadas em sistemas de transfecção *in vitro.*

g) Avaliar a associação da expressão dos cell-free miRNAs selecionados e seus genes alvos correspondentes por RT-qPCR, identificando aqueles miRNAs que possivelmente contribuem para a patogênese do CaP.

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4. REVISÃO BIBLIOGRÁFICA

4.1. CAPÍTULO 1: The potential of cell free and extracellular vesicles microRNAs as biomarkers liquid biopsy in serum and plasma in patients with prostate cancer.

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REVIEW – CANCER RESEARCH

The potential of cell-free and exosomal microRNAs as biomarkers in liquid biopsy in patients with prostate cancer

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Abstract

Purpose Prostate cancer (PCa) is the 4th most diagnosed cancer and the 8th leading cause of cancer-related death worldwide. Currently, clinical risk stratification models including factors like PSA levels, Gleason score, and digital rectal examination are used for this purpose. There is a need for novel biomarkers that can distinguish between indolent and aggressive pathology and reduce the risk of overdiagnosis/overtreatment. Liquid biopsy has a non-invasive character, can lead to less morbidity and provide new biomarkers, such as miRNAs, that regulate diverse important cellular processes. Here, we report an extended revision about the role of cell-free and exosomal miRNAs (exomiRNAs) as biomarkers for screening, diagnosis, prognosis, or treatment of PCa.

Methods A comprehensive review of the published literature was conducted focusing on the usefulness, advantages, and clinical applications of cell-free and exomiRNAs in serum and plasma. Using PubMed database 53 articles published between 2012 and 2021 were selected and discussed from the perspective of their use as diagnostic, prognostic and therapeutic biomarkers for PCa.

Results We identify 119 miRNAs associated with PCa development and the cell-free and exosomal miR-21, miR-141, miR-200c, and miR-375 were consistently associated with progression in multiple cohorts/studies. However, standardized experimental procedures, and well-defined and clinically relevant cohort studies are urgently needed to confirm the biomarker potential of cell-free and exomiRNAs in serum or plasma.

Conclusion Cell-free and exomiRNAs in serum or plasma are promising tools for be used as non-invasive biomarkers for diagnostic, prognosis, therapy improvement and clinical outcome prediction in PCa patients.

Keywords Non-coding RNAs · Serum and plasma · Biomarkers · Preclinical

Introduction

Prostate cancer (PCa) is the most common disease in older men, the second most diagnosed solid-organ malignancy worldwide and the fifth leading cause of cancer death among men in 2020 (Sung et al. 2021). Currently, there are several factors that are well established in relation to the risk of developing this tumor, the most important of which are age, a family history of cancer, and ancestry (Rebbeck 2017; Nóbrega et al. 2020).

As with most cancers, genomic instability is also a hallmark of PCa, being important for orchestrating and accelerating the acquisition of various characteristics of the disease, such as supporting proliferative signaling, resistance to cell death, induction of angiogenesis, and invasion and metastasis activation, among others (Leongamornlert et al. 2014; O'Connor 2015). Therefore, the high incidence of PCa is related to the number of first-degree relatives affected, age at diagnosis (Lynch et al. 2015), accumulation of DNA damage, and decline in DNA repairome during aging (Lockett et al. 2005; Yadav et al. 2020). It is estimated that the risk of developing PCa increases to 20% when men with a family

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history are exposed to certain environmental carcinogens and a common lifestyle (Rawla 2019).

The number of known genes for which germline mutations clearly predispose to PCa is small, and most of them are involved in DNA repair, including *BRCA2*, *BRCA1*, *CHEK2*, *NBS1*, *HOXB13*, *ATM*, *BRIP1*, *MSH1*, *MLH1*, *MSH2*, *MSH6*, *EXO1*, *ERCC6*, *POLQ*, *NEIL3*, and *ATR* (Leongamornlert et al. 2014). Aside from high-penetrance mutations, more common alleles (SNPs) associated with PCa can occur in up to 5% of the population (Eeles and Raghallaigh 2017; Wang et al. 2018).

Overall, PCa incidence and mortality rates vary markedly according to racial and ethnic groups (Siegel et al. 2014). European–American men carry a one in eight probability of being diagnosed with PCa during their lifetime, while African–American men present one in six chance (Sathianathen et al. 2018). Despite the high incidence of PCa, its etiology, the molecular mechanisms involved in carcinogenesis, and its progression are not well-known (Rawla 2019). However, sequenced primary PCa data from the "The Cancer Genome Atlas" (TCGA) showed great genomic heterogeneity in addition to molecularly distinct subtypes which can subclassify 74% of new cancer cases (Sanhueza and Kohli 2018), confirming that this is a heterogeneous disease, with different biological behaviors (Souza et al. 2017).

The process of malignant transformation of the prostate tissue is multistep (Wang et al. 2018) and the tumor stages can be identified by microscopic analyses based on the histological appearance of prostate cells, which is currently the most commonly used classification system to define the prognosis of PCa (Epstein et al. 2016). Metastatic disease is the leading cause of PCa associated deaths (Wang et al. 2018).

Screening for PCa is performed by determination of levels of prostate-specific antigen (PSA) in blood and/or physical examination (digital rectal examination) of the prostate gland. Suspicious results are submitted to a transrectal ultrasound-guided systemic biopsy (Epstein et al. 2016). Although tissue biopsies are considered the "gold standard" for stratification of PCa risk, diagnosis, and treatment management, the technique presents many limitations, including the fact that biopsies are invasive, uncomfortable, and can be a risk factor for hematuria, rectal bleeding, pain, and infection (Hoey and Liu 2019).

After the inclusion of the PSA test, early detection campaigns caused problems related to overdiagnosis and unnecessary treatment for many men with the indolent disease, in addition to adverse effects and significant costs (Berman and Epstein 2014).

There is considerable variation between studies regarding the treatment for PCa, which depends mainly on the stage of the disease (Mottet et al. 2017). The treatment options can include active surveillance (AS), surgery (radical prostatectomy), chemotherapy, radiation therapy, brachytherapy, antiandrogen therapy, androgen deprivation therapy by castration (ADT), immunotherapy, radionuclide therapy, and PARP inhibitor therapy (Mottet et al. 2017; Paschalis and Bono 2020). Although most patients respond well to ADT and tumor regression is observed after 1–3 years, many will cease to respond and PCa will recur as castration-resistant prostate cancer (CRPC) (Katsogiannou et al. 2015; Endzeliņš et al. 2016).

Methods to accurately predict the biological behavior of the tumor and accelerate the diagnosis, prognosis, and therapeutic response for stratifications within the same group of individuals have been investigated (Sathianathen et al. 2018). Among the new advances to elucidate the evolution of the disease and contribute to the development of new therapies, the validation and clinical qualification of non-invasive substitute biomarkers is a high priority in PCa research. In this sense, circulating biomarkers have been included and shown promise (Oliver and Bono 2018; Wang et al. 2018; Paschalis and Bono 2020).

Liquid biopsy is a technique in which it is possible to analyse, in body fluids, circulating DNA and RNA and exosomes derived from cancer cells. Therefore, analysis of cell-free miRNAs (cfmiRNAs) or those contained in exosomes circulating in the plasma provide genetic signatures for cancer (Rubis et al. 2019).

In this review, we will briefly introduce cell-free nucleic acids (cfNAs), exosomes, and their characteristics, focusing on circulating miRNAs: those that are associated with protein (Argonaute2—AGO2), (nucleophosmin—NPM1) or with high-density lipoproteins (HDL) and are defined as circulating cfmiRNAs; and those that are packed into extracellular vesicles (EVs), defined as exosomal miRNAs (exomiRNAs).

We retrieved the relevant literature from the PubMed database and we will show what has been published about the role of miRNAs in PCa in the period from 2012 to August 2021. The search language was limited to English and this review was carried out to identify studies that examined relevant biomarkers of PCa. A total of 105 articles were obtained and critically reviewed. References were considered eligible for evaluation if authors assessed any clinical relationship between PCa, and circulating cell-free and exomiRNAs obtained in serum or plasma. Among the literature searched, some studies were removed because they were duplicated; were reviews of in silico studies or meta-analysis; could not be accessed; the biological sample wasn't serum or plasma; were not PCa studies. A detailed flow diagram showing the selection process of our review is reported in Fig. 1.

Fig. 1 A detailed flow diagram showing the selection process of our review is reported in

Identification

Total records identified through PubMed (n=105) Mesh terms:

Circulating cell-free microRNA, prostate cancer patients, plasma (n = 32)
Circulating cell-free microRNA, prostate cancer patients, serum (n = 36)
Exosomal microRNA, prostate cancer patients, plasma (n = 15)
Exosomal microRNA, prostate cancer patients, serum (n = 22)

Screening

Total records removed (n = 52)

Duplicates (n = 20)
Review (n = 13)
Not available (n = 1)
Inappropriate methodology (n = 18)

Inclusion

Studies included for definitive analysis (n = 53)

Circulating cell-free microRNA, prostate cancer patients, plasma (n = 22)
Circulating cell-free microRNA, prostate cancer patients, serum (n = 18)
Exosomal microRNA, prostate cancer patients, plasma (n = 4)
Exosomal microRNA, prostate cancer patients, serum (n = 9)

Liquid biopsy

The measurement of tissue biomarkers for solid tumors is usually limited by difficult access and associated with significant clinical risk (Marrugo-Ramírez et al. 2018). Nowadays, efforts have been made to discover non-invasive methods for the comprehension of the genomic, transcriptomic, epigenomic, and other possible biomarkers that could lead to early detection and monitoring of tumor evolution and response in real time. In this field, liquid biopsy has received significant attention (Han et al. 2017).

Liquid biopsy is a non-invasive tool for biomarker discovery and involves the collection of body fluids, such as amniotic fluid, breast milk, bronchoalveolar lavage, cerebrospinal fluid, colostrum, tear fluid, saliva, pleural effusion, blood, ascites peritoneal lavage, urine, and seminal fluid (Weber et al. 2010; Ortiz-Quintero 2016; Liskova et al. 2020). This tool is associated with lower morbidity and can provide a genetic overview of cancerous lesions (primary and metastatic), as well as offering instant assessment of the disease at successive time points, in addition to systematically tracking its genomic evolution (Crowley et al. 2013; Di Meo et al. 2017).

The concept of liquid biopsy applied for "precision medicine" is considered a new era in cancer management (Di Meo et al. 2017). The goal of precision medicine is to eliminate the "one size fits all" model of patient management and this concept can benefit from the broad range of applications of liquid biopsy, including screening, early diagnosis, and classification of patients into precise groups by their probable disease risk, prognosis, and/or prediction of response and resistance to treatment. Therefore, understanding the molecular profiles of patients, together with traditional clinical information, enables the provision of individualized medical care with the greatest benefit, while minimizing risk (Diamandis et al. 2010; Vargas and Harris 2016).

Approaches involved in liquid biopsy include the analysis of circulating cfNAs such as circulating tumor DNA (ctDNA), circulating tumor RNA (ctRNA), circulating tumor cells (CTCs), protein and tumor-derived EVs that are released from tumors into body fluids (Siravegna et al. 2017) (Fig. 2). Several factors can be analyzed, including DNA integrity, DNA alterations such as amplification and deletion, DNA methylation, and RNA expression, among others, depending on the nature of the tumor and clinical application proposed (Swarup and Rajeswari 2007; Schwarzenbach et al. 2011).

cfNAs originate from apoptotic or necrotic cells (normal host and tumor cells) that release them into the bloodstream and other biofluids. The ctDNA and ctRNA fragments originated from tumor cells represent a small fraction of released nucleic acid and contain the alterations found in the tumor cells they originate from (Alix-panabières and Pantel 2016; Bardelli and Pantel 2017).

CTCs are a population of tumor cells that are mostly shed from primary lesions during their formation and early growth. They circulate through the bloodstream to potential metastatic sites, either as a single cell or in clusters, becoming the main mechanism for metastasis (Ferreira et al. 2016).

Fig. 2 Circulating biomarkers in prostate cancer. Liquid biopsy is a non-invasive, low risk procedure, easily and repeatedly obtained, and allows the evolving landscape of prostate cancer in real time detection during disease. In blood vessels, cell-free DNA, cell-free RNA, and circulating tumor cells are released through apoptosis and necrosis. The cell-free RNAs can be cell-free miRNAs associated proteins, packaged in high-density lipoprotein complexes or present inside EVs and can be obtained in blood analysis. *CTC* circulating tumor cell, *AGO2* Argonaute 2, *NPM1* nucleophosmin 1, *HDL* high-density lipoprotein, *miRNAs* microRNAs

The constituents of EVs provide a 'molecular fingerprint' of the tumor cells of origin, and their DNA, RNA, and protein (both surface and intraluminal) content provide a rich source of cancer biomarkers. ctRNA, including EV-associated circulating RNA, includes different RNA classes. Among these, long non-coding RNA (lncRNA) expression and microRNA (miRNAs) expression panels are good sources of quantitative biomarker information (Rubis et al. 2019).

Biogenesis and function of microRNA

miRNAs are small non-coding RNAs acting as guide molecules in the downregulation of target RNAs (Ha and Kim 2014). miRNAs are synthesized from miRNA-specific genes or non-coding regions by polymerase II, resulting in a long primary miRNA (pri-miRNA). The maturation of miRNA involves a complex metabolic pathway that begins in the nucleus, where it is processed into pre-miRNA by the microprocessor complex, consisting of a DGCR8 and the ribonuclease III enzyme, DROSHA (Ludwig and Giebel 2012; Yu et al. 2015; O'Brien et al. 2018). Through exportin 5 and the RAN-GTP complex, the pre-miRNA is translocated from the nucleus to the cytoplasm where it is further processed by another RNAse III, known as DICER, resulting in a mature miRNA duplex (Moustafa et al. 2018; O'Brien et al. 2018; Berti et al. 2019). The double miRNA duplex is composed of a functional strand (which is complementary to the target mRNA) and the passenger strand (which is subsequently degraded). The RISC complex and Argonaute 2 (RISC-AGO2 complex) guides the functional strand to target the

3'UTR of the target mRNA, causing inhibition or promoting degradation. The imperfect complementary (miRNA:RNA) leads to repression of translation of the target mRNA and is the main mechanism of miRNA regulation in animals, whereas perfect complementarity induces degradation of the target mRNA and is mainly detected in plants (Li et al. 2018).

A single miRNA can have hundreds of target mRNAs, highlighting the importance of this gene regulation system in cellular functions (Majid et al. 2013). MiRNA gene expression control is critical for the cellular response to environmental stresses, such as starvation, hypoxia, oxidative stress, and DNA damage, largely implicated in cancer (Otmani and Lewalle 2021).

miRNAs involved in cancer are divided in two groups: the oncomiRs that directly or indirectly can induce tumor initiation and progression; are upregulated in cancer cells. The second group is tumor suppressor miRNAs, which prevent cancer beginning and progression of cancer through suppressing the expression of various oncogenes; are downregulated in cancer cells (Aghdam et al. 2019; Otmani and Lewalle 2021). Therefore, the dysregulation of miRNA expression is closely associated with several stages of the tumorigenesis process, such as cancer initiation, progression, and metastasis (Otmani and Lewalle 2021).

Although miRNA biogenesis is a tightly regulated process, alterations in the biogenesis pathway proteins, including DROSHA, DICER, and AGO2 can lead to dysregulation of miRNAs (Rupaimoole et al. 2016). In addition, several miRNA genes are in fragile chromosomal loci and, therefore, subject to changes such as deletions, genomic amplifications, and chromosomal rearrangements and this makes miRNAs undergo dysregulation and, consequently, causes several diseases, including cancer (Khan et al. 2019).

Circulating miRNAs as biomarkers in prostate cancer

Circulating miRNAs participate in numerous regulations of biological processes and are expressed aberrantly under abnormal or pathological status. The changes in quality and quantity of circulating miRNAs are associated with playing a key role in the initiation, tumor progression, and metastasis of cancer (Armand-Labit and Pradines 2017; Cui et al. 2019).

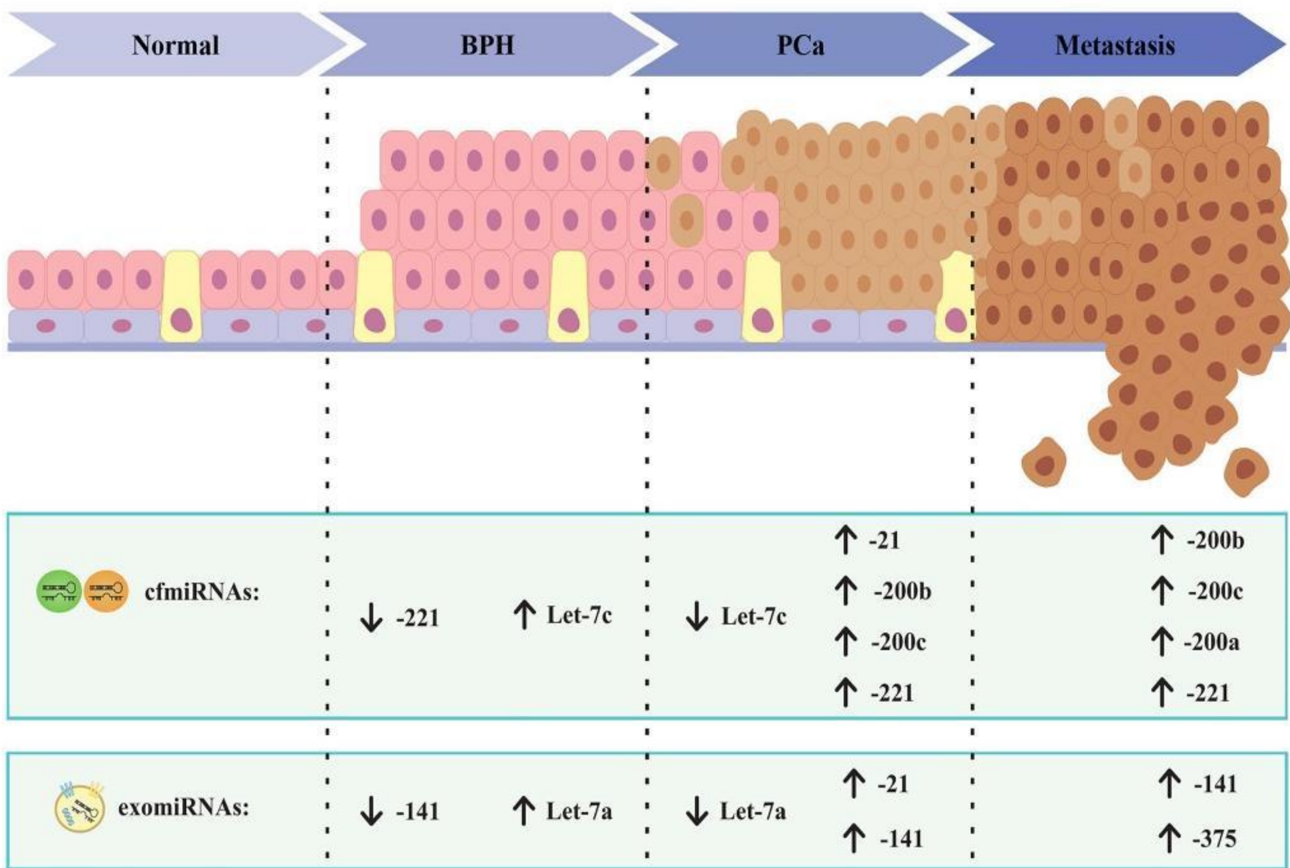


Fig. 3 miRNAs involved in prostate cancer clinical outcome. The most described cfmiRNAs and exomiRNAs associated with various stages of prostate cancer. The symbol ↑ indicates upregulated miRNAs and ↓ indicates downregulated miRNAs

The first study on the expression profile of miRNAs in PCa of which we are aware is that of Porkka et al. 2007, in which the authors suggested that miRNAs could be used as diagnostic and prognostic tools for the disease. Since then, numerous studies have been carried out aimed at elucidating the clinical value of cfmiRNAs and exomiRNAs in the serum and plasma of PCa patients (Table SI).

Nowadays more than 4800 human mature miRNAs are recorded in miRBase v22 (http://www.mirbase.org). In PCa, altered expression of miRNAs has been frequently reported in benign prostate hyperplasia (BPH), primary PCa, metastasis (Fig. 3) and in castrate-resistant prostate cancer (CRPC), showing their deregulation in different processes, such as cell proliferation, differentiation, progression, aggressiveness, metastasis, and recurrence (Spahn et al. 2010; Sekhon et al. 2016).

Different RNA molecules are known to be highly unstable; many studies have indicated that miRNAs are stable in biological samples, and remain stable under some extreme conditions, such as multiple freeze and thaw cycles, as well as extreme pH (Mitchell et al. 2008; Glinge et al. 2017), as they can be circulating molecules associated with proteins, lipids, or encapsulated in EVs and as a result, they are protected from endogenous RNase activity, making them strong candidates for biomarkers in several diseases (Mitchell et al. 2008).

The release of nucleic acids into the blood is thought to be related to the apoptosis and necrosis of cancer cells in the tumor microenvironment (Schwarzenbach et al. 2011) and in liquid biopsy the screening of miRNAs from blood samples is the most commonly used approach (Campos-Fernández et al. 2019).

There are three well-established and sensitive methods of analysis that provide good information on miRNAs detection: RT-qPCR, microarray, and NGS (Fernández-Lázaro et al. 2020).

Wang et al. (2012), when comparing the microRNA spectrum, observed higher miRNA concentrations in serum samples when compared to the corresponding plasma samples from the same individuals. The difference showed some associations with miRNA from platelets, which could indicate that the coagulation process may affect the spectrum of extracellular miRNA in blood.

Circulating cell-free miRNA in prostate cancer

cfmiRNAs are an important source of information for understanding PCa, as well as for diagnosis and prognosis purposes. Most studies have focused on the identification of cfmiRNAs to differentiate patients with PCa and control individuals or individuals with benign prostatic hyperplasia (BPH). Studies investigating the clinical value of cfmiRNAs in PCa are summarized in Table 1.

The first cfmiRNAs in patients were described by Mitchell et al. (2008) in serum and plasma of PCa patients. The authors suggested that cfmiRNAs could be used as blood-based biomarkers. Among six candidates (miR-100, miR-125b, miR-141, miR-143, miR-205, and miR-296), it was shown that five miRNAs presented increased expression in PCa serum when compared with healthy group serum. Only miR-205 did not show a conclusive result, whereas overexpression of miR-141 was observed in the serum of PCa patients, distinguishing them from healthy controls. Several subsequent studies corroborated the upregulation of miR-141 in serum and plasma of PCa patients (Cheng et al. 2013, 2018; Egidi et al. 2013; Nguyen et al. 2013; Selth et al. 2013; Westermann et al. 2014; Osipov et al. 2016; Porzycki et al. 2018; Ibrahim et al. 2019) showing the deregulation of this cfmiRNA in different populations around the world.

Other miRNAs have been highlighted across multiple studies in PCa in serum or plasma. There are 79 upregulated miRNAs (Table 1) described for different stages of PCa compared to individuals without cancer. Of the 79 cfmiRNAs upregulated in PCa, only 26 have been described by at least two authors, and of these 26, only 21 presented compatible results in all the available studies analyzed.

Regarding downregulated cfmiRNAs, only 3three were reported by at least two authors Let-7c (Kachakova et al. 2015; Shkurnikov et al. 2016), miR-34a (Porzycki et al. 2018; Gandellini et al. 2021), and miR-375 (Kachakova et al. 2015; Al-Qatati et al. 2017; Zedan et al. 2019).

miRNAs let-7c, miR-30c, miR-141, miR-221, and miR-375 presented controversial data and Andl et al. (2020) alert that current obstacles faced when using miRNA data, such as reproducibility and tissue-specific delivery, need to be resolved and that these nucleic acids may soon have significant impacts on the treatment of PCa.

Exosomal miRNA in prostate cancer

Cells release several types of EVs which differ in size, including apoptotic bodies (1000–5000 nm), intermediate-size microvesicles (MVs, 200–1000 nm), and the smallest, exosomes (30–150 nm) (Sokolova et al. 2011; Brinton et al. 2015; Rajagopal and Harikumar 2018).

Exosomes are small EVs produced by bacteria, plants, and animals, including humans (Berleman and Auer 2013; Raposo and Stoorvogel 2013). Human exosomes are produced by all cell types and are present in all body fluids (Keller et al. 2011; Willms et al. 2016). Initially they were considered "garbage bags" that exported residual molecules that were not useful to the cell (Harding et al. 1983; Théry

Table 1 Circulating whole blood cell-free miRNAs identified as biomarker useful in preclinical studies

First author, year	Country	Methodology	Sample size	Biologic sample	Applications	Significant miRNAs	
						Downregulated	Upregulated
Al-Qatati et al. (2017)	Canada	Microarray	79 PCa 33 HC	Plasma	Diagnostic/prognostic	miR-375; miR-183	Let-7c, let-7f, miR-15b, miR-16, miR-20a, miR-21, miR-25, miR-26a, miR-26b, miR-30c, miR-100, miR-106b, miR-130b, miR-148a, miR-181a, miR-194, miR-195, miR-200b, miR-200c, miR-210, miR-221, miR-224, miR-331, miR-361, miR-425, miR-494
Alhasan et al. (2016)	USA	Scano-miR; RT-qPCR	18 PCa 10 HC	Serum	Diagnostic	None	miR-106a, miR-135a, miR-200c, miR-433, miR-605
Benoist et al. (2020)	Netherlands	RT-qPCR	40 mCRPC 30 HC	Plasma	Prognostic/ Predictive	None	miR-375; miR-3687
Cheng et al. (2013)	Australia	Microarray	46 mCRPC 45 HC	Plasma	Prognostic	None	miR-141, miR-200a, miR-200c, miR-210 e miR-375
Cheng et al. (2018)	USA	RT-qPCR	25 mCRPC 25 HC	Serum	Prognostic	None	miR-141, miR-200a, miR-200c, miR-210, miR-375
Egidi et al. (2013)	Italy	RT-qPCR	38 PCa 40 HC	Serum	Predictive	None	miR-21 and miR-141
Endzelinš et al. (2017)	Latvia	RT-qPCR	50 PCa 22 BPH	Plasma	Diagnostic	None	miR-375
Farran et al. (2018)	USA	RT-qPCR	144 PCa	Plasma	Prognostic	miR-181a	miR-17, miR-22, miR-150a and miR-192
Fredsøe et al. (2020)	Denmark	miRNA RT-PCR	407 PCa 144 BPH 57 APC; 63 TRUSbx benigna; 82 TRUSbx maligna	Plasma	Diagnostic	miR-130a, miR-146a, miR-154, miR-376c and miR-410	miR-26a, miR-142, miR-215, miR-375 and miR-451
Gandellini et al. (2021)	Italy	PCR-based TaqMan OpenArray	386 PCa	Plasma	Diagnostic	miR-128a, miR-142, miR-181c, miR-199a, miR-204, miR-330, miR-337, miR-361, miR-422a, miR-502, miR-511, miR-572 miR-598 and miR-1255	miR-27b, miR-122, miR-324 and miR-361
Ge et al. (2020)	China	RT-qPCR	66 PCa 63 BPH	Plasma	Diagnostic	None	Let-7f and miR-103a
Ibrahim et al. (2019)	Egypt	RT-qPCR	30 MPCa 50 PCa 30 BPH 50 HC	Plasma	Diagnostic/Prognostic	None	miR-18a, miR-21, miR-141, miR-221

Table 1 (continued)

First author, year	Country	Methodology	Sample size	Biologic sample	Applications	Significant miRNAs	
						Downregulated	Upregulated
Jones et al. (2018)	USA	RT-qPCR	10 PCa 5 HC	Serum	Diagnostic	None	miR-186
Kachakova et al. (2015)	Bulgaria	RT-qPCR	59 PCa 16 BPH	Plasma	Diagnostic	let-7c; miR-30c; miR-141; miR-375	None
Shkurnikov et al. (2016)	Russian	Microarray	152 PCa 40 BPH	Plasma	Prognostic	Let-7c; let-7b	miR-619 and miR-1184
Kotb et al. (2014)	Egypt	RT-qPCR	10 PCa 10 HPB	Serum	Diagnostic	None	miR-21 and miR-221
Lin et al. (2014)	Australia	Microarray	97 CRPC	Serum/Plasma	Predictive	miR-200b and miR-200c	miR-20a, miR-20b, miR-146a, miR-200a, miR-222, miR-301b and miR-429
Lin et al. (2017)	Australia	RT-qPCR	89 CRPC	Plasma	Predictive	None	miR-132; miR-200a, miR-200b, miR-200c, miR-222, miR-301b, miR-375 and miR-429
Liu et al. (2018)	Canada	RT-qPCR	133 PCa	Serum	Predictive	None	miRNA-24, miRNA-223, and miRNA-375
McDonald et al. (2019)	USA	Microarray	66 PCa	Plasma	Prognostic	miR-28; miR-100; miR-942;	miR-128a, miR-185, miR-202, miR-374, miR-376c, miR-708, miR-886 and miR-1298
Mello-Grand et al. (2020)	Italy	RT-qPCR	60 PCa, 51 BPH 9 HC	Plasma	Diagnostic	None	let-7a and miR-103a
Mihelich et al. (2015)	USA	RT-qPCR	100 PCa 50 BHP	Serum	Diagnostic/Prognostic	None	Let-7a, miR-24, miR-26b, miR-30c, miR-45, miR-93, miR-100, miR-103, miR-106a, miR-107, miR-130b, miR-146a, miR-223 and miR-451
Torbati et al. (2019)	Iran	RT-qPCR	40 PCa 40 HC	Serum	Predictive	None	miR-20a
Nguyen et al. (2013)	USA	RT-qPCR	26 CRPC 58 PCa	Serum	Prognostic	miR-409	miR-141, miR-375 and miR-378
Osipov et al. (2016)	Russian	RT-qPCR	48 PCa 47 HC	Plasma	Prognostic	None	miR-141 and miR-205
Osip'yants et al. (2017)	Russian	Microarray	18 MPCa 18 PCa	Plasma	Prognostic	None	miR-19b, miR-297, miR-320c, miR-328, miR-5680 and miR-6805
Pastor-Navarro et al. (2020)	Spain	RT-qPCR	40 PCa 45 HC	Serum	Diagnostic	None	miR-21, miR-125b, miR-141, miR-182, miR-375

Table 1 (continued)

First author, year	Country	Methodology	Sample size	Biologic sample	Applications	Significant miRNAs	
						Downregulated	Upregulated
Porzycki et al. (2018)	Poland	RT-qPCR	20 PCa 8 HC	Serum	Diagnostic	miR-34a	miR-21, miR-106b, miR-141-3p and miR-375
Rajendiran et al. (2021)	USA	RT-qPCR	32 PCa 25 HC	Serum	Diagnostic	None	miR-940
Shkurnikov et al. (2017)	Russian	Microarray	65 PCa	Plasma	Prognostic	None	miR-619
Souza et al. (2017)	Brazil	RT-qPCR	102 PCa 50 HC	Plasma	Diagnostic/ Prognostic	None	miR-200b and miR-200c
Tang et al. (2019)	China	RT-qPCR	35 PCa 35 HC	Serum	Diagnostic	miR-212	
Tinay et al. (2018)	USA	RT-qPCR	25 CRPC 25 MPCa 25 PCa 20 HC	Serum	Diagnostic	None	miR-9, miR-330 and miR-345
Urabe et al. (2019)	Japan	Microarray	384 PCa 100 HC	Serum	Diagnostic	miR-1228 and miR-1343	miR-17, miR197, miR-320b, miR-422a, miR-1185–1, miR-1202, miR-3185, miR-4417, miR-4433a, miR-5698, miR-6076, miR-6741, miR-6819, miR-6877 and miR-8073
Wang et al. (2016)	China	RT-qPCR	149 PCa 121 BHP 57 HC	Serum	Diagnostic	None	miR-410
Westermann et al. (2014)	Germany	RT-qPCR	54 PCa 79 HC	Serum	Prognostic	None	miR-141
Zedan et al. (2019)	Denmark	RT-qPCR	149 PCa	Plasma	Predictive	miR-125b and miR-221	miR-93
Zedan et al. (2020)	Denmark	Microarray	84 CRPC	Plasma	Predictive	miR-141 and miR-375	None
Zidan et al. (2018)	Egypt	RT-qPCR	70 PCa 70 BPH 30 HC	Serum	Diagnostic	miR-15a and miR-16–1	None

CRPC castrate-resistant prostate cancer, *MPCa* metastatic prostate cancer, *PCa* prostate cancer, *BCR* biochemical recurrence, *BPH* benign prostatic hyperplasia, *HC* health control, *NGS* next-generation sequencing, *RT-qPCR* reverse transcription quantitative polymerase chain reaction, *miR* microRNA

Table 2 Circulating whole blood miRNAs derived of exosomes identified as biomarker useful in preclinical studies

First author, year	Country	Methodology	Sample size	Biologic sample	Type of biomarker	Significant miRNAs	
						Downregulated	Upregulated
Bhagirath et al. (2018)	USA	RT-qPCR	44 PCa 4 BPH 8 HC	Serum	Prognostic	None	exomiR-1246
Bryant et al. (2012)	USA	RT-qPCR	78 PCa 28 HC	Plasma/Serum	Prognostic	None	exomiR-141, exomiR-200b and exomiR-375
Endzelinš et al. (2017)	Latvia	RT-qPCR	50 PCa 22 BPH	Plasma	Diagnostic/Prognostic	Let-7a-5p	exomiR-21 and exomiR-200c
Huang et al. (2015)	USA	NGS and RT-qPCR	23 screening 100 follow-up	Plasma	Prognostic	None	exomiR-375 and exomiR-1290
Li et al. (2015a)	USA	RT-qPCR	8 MPCa 6 PCa 10 HC	Serum	Diagnostic	None	exomiR-21, exomiR-375 and exomiR-574
Li et al. (2016)	China	RT-qPCR	20 PCa 20 BPH 20 HC	Serum	Diagnostic	None	exomiR-141
Li et al. (2020)	China	RT-qPCR	31 PCa 19 HC	Plasma	Diagnostic	exomiR-125	exomiR-141
Matsuzaki et al. (2021)	Japan	RT-qPCR	28 PCa 25 HC	Serum	Diagnostic	None	exomiR-30b and exomiR-126
Malla et al. (2018)	Switzerland	RT-qPCR	11 PCa 3 HC	Serum	Predictive	None	Exo-let-7a and exomiR-21
Panigrahi et al. (2018)	USA	RT-qPCR	12 PCa 7 HC	Serum	Predictive	exomiR-521	exomiR-324 and exomiR-885
Yu et al. (2018)	China	NGS	8 PCa	Serum	Predictive	None	exomiR-200c, exomiR-323a, exomiR-379, exomiR-409, exomiR-411, exomiR-493, exomiR-494, exomiR-543 and exomiR-654
Zhang et al. (2021)	China	Exiqon and RT-qPCR	24 PCa 24 HC	Serum	Diagnostic	None	exomiR-24, exomiR-93 and exomiR-146a
Zhou et al. (2020)	China	Sequencing and RT-qPCR	10 PCa 10 HC	Plasma	Diagnostic	exomiR-519a, exomiR-606, exomiR-148a, exomiR-506 and exomiR-23b	exo-let-7d, exomiR-127, exomiR-147a, exomiR-217, exomiR-422a, exomiR-520a and exomiR-659

CRPC castrate-resistant prostate cancer, *PCa* prostate cancer, *BCR* biochemical recurrence, *BPH* benign prostatic hyperplasia, *NGS* next-generation sequencing, *RT-qPCR* reverse transcription quantitative polymerase chain reaction, *exomiR* exosomal microRNA

et al. 2018). Currently, these vesicles are known to play an important role in cell–cell communication, transferring bioactive molecules to neighboring or distant cells (Ludwig and Giebel 2012; Yu et al. 2015). But so far, the exosome's normal physiologic functions well established are normal synaptic physiology and modulation of immune response. So, much has still to be investigated about this (Ruivo et al. 2017).

The molecular content of exosomes can be altered significantly based on the physiological conditions and original cell type (Broe et al. 1977). It is important to highlight that the composition of an exosome is not a mere reflection of the donor cell, and it has been shown that the profile of exosomal cargo can be substantially different from the originating cell, which indicates the existence of a highly controlled sorting process (Villarroya-Beltri et al. 2014). In the blood, the circulating RNAs can be found packaged in cell-derived EVs, including exosomes. RNA sequencing analysis of plasma-derived exosomes revealed that miRNAs are the most abundant exosomal RNA species (Huang et al. 2013; Valentino et al. 2017; Elewaily and Elsergany 2021).

Higher levels of exosomes are found in the plasma of cancer patients compared to control individuals, suggesting that tumor cells secrete more exosomes than normal proliferating cells (Whiteside 2016; Sharma et al. 2020). Cancer cell exosomes carry malignant information and can reprogram recipient cells. In this way, exosomes contribute to major steps of disease progression, such as tumor progression, remodeling of the tumor microenvironment (epithelial-to-mesenchymal transition), modulation of immune response, angiogenesis, metastasis, and chemoresistance (Ruivo et al. 2017; Lorenc et al. 2020). Therefore, the associated possibilities of interacting with cells make exosomes multifaceted regulators of cancer development (Lorenc et al. 2020).

In the last 10 years Bryant et al. (2012), have begun to intensively investigate the clinical relevance of exomiRNAs as markers for PCa. Until now, there are 21 upregulated and seven downregulated exomiRNAs, but with different results obtained for the same miRNA (exo-let-7a) (Table 2).

Circulating cell-free and exosomal microRNA in diagnosis and prognosis

Among the miRNAs, the one that seems to be the most promising is miR-21, which appears repeatedly in several studies and is characterized as being highly overexpressed in PCa, playing an important role in growth processes of this tumor. Several authors studied circulating cell-free and exosomal miR-21 Al-Qatati et al. (2017), Ibrahim et al. (2019), Egidi et al. (2013), Kotb et al. (2014), Porzycki et al. (2018), Pastor-Navarro et al. (2020), Li et al. (2015a), Endzelinš et al. (2017) and Malla et al. (2018) confirmed its potential as a biomarker for PCa diagnostics.

miR-21 is located in a chromosomal region (17q23.2) normally amplified in PCa, mainly in CRPC (Kasahara et al. 2002). MiR-21 is an important element to understand the androgen-dependent and androgen-independent PCa growth, because it has been observed that the activated androgen receptor (AR) interacts directly with regulatory regions of miR-21, indicating direct transcriptional induction (Ribas and Lupold 2010). Because the action of androgens is manifested through activation of the *AR*, overexpression or mutations in this gene are two of the main events that have been suggested to lead to CRPC, making the *AR* gene an important target for metastatic PCa or CRPC therapy (Thieu et al. 2014).

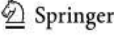
The let-7 family, highly conserved across multiple animal species, is one of the well-known miRNA clusters. Let-7 has emerged as a central regulator of systemic energy homeostasis and it displays remarkable plasticity in metabolic responses to nutrient availability and physiological activities (Jiang 2019). Some groups have demonstrated the diagnostic value of upregulated cell-free let-7f in PCa patients *versus* controls (Al-Qatati et al. 2017) or BPH (Ge et al. 2020). Besides, Ge et al. (2020) showed that the combination of let-7f-5p and PSA levels had a better discernibility of PCa and BPH patients.

The oncogenic miR-17 family consists of six members that share the same seed region AAAGUG (miR-17, -20a, -20b, -106a, -106b, and -93); among these, the upregulation of four members (miR-17, -20a, -106a, and 106b) was pointed out as a diagnostic by at least two authors (Table 1).

Members of the miR-130 family (miR-130a-3p, 130b-3p, -301a-3p, and -301b-3p) share common seed sequences and perform similar biological functions. They have been reported to promote cell proliferation and upregulation in several types of cancer. In this review, the expression of the -130a-3p, 130b-3p family members was evaluated in PCa patient blood and showed that it can effectively distinguish patients from controls, indicating it as a possible biomarker for diagnosis.

miR-103a was confirmed as an oncomiR by two authors (Ge et al. 2020; Mello-Grand et al. 2020). Mello-Grand and colleagues 2020 showed that miR-103a was upregulated in the comparison between PCa and BPH. This result was also confirmed by Ge et al. 2020 who analyzed the profile of expression of miR-103a in different ways, using the GEO database, TCGA RNAseq dataset, microarray, and RNAseq analysis, and the dysregulation of the expression level of miR-103a-3p was significantly higher in PCa patients than in cancer-free controls.

The miR-200 family consists of five members (miR-200a, -200b, -200c, -141, and -429), which are clustered and expressed as two separate polycistronic pri-miR transcripts (-141, miR-200b, -200c, -429, and miR-200a), located on human chromosomes 1 and 12, respectively. Due to the similarity in their seed sequences, they may have similar target genes, including the *Notch*, *Wnt*, and transforming growth factor β pathways; thus, inhibiting migration, tumor cell adhesion,



epithelial-to-mesenchymal transition, and angiogenesis (Chen and Zhang 2017). Among the miRNAs of this family, miR-200a, miR-200b, miR-200c, and miR-429 are present in several studies in this review (Alhasan et al. 2016), exhibiting differential expression levels in both cell-free and exosomal serum or plasma in PCa. Li et al. (2015b) showed that exomiR-141 was upregulated in patients with PCa when compared with patients with BPH and health controls, but in cell-free samples the results are contradictory. Bryant et al. (2012) showed altered concentrations of exomiR-200b in metastatic PCa patients compared with non-metastatic cases.

miR-210 is an important miRNA in PCa. In three studies, cell-free miR-210 levels in plasma (Cheng et al. 2013; Al-Qatati et al. 2017) or serum (Cheng et al. 2018) were found to be significantly higher in PCa patients with localized disease or metastatic and decreased health controls or BPH, indicating a diagnostic or prognostic role.

miR-221 and miR-222 are found on the X chromosome; are expressed from a single transcript; are overexpressed in blood of PCa patients; and are considered oncomiRs for many cancer types (Howe et al. 2012). The cell-free miR-221 upregulation has been suggested as diagnostic (Kotb et al. 2014; Al-Qatati et al. 2017) and as a prognostic (Ibrahim et al. 2019) biomarker in PCa.

Available data show that miR-375 defies simple classification as a tumor suppressor miRNA or oncomiR (Souza et al. 2017; Andl et al. 2020; Benoist et al. 2020). Souza et al. 2017 using TCGA, showed that miR-375 is highly expressed in tissues of PCa when compared to normal prostate; however, when validated in plasma, the authors did not find differences between the groups. Andl et al. (2020) conducted an extensive review and described contradictions in 24 publications about miR-375. However, the authors affirmed that this miRNA, when encapsulated in EVs, has the potential to be used as a prognostic biomarker, as it is upregulated in metastatic PCa patients compared with non-metastatic cases (Bryant et al. 2012).

The role of miR-619-5p was reported by Knyazev et al. (2016) and Shkurnikov et al. (2017). The miR-619-5p host gene is *SSH1*, which encodes a phosphatase dephosphorylating and activating cofilin protein, responsible, in turn, for disassembly of actin filaments, concentrated in lamellipodia of migrating cells (Takahashi et al. 2014). The first study about this miRNA was performed by Knyazev et al. (2016) who showed that the expression of cell-free miR-619 is fivefold higher in PCa vs. BPH. Subsequently, Shkurnikov et al. (2017) showed that plasma specimens from 65 patients with PC in stages pT2, pT3, and pT4, were associated with an increase in the concentrations of cell-free miR-619-5p from stage to stage and the authors suggested that this increase in the production of cell-free miR-619 is caused by the growth of prostatic malignant tumor.

Circulating cell-free and exosomal microRNA in therapy response

The treatment approach is based on risk stratification using traditional clinical-histopathological parameters and tumor stage, as well as considering patient overall health and personal preferences (Schaeffer et al. 2021) Importantly, therapy follow-up is essential to observe patient response and recurrence events. In this context, liquid biopsy is an interesting tool to be used in personalized medicine, allowing physicians to choose the best therapy approach based on each patient tumor (Ozawa et al. 2020). The field of miRNAs as therapeutics and biomarkers for PCa is still an attractive and growing area of research; this minimally invasive method can also be performed several times over the course of the treatment (Fig. 4).

Despite the numerous therapeutic options recently proposed, including inhibitors of androgen signaling (enzalutamide) or synthesis (abiraterone); taxane-based chemotherapy (docetaxel and cabazitaxel); bone-targeting radiotherapy (radium-223); and immunotherapy (sipuleucel-T), the medical management of CRPC still represents an important clinical issue (Arrighetti and Beretta 2021) and the blood levels of miRNAs have already been associated with therapy

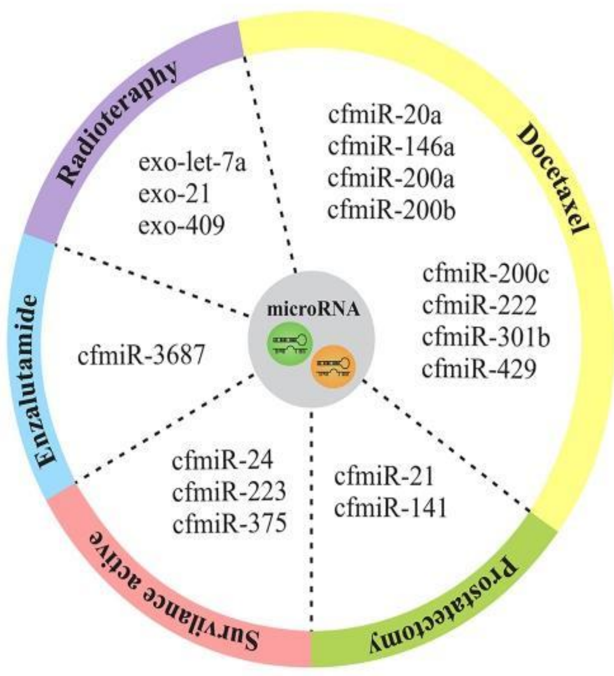


Fig. 4 miRNAs involved with therapeutic outcome in patients with prostate cancer. Illustrative overview of promising exosomal and cell-free microRNAs associated with therapeutic response

response, drug resistance, and survival, providing opportunities to adjust the therapeutic approach to increase patient survival. In this way, several studies have demonstrated the relationship between the levels of a diverse group of miRNAs with response and resistance to docetaxel, ADT (Zedan et al. 2019), radiotherapy (Zedan et al. 2019); abiraterone (Zedan et al. 2020); and cixutumumab (Cheng et al. 2018).

Malla et al. (2018) evaluated miR-21-5p derived from exosomes by RT-qPCR and showed that the radiation response was higher in the high-risk patients compared to the intermediate group. The overexpression of miR-21 enhanced tumor xenograft growth and was sufficient to support androgen-independent proliferation following surgical castration (Ribas and Lupold 2010). Guan et al. (2016) showed that the overexpression of miR-21 is associated with a poor response to ADT, which might be helpful in understanding the role of miR-21 as a predictive biomarker and in guiding therapeutic decisions.

Lin et al. (2014) reported that the baseline levels of cell-free miR-200b and miR-200c post-docetaxel were different between responders and non-responders. Patients with CRPC treated with docetaxel were associated with poorer survival outcome (Lin et al. 2017). In this context, higher levels of cell-free miR-200a, miR-200b, and miR-200c have the potential to be biomarkers of an early therapeutic response to docetaxel.

Bryant et al. (2012) compared exosomal plasma miRNAs of recurrent patients with PCa with non-recurring patients, indicating miR-141 as an oncomiR in patients with recurrent metastatic PCa.

However, miRNAs could have a predictive function in PCa therapy, whereby their dysregulation could be indicative of the patient's response to treatment manifested as either resistance or sensitivity to therapy. In addition, exosomes can be regarded as vehicles for loading miRNAs, targeting and combining fundamental genetic molecules in the pathways mediating chemotherapy, radiotherapy, and targeted therapies.

Challenges and perspectives of liquid biopsy

Liquid biopsy has emerged as a potential non-invasive approach for precision medicine. This review aimed to provide a head-to-head comparison of circulating miRNA, including cell-free and exomiRNAs in PCa. Based on the data presented, we demonstrated the importance and high potential of miRNAs as cancer biomarkers. The accumulation of studies in this area has shown that the transcriptional profiles of miRNAs are able to classify different types of tumors compared to the mRNA profiles, including more reliably identifying metastatic cancer of unknown primary origin.

This report should improve the understanding of PCa biology, focusing on the recent research in miRNA transcripts. These transcripts, integrated in the register of liquid biopsies, can provide rapid results with minimal invasiveness that can easily be integrated in clinical practice. Research data based on performing liquid assays demonstrate that this process is feasible and can be performed repeatedly at any moment of the disease surveillance. However, more studies are needed, because although many authors have identified the miRNAs in PCa, some miRNAs differ substantially from each other, probably due to the various types of samples and methodologies used, the cohort size, ethnic differences between the studies, and differences in the characterization between local and advanced PCa (Stuopelyte et al. 2016; Endzelinš et al. 2017; Hoey and Liu 2019).

In this review we showed that comprehensive studies are needed about miRNA expression in PCa. We suggest some insights to overcome the limitations of miRNA analysis approaches, including desirable features in preanalytical (sample collection) and analytical phases (miRNA analysis). In the preanalytical phase are relevant (i) the sample type: it is expected that different types of biofluids (serum or plasma) have different spectra of miRNA. Processing of plasma to remove subcellular/cellular components reduces miRNA concentrations in relation to those of serum (McDonald et al. 2011), but the cfmiRNA analysis in plasma sample could be better because in serum there is an increase of RNA released by blood cells and platelets during the blood coagulation process (Lee et al. 2017); (ii) the time interval between blood drawing and the sample process must be as quickly as possible (Mussbacher et al. 2020). Ideally, the sample process should occur within 2 h of collection to ensure peripheral blood mononuclear cells do not lyse and release either DNA or RNA, which could interfere with subsequent analyses (Page et al. 2013); (iii) the centrifugation speed: centrifugation force used in plasma processing can affect levels of cfmiRNAs due to the amount of platelets remaining in the sample (Salvianti et al. 2020) and last but not least important, (iv) the immediate storage at −80 °C after centrifugation is necessary to preserve the sample. In the analytical phase are relevant (i) choice of qPCR chemistry: it is important to use a probe and primer set that offer high specificity, reproducibility, and sensitivity; (ii) data normalization: there is lack of consensus about this theme that has resulted in the generation of various normalization strategies. Regarding the cohort size, we suggest that the research groups use ISUP grading from the International Society of Urological Pathology to standardize the PCa groups.

Therefore, standardized experimental procedures, and well-defined and clinically relevant cohort studies are urgently needed to investigate the biomarker potential of cfmiRNAs and exomiRNAs in serum or plasma (Egidi et al. 2013; Farina et al. 2014; Westermann et al. 2014; Wang et al. 2016; Al-Qatati et al. 2017; Farran et al. 2018; Urabe et al. 2019; Fredsøe et al. 2020).

Finally, over 2 decades of research on miRNAs as regulators of gene expression have shown that biological processes, including the occurrence of pathological conditions, involve many miRNA species at once in complex regulatory networks. Thus, the exploitation of these small RNAs as diagnostic and prognostic biomarkers generally requires quantification of the expression level of a suitable collection of miRNA sequences, which needs to be identified. In other words, miRNA quantification strategies should be amenable to parallel analysis of several miRNA species at once. This, however, does not mean that each technology should deliver high-throughput miRNA profiling.

We believe that microRNAs will soon be used in PCa for clinical outcome prediction and therapy improvement, facilitating disease classification, monitoring of its progression, and therapeutic use for patients with treatment-resistant disease.

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5. ARTIGOS EM PREPARAÇÃO

5.1 CAPÍTULO 2: Potential clinical application of cell-free miRNA-25-3p in prostate cancer and its functional roles in LNCaP cells.

Title: Potential clinical application and functional roles of cell-free miRNA-25-3p in prostate cancer.

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Abstract

MicroRNAs (miRNAs) in plasma are ideal biomarkers, as they are low-cost, stable, and their analysis are minimally invasive compared to tissue biopsy. Alteration in their expression levels have the potential to be used in the clinic as a diagnostic, prognostic or predictive biomarker. Four overexpressed miRNAs in prostate cancer were selected from The Cancer Genome Atlas database (TCGA): miR-25-3p, miR-92a-1-5p, miR-92a-2-5p, and miR-148a-3p to be investigated in this study. Plasma samples of 80 prostate cancer (PCa) patients and 40 controls were analyzed to validate candidate circulating cell-free miRNA as biomarkers for PCa and their expression were analyzed using RT-qPCR. The plasma miRNA expression was associated with high Prostatic Specific Antigen (PSA) levels and poor outcome clinicopathological parameters. The miR-25-3p was overexpressed in plasma of PCa patients when compared to controls ($p=0.013$). The patients with International Society of Urological Pathology (ISUP) $\geq$ 3 presented higher expression levels of miR-25-3p ($p<0.0001$) than patients with ISUP $\leq$ 2. Next, we determined the regulatory role of miR-25-3p performing transient transfection in the LNCaP cell line with miR-25-3p mimic and negative control systems. Overexpression of miR-25-3p significantly increased the cell proliferation, colony formation, migration and invasion capacity, and modulated the expression of *BCL2L11, CDH1, CDKN1C, EHZ2* and *TP53* genes, which are direct targets of miR-25-3p. We demonstrated that miR-25-3p differentiated patients with PCa from controls. This miRNA was also associated with tumor aggressiveness as ISUP-grading. We showed that the miR-25-3p has an oncogenic role in PCa, being a potential marker to assess prostate cancer development and progression using liquid biopsy, a minimally invasive method.

Keywords: prostate cancer, liquid biopsy, circulating microRNA, miR-25-3p, biomarker,

Abbreviations:

- *BCL2L11:* B-cell lymphoma 2 Like 11;
- *CDH1:* Cadherin 1;
- *CDKN1C:* Cyclin Dependent Kinase Inhibitor 1C;
- DRE: Digital Rectal Examination;
- EDTA: Ethylenediaminetetraacetic Acid;
- EMT: Epithelial to Mesenchymal Transition;
- *EZH2:* Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit;
- FC: Fold-Change;
- FDR: False Discovery Rate;
- GS: Gleason Score;
- ISUP: International Society of Urological Pathology;
- LB: Liquid Biopsy;
- LNCaP: Lymph Node Carcinoma of the Prostate;
- *MCM7:* Minichromosome Maintenance Complex Component 7;
- *MDM2:* MDM2 Proto-Oncogene;
- miRNAs: microRNAs;
- mRNA: Messenger RNA;
- *MYC:* Avian myelocytomatosis viral oncogene homolog;
- ncRNAs: Non-coding RNAs;
- oncomiR: Oncogenic microRNA;
- PCa: Prostate cancer;
- PSA: Prostate-Specific Antigen
- RNA: Ribonucleic Acid;
- ROC: Receiver Operating Characteristic;
- RT-qPCR: Quantitative reverse transcription PCR;
- TCGA: The Cancer Genome Atlas;
- *TP53:* Tumor Protein P53;
- UTR: Untranslated Regions;
- *WDR4:* WD repeat domain 4.O Piva está alugando o delel

INTRODUCTION

Prostate cancer (PCa) represents the fourth most diagnosed cancer worldwide and the eighth cancer related to death [1]. Besides the high incidence, the pathogenesis of PCa has not yet been clearly defined [2, 3]. PCa includes several phenotypes, from indolent to highly aggressive cancer. Currently, diagnostic and prognostic tools have several limitations such as false-negative or positive results, inaccuracy of biopsy, overdiagnosis, cost and invasiveness and there is a need for new biomarkers to stratify patients and assign them optimal therapies by taking into account genetic and epigenetic alterations [4].

Minimally invasive methods, such as liquid biopsy (LB), which involve the analysis of biofluids, have the potential to improve early diagnosis and reduce treatment costs. LB also allows for continuous monitoring through repeated sampling, personalized therapeutic regimens, and screening for therapeutic resistance [5]. MiRNAs are one of molecules that can be released into the bloodstream by cancer cells, and their detection in the fluids of cancer patients can provide valuable information about the cancer's location, stage, and grade. Additionally, miRNAs are highly stable under varying conditions and resistant to endogenous RNases, making them ideal markers for liquid biopsies. Each type of cancer has a specific and altered miRNA expression pattern [9], and for prostate cancer, various cohorts [2] and public datasets have identified miRNAs expression signature [10–12].

MiRNAs are single-stranded non-coding RNAs (ncRNAs) fragments of 19–25 nucleotides [6]. The miRNAs have a role in regulating protein-coding genes, which has been estimated to control the activity of more than 50% of genes in mammals. Most of them act as translational repressors by binding to complementary sequences in the 3′ untranslated regions (3’ UTR) of their target mRNAs. By inhibiting translation or degrading target mRNA, miRNAs are repressing protein expression [7]. Additionally, as evidence continues to grow, miRNAs may activate gene expression by targeting gene regulatory sequences [8].

The present study aimed to investigate the expression of four overexpressed miRNAs selected from The Cancer Genome Atlas database (TCGA): miR-25-3p, miR-92a-1-5p, miR-92a-2-5p, and miR-148a-3p in plasma samples of 80 prostate cancer (PCa) patients and 40 controls to validate candidate circulating cell-free miRNA as diagnostic biomarkers in PCa. In addtion, the effect of the upregulated hsa-miR-25-3p in PCa patients was functionally investigated in LNCaP cell line, confirming the potential of this miRNA to be used as a biomarker in liquid biopsy, as well as an oncogenic capacity in prostate cancer cell line.

MATERIALS AND METHODS

Ethics statement

Study approval was granted by the Research Ethics Committee of the State University of Londrina (CAAE19769913.0.0000.5231). All participants provided written informed consent and answered a modified questionnaire based on Carrano and Natarajan [6].

Clinical Samples

In the period 2014 to 2016, a total of 80 patients with histopathological confirmation of PCa were enrolled at the Londrina Cancer Hospital (Londrina/PR, Brazil). Clinicopathological data were obtaneid from medical and pathological reports. 40 cancer-free individuals enrolled at the CISMEPAR (Londrina/PR, Brazil), without urinary disease symptoms and Prostate Specific Antigen (PSA) levels ≤4.0 ng/mL were included as control group. Demographic, clinical, and histopathological characteristics of PCa patients and cancer-free controls are shown in Table 1.

Table 1 - Demographic and clinical characteristics of prostate cancer patients and cancer-free controls

Characteristics		PCa	Controls	*p*
		N (%)	N (%)	
Age	< 64	31 (38.8)	21 (52.5)	
	> 65	49 (61.3)	19 (47.5)	0.154
Ancestry	Afro-descendant	17 (21.3)	7 (17.5)	
	Euro-descendant	63 (78.8)	33 (82.5)	0.631
Family history of cancer	No	32 (40.0)	23 (57.5)	
	Yes	34 (42.5)	15 (37.5)	0.168
	Yes, prostate	14 (17.5)	2 (5.0)	**0.044***
Vasectomy	No	78 (97.5)	40 (100.0)	
	Yes	2 (2.5)	0 (0.0)	0.999
Occupational exposure	No	20 (25.0)	11 (27.5)	
	Yes, agrochemicals	42 (52.5)	19 (47.5)	0.670
	Yes, others	18 (22.5)	10 (25.0)	0.985
PSA (ng/mL)	< 4.0	8 (10.0)	40 (100.0)	
	4.1 to 10.0	32 (40.0)	0 (0.0)	
	> 10.0	40 (50.0)	0 (0.0)	
Treatment	Active surveillance	33 (41.3)	n.a.	
	Prostatectomy	46 (57.5)	n.a.	
	TURP	1 (1.3)	n.a.	
ISUP-grading (Radical prostatectomy)	Grade 1 (GS=6)	14 (17.5)	n.a.	
	Grade 2 (GS=3+4)	25 (31.3)	n.a.	
	Grade 3 (GS=4+3)	6 (7.5)	n.a.	
	Grade 4 (GS=4+4)	2 (2.5)	n.a.	
	Grade 5 (GS=9,10)	0 (0.0)	n.a.	
	NS	33 (41.2)	n.a.	
Tumor laterality	No	29 (36.3)	n.a.	
	Yes	17 (21.3)	n.a.	
	NS	34 (42.4)	n.a.	
Seminal vesicle	No	44 (55.0)	n.a.	
	Yes	3 (3.8)	n.a.	
	NS	33 (41.2)	n.a.	
Perineural invasion	No	43 (53.8)	n.a.	
	Yes	3 (3.8)	n.a.	
	NS	34 (42.4)	n.a.	
Lymph-node metastasis	No	45 (56.3)	n.a.	
	Yes	1 (1.3)	n.a.	
	NS	34 (42.5)	n.a.	
Bone metastasis	No	65 (81.3)	n.a.	
	Yes	11 (13.7)	n.a.	
	Not evaluated	4 (5.0)	n.a.	

PCa, Prostate Cancer; Yes: Presence; No: Absence; TURP: transurethral resection of the prostate; NS: Not surgery;PSA: ; ISUP: n.a.: not applicable.

miRNAs selection and target prediction

Four miRNAs found to be overexpressed in PCa tissue when compared to normal prostate tissue were selected, to have their potential exploited by LB, using the PCa data of the public dataset The Cancer Genone Atlas (TGCA) obtained from our previous study [11]. The criteria selection was based on the following parameters: fold change (FC) > 2, adjusted P < 0.001, and false discovery rate (FDR) < 0.001 (Supplementary Table 1). Of note, although those miRNAs have been already identified in PCa tissue, their role in LB has not been explored yet.

We then evaluated predicted miRNAs targeted genes using MIENTURNET (MicroRNA ENrichment TURned NETwork) that identify miRNA-target interactions experimentally validated using miRTarBase [14]. Then it filters based on the statistical significance resulting from a miRNA-target enrichment analysis [15].

Sample collection and cell-free RNA isolation

Peripheral blood samples were collected through BD Vacutainer® tubes containing 6% ethylenediaminetetraacetic acid (EDTA). Whole blood samples were centrifuged at 700 x g for 10 minutes for plasma separation within 2 hours after collection. Next, the cell-free plasma was centrifugated at 2,000 x g for 10 minutes at 4ºC and stored at -80ºC until use [11, 16]. Isolation of total RNA, including small RNAs, was performed using the miRNeasy Mini kit (217004, Qiagen, Hilden), according to Souza et al. [11]. Samples were quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Cell culture conditions

The prostate cancer cell line androgen-dependent (Lymph Node Carcinoma of the Prostate - LNCaP) was cultured in RPMI 1640 Medium (11875093, Gibco, USA) containing 15 % fetal bovine serum (12483-020, Gibco, Canada) and 1% gentamicin reagent solution (15710-064, Gibco, China). The cells were cultured in a humidified incubator at 37°C and 5% CO2.

Analysis of miRNA expression with RT-qPCR

For PCa and controls, the expression patterns of miRNAs (hsa-miR-25-3p [000403], hsa-miR-92a-1-5p [002137], hsa-miR-92a-2-5p [002138] and hsa-miR-148a-3p [000470]) were performed using 5 ng of total RNA and TaqMan miRNA Reverse Transcription kit (PN4427975, Applied Biosystems, USA), following the manufacturer's instruction. A 1:4-fold dilution of miRNA cDNA was used as input for the qPCR reaction. The qPCR reaction was performed using 5.5 µL of TaqMan 2X Universal PCR Master Mix (4440038, Applied

Biosystems, USA), 0.45 µL of miRNA-specific TaqMan Probe (PN4427975, Applied Biosystems, USA), and 7 µL of diluted miRNA cDNA on the Techne Quantum™ Real-Time PCR Cycler System (UK) at 50ºC for 2 min, 95ºC for 10 min and 50 cycles at 95ºC for 15 seconds and 60ºC for 1 minute. Two miRNAs (U6 snRNA [001973] and RNU48 [001006]) were used as endogenous controls for cell-free RNA, and a pool of samples was used as calibrator in every run.

miR-25-3p *Cell transfection*

The oligonucleotides miR-25-3p mimic (4464066, #MC10584, Ambion, USA) and mirVana™ miRNA Mimic, Negative Control #1 (4464058, Ambion, USA) were transfected to each assay at a final concentration of 10 µM using Lipofectamine 2000 (11668-027, Invitrogen, USA) according to the manufacturer's protocol. LNCaP cells were grown in a transfection medium for 24, 48 and 72 hours in a CO2 incubator at 37°C and 5 % CO2 atmosphere and then submitted to gene expression and functional assays. For cell line, the miR-25-3p relative expression was performed according to the above mentioned protocol.

Gene expression analysis (RT-qPCR)

For analysis of gene expression of target genes of hsa-miR-25-3p (*BCL2l1, CDH1, CDKN1C, EZH2, MDM2,* and *TP53)*, LNCaP cells ($5x10^3$) were seeded in triplicate at 96-well plates, transfected and maintained with the mimic and controls for a period of incubation of 48 hours. The total RNA was isolated from cells using Qiazol® Lysis Reagent according to the manufacturer's protocol (79306, Invitrogen, USA) and quantified by spectrophotometry using NanoDrop 2000C (Thermo Fisher Scientific, USA).

Briefly, first-strand cDNA synthesis was performed with the Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche Applied Science, Germany) using 1µg of total RNA according to manufacturer's instructions on a Mastercycler Nexus Gradient PCR machine (Eppendorf, Germany), under the following conditions: 25°C for 10 min, 30°C for 30 min, and the reaction terminated at 85°C for 5 min. The resulting cDNA was diluted in 120 µL nuclease-free water. The specific primer sets used in RT-qPCR are listed in Supplementary Table 2. Reactions were performed using SensiFAST™ SYBR® No-ROX Kit (BIO-98005, Bioline, UK) according the protocol in a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The housekeeping β-Actin gene was used as an endogenous control.

Cell Proliferation assay

Proliferation assays to compare transfected cells with NC and miR-25-3p were performed in 96-well dishes with 2×10^3 LNCaP cells seeded per well with 10 replicates per condition with one field imaged per well under 4× magnification every 24 h for a total of 96 h. Proliferation was measured as a function of cell confluence by live-cell microscopy. Data were analyzed using the IncuCyte® version S3 software (Essen Biosciences, USA), which quantified cell surface area coverage as confluence values.

Colony formation assay

The clonogenicity of cells was measured by the colony forming assay. Transfected cells were detached and 2500 cells per well were plated in a 6-well plate with 10% FBS for 14 days to form colonies. Then, cells were washed twice with PBS, immediately fixed with 1 mL methanol at room temperature for 15 min, and stained with 5% Giemsa solution (Solarbio, China) for 30 min at room temperature.

We dissolved the 5% Giemsa stain for 20 minutes of incubation in acetic acid 10% and we measured the absorbance of the clonogenic assay at 590 nm using a microplate reader (FLUOstar Omega; BMG Labtech, Germany).

Cell Migration and Invasion Assay

The migration and invasion of PCa cells were analyzed in 24-well transwell devices with 8 µm pore size polycarbonate membranes (662638, Greiner Bio-One, Germany). A total of $5\text{x}10^4$ PCa cells suspended in 200 µL serum-free medium were added into the upper chamber while in the lower chamber, medium containing 10% FBS as cell chemo-attractant was added. Matrigel-coated membrane was used for invasion assays (354480, BD BioCoat™, USA) under the same conditions as described. Following 48 hours of cell seeding, the upper surface of membranes was wiped clean with cotton swabs, while the lower surface of the membranes was fixed in methanol and stained with crystal violet. Using a light microscope with a magnification of 10×, the number of cells was counted in four random fields.

Statistical analysis

A univariate regression logistic analysis was carried out using IBM SPSS Statistics 22.0 (IBM Corp., USA) to analyze the demographic data. The miRNA and mRNA expression data was performed by delta–delta cycle threshold to obtain the relative expression data [17]. Data were reported as mean values ± SD of at least technical three replicates. The data were analyzed by Student’s t-test or two-way ANOVA using GraphPad Prism software, version 8.0.2 (GraphPad, USA). A value of $p< 0.05$ was considered statistically significant. The diagnostic

values were evaluated by receiver operating characteristic (ROC) curves analysis (GraphPad, USA).

RESULTS

Clinical information

The total sample was composed of 120 men (80 PCa patients and 40 controls), of which 80.6 % were European descendants and 19.4 % Afro-descendants. The average age of patient was 70 (range 48−87 years) and controls 64 (range 51-75 years). There was no difference between PCa patients and controls for most of the studied characteristics, except for family history of prostate cancer (p= 0.044). The PSA average in PCa was 38 ng/mL (range 1.51−933.9). Additionally, a detailed description of treatment, ISUP-grading (International Society of Urological Pathology), tumor laterality, seminal vesicle invasion, perineural invasion, lymph node invasion, and bone metastases are described in Table 1.

Plasma of PCa patients presented upregulation of miR-25-3p when compared to controls.

In the validation phase using RT-qPCR, we evaluated the expression of miR-25-3p, miR-92a-1-5p, miR-92a-2-5p and miR148a-3p from plasma samples of patients with PCa and control subjects (Fig. 1). We found that the miR-25-3p was significantly overexpressed in plasma of patients with PCa (p= 0.013; FC= 2.15) (Fig. 1A) when compared with control subjects. Since this miRNA was significantly increased, a receiver-operating characteristic (ROC) curve analysis was performed to determine its usefulness to separate the two different groups of blood donors, showing an area under curve (AUC) of 0.629 (95% CI, 0.520-0.737; p< 0.023) (Fig. 1F). Neither the miR-92a-1-5p (Fig. 1B), miR-92a-2-5p (Fig. 1C) nor miR148a-3p (Fig. 1D) showed statistically significance in relation to controls.

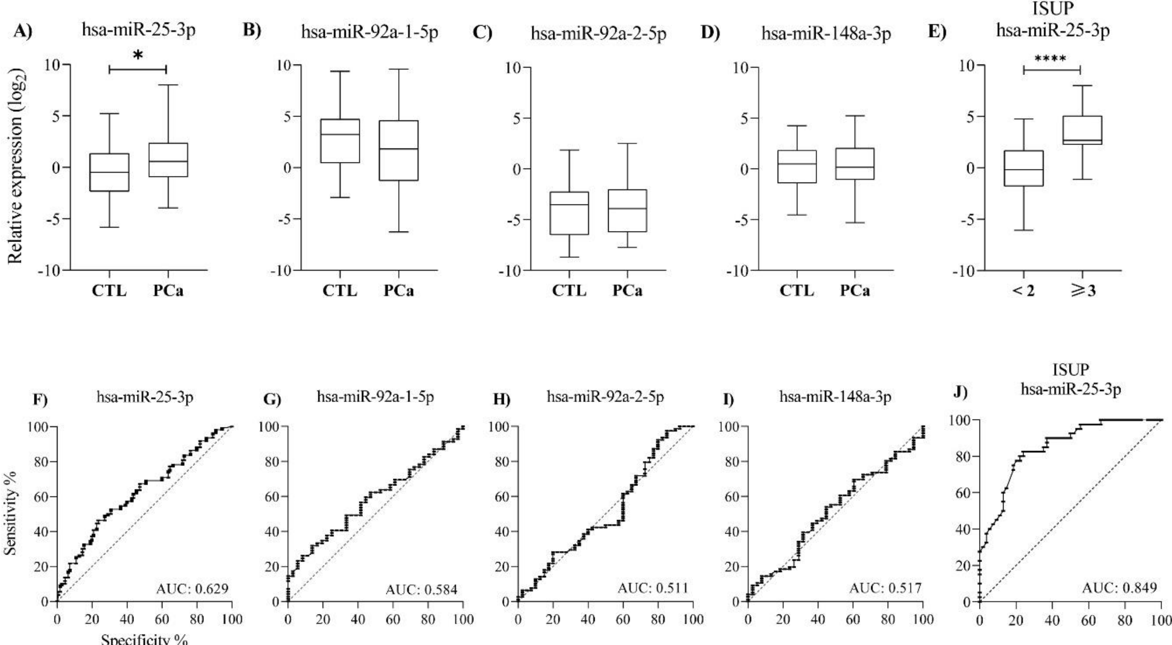

Figure 1 - Circulating cell-free miRNAs levels in patients with PCa and controls.

Differential expression of cell-free miRNAs in plasma samples of controls (CTL) and patients with prostate cancer (PCa) (A-D), and patients with prostate cancer according to the ISUP-grading (E) and their respective ROC curves (F-J). ISUP-grading= International Society of Urological Pathology; ROC = receiver operating characteristic, AUC = area under the curve; *p < 0.05, ****p < 0.00001.

The upregulation of miR-25-3p was also related to poor outcomes in prostate cancer. Patients with ISUP ≥ 3 exhibited miR-25-3p level twice higher than those detected in patients with ISUP ≤ 2 (p< 0.001; FC= 3.28) with AUC 0.849 (95% CI, 0.574-0.913; p< 0.003) (Fig. 1E, 1J). The miRNA expression profiles were combined to several other characteristics of worse tumor prognosis, but the results were not statistically significant (Supplementary Table 3).

Cell proliferation, colony formation, migration, and invasion are enhanced by miR-25-3p overexpression.

To test the functional role of miR-25-3p, we selected the prostate cancer cell line LNCaP. This cell line does not express the miR-25-3p, being then a suitable model to increase the expression of this miRNA. The transfection process with the mimic (miR-25-3p) compared to NC was efficient in all three times assessed (24, 48, and 72 hours), however, 48 hours was chosen because it showed a slight overexpression compared to the others (Fig. 2A).

We evaluated the proliferation rate of LNCaP cells after 24 hours of transfection with NC and miR-25-3p. Cell proliferation images were capture every 8 hours for 72 hours after transfection using a Incucyte system. We then applied a confluence mask in all images using

the Incucyte software S3 (version 2019) and calculated the cell surface area coverage. The results presented in Fig. 2B demonstrated that overexpression of miR-25-3p significantly increased cell proliferation after 48h and 72h of transfection (p=0.007 and p=0.0002, respectively).

As shown in Fig. 2C, miR-25-3p overexpression in LNCaP cells increased colony formation relative to NC cells. The relative colony-formation efficiency significantly increased the absorbance rate from 0.09 to 0.19, when the miR-25-3p was overexpressed. In addition, Boyden-chamber transwell migration and invasion assay, revealed that migration (average NC: 22.30 vs miR-25-3p: 54.17; p< 0.0001) (Fig. 2D) and invasion (average NC: 16.83 vs miR-25-3p: 31.89; p= 0.029). (Fig. 2E) were enhanced in LNCaP cells after 48h of transfection.

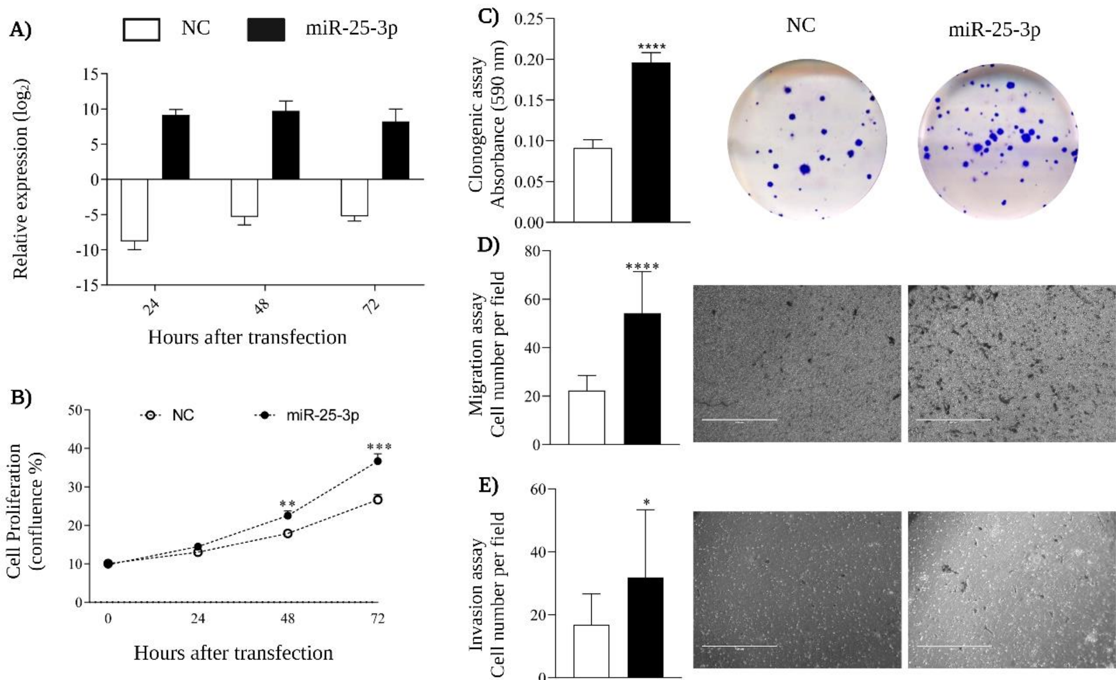

Figure 2 - Functional validation of the miR-25-3p.

A) LNCaP cell line transfected with mimic miR-25-3p in relation to the NC to assess the transfection efficiency by RT-qPCR in different times points. **B)** The proliferation was determined by IncuCyte® live-cell analysis; **C)** The clonogenic assay was used to assess the ability of cells to form colonies. Magnification: 1×; **D)** The migratory capacity was checked using Boyden chamber assay. Magnification: 10×; **E)** Cellular invasion, was measured using transwell assay coated with Matrigel. Magnification: 10×. NC: Mimic negative control.

High expression of miR-25-3p correlates with downregulation of BCL2L11, CDH1, CDKN1C, EHZ2, and TP53 in LNCaP cells.

To investigate how miR-25-3p contributes to PCa development and progression, the *BCL2L11*, *CDH1*, *CDKN1C*, *EHZ2*, *MDM2*, and *TP53* genes were selected based on their strong evidence to be target of miR-25-3p (Fig. 3A). The expression levels of *BCL2L11, CDH1, CDKN1C, EZH2* and *TP53* were significantly reduced in LNCaP cells after increasing the expression of miR-25-3p. However, *MDM2* was not affected by miR-25-3p overexpression in LNCaP cells (Fig. 3B).

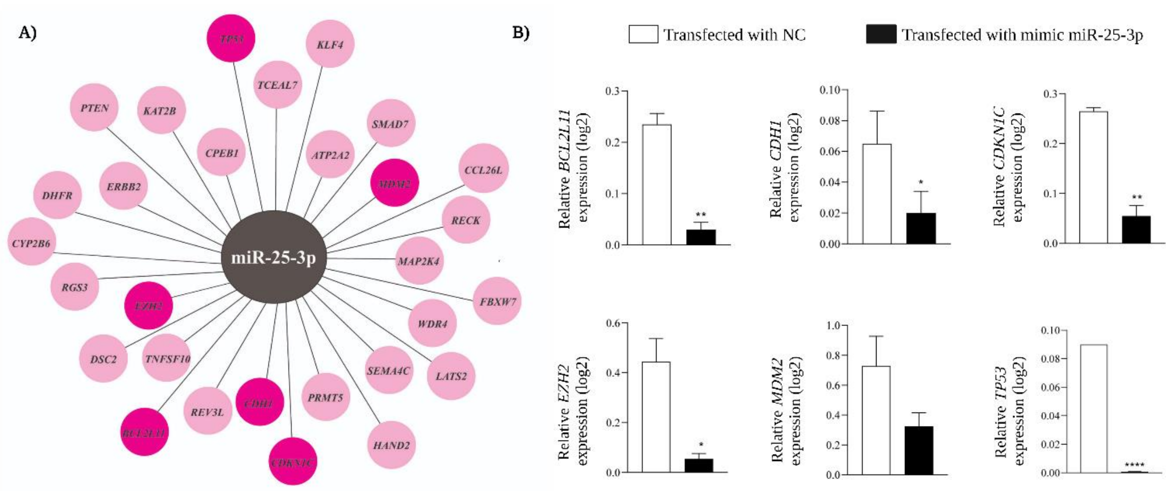

Figure 3 - MiR-25-3p/mRNA target interaction.

A) MiR-25-3p (grey color) interaction with mRNA experimentally validated targets (light pink) and interaction with mRNA validated by RT-qPCR in this study (fuchsia). Figure generated in MIENTURNET and modified using **BioRender.com**. **B)** Gene expression of miRNA-25 targets using RT-qPCR after transfection of LNCaP cells with NC and miR-25-3p. Data are presented as mean ± SD. NC: Negative Control; **$p < 0.01$; ***$p < 0.001$.

DISCUSSION

Current screening methods for PCa include measuring PSA levels in the blood and digital rectal examination (DRE). In case of any changes in the result of these exams, will be requested a prostate biopsy to confirm the clinical suspicion [18]. Although tissue biopsies are considered the "gold standard" for stratification of PCa risk, diagnosis, and treatment management, this technique presents many limitations. The procedure is invasive, uncomfortable, and can lead to hematuria, rectal bleeding, pain, and secondary infections associated to the procedure [19]. To overcome these limitations, extensive research is being conducted to find reliable non-invasive markers that will not only increase the precision of

diagnosis, prognosis and therapeutic methods but also reduce patient discomfort.

In addition to the benefits of liquid biopsy, studies show that miRNAs are stable in biological fluids and therefore have been investigated as potential circulating cell-free biomarkers [20].

These miRNAs regulate the expression of their target genes by degrading mRNA transcripts or by inhibiting mRNA translation, and they are categorized according to their expression and specificity as oncogenic or tumor suppressor miRNA. Oncogenic miRNAs are often overexpressed by inhibiting tumor suppressor genes, increasing cell proliferation and cell survival that promote cancer progression. Tumor suppressor miRNAs are often downregulated, and their loss of function can result in induction of apoptosis, endothelial to mesenchymal transition, and the cell proliferation [21, 22].

miR-25-3p belongs to an intragenic cluster (named microRNA-106b-25 cluster) located in intron 13 of the *MCM7* gene on chromosome 7 (7q22.1) [23]. This locus is frequently amplificated in PCa patients and this amplification leads to an upregulation of the miR-25-3p [24]. The oncogenic properties of miR-25 and its upregulation has been recently identified on multiple human cancers [25], such as prostate [26], breast [27], gastric [28, 29], non-small cell lung [30, 31], squamous cell carcinoma [32], endometrial [33], esophageal [34], glioma [35], hepatocellular [36, 37], and ovarian [38]. Therefore, miR-25-3p is an important oncogenic miRNA in carcinogenesis.

Based on TCGA data, in our previous study [11], we showed that the miR-25-3p is highly expressed in PCa. The high expression of miR-25-3p in PCa was also reported by Walter et al. [39] and Poliseno et al. [40], both studies using PCa tissue. The present study shows the first finding of cell-free miR-25-3p in the plasma of PCa patients, providing new information that could help guide PCa care in the future, suggesting the potential of miRNA to reflect tumor dynamics and be used as a non-invasive biomarker.

PCa risk stratification is crucial to making clinical decisions [41]. The present study revealed higher abundance of plasmatic cell-free miR-25-3p in patients with ISUP ≥3, highlighting its potential role as prognostic biomarker. While the miRNA expression can reflect differences in the underlying and unique molecular changes for tumors risk according with ISUP-grading, it can be used to support both the diagnosis, and the detection of aggressive cases.

Complex regulatory networks are orchestrated by miRNAs across a wide range of biological pathways [42]. Each miRNA can regulate dozens of mRNA transcripts; and conversely, a single mRNA transcript can be regulated by several miRNAs. Interestingly, a

single miRNA can exert multiple effects in various biological systems [42–44]. A functional pattern of miRNA-mRNA regulatory network has been designed to bring insights about the increased proliferation, colony formation, migration and invasion seen in prostate cell line in experimental validation. In our analysis, *in vitro* transfections followed by RT-qPCR, showed that the *BCL2L11*, *CDH1, CDKN1C*, *EZH2*, *and TP53* were downregulated when miR-25-3p was upregulated.

Therefore, miR-25-3p showed an ability to modulate important genes of belonging to different pathways, including epigenetic modifiers (*EZH2*); inhibitors of metastasis, cellular migration and invasion (*CDH1*); pro-apoptotic gene (*BCL2L11*) and genes related to cell cycle arrest (*TP53* and *CDKN1C*). It is interesting to note that miR-25-3p has common target mRNAs in different tissue and cell types that regulate the same biological process [45]. The expressional changes of these common target mRNAs could result in different diseases, including various types of cancer. These diseases share common general mechanisms, through related genes, e.g., *CDH1*, *CDKN1C*, *PTEN and TP53*, and which can be explored in relation to potential therapeutic targets for other cancers [25, 45] beyond prostate.

In summary, we demonstrated that cell-free miR-25-3p is increased in the circulation of PCa patients compared with cancer-free controls. In particular, this miRNA is also significantly associated with more aggressive PCa (Fig. 4A). Functional assays showed that the miR-25-3p can act in different pathways helping the tumor progression (Fig. 4B) and is an oncogenic miRNA in PCa, inducing a cell proliferation, migration, and invasion in LNCaP cells (Fig. 4C). The insights provided about miR-25-3p expression, function, and targets genes, contribute to a better understanding of its role in PCa.

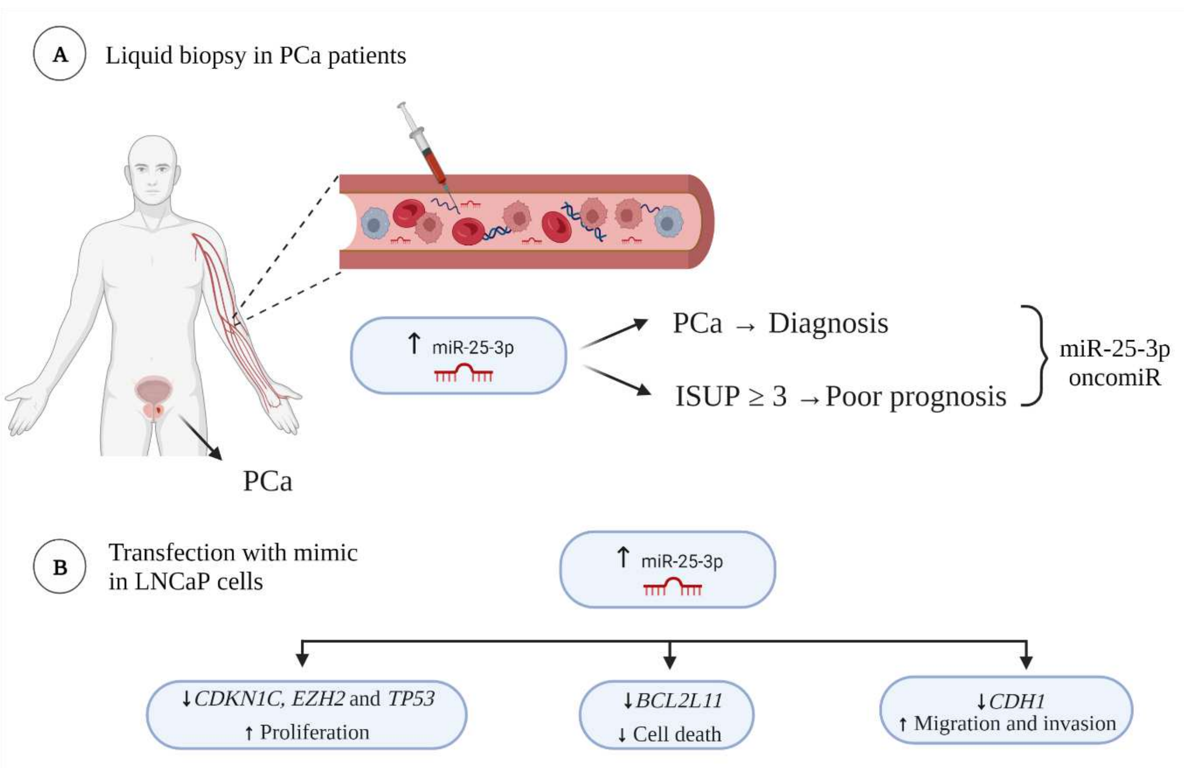

Figure 4 - The role of the miR-25-3p in PCa.

A) miR-25-3p was overexpressed in plasma of PCa patients when compared to controls, and also patients ISUP ≥3; **B)** Overexpression of miR-25-3p decreased the expression of its targets genes and increased the cell proliferation, migration and invasiveness observed in the functional analysis confirmed the oncogenic activity of miR-25-3p. miR-25-3p: hsa-miR-25-3p Figure created with **BioRender.com**

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SUPPLEMENTARY MATERIAL

1 Table Supplementary 1 - Differentially expressed miRNA in the in silico analysis selected from Souza et al. 2017.

microRNA ID	Parametric P value	FDR	R-CaP	R-CTL	Fold-Change
hsa-miR-92a-2	< 1e-07	< 1e-07	26858.16	12931,03	2,08
hsa-miR-25	< 1e-07	< 1e-07	26109,99	11659,61	2,24
hsa-miR-148a	< 1e-07	< 1e-07	236706,14	85496,45	2,77
hsa-miR-92a-1	< 1e-07	< 1e-07	2827,86	698,8	4,05

hsa: Human Species; **FDR:** false discovery rate; **R-CaP:** geometric mean of intensities in class tumor; **R-CTL:** geometric mean of intensities in class normal.

2 Supplementary Table 2. Primer sequences used for RT-qPCR

Gene	Primer sequence (5′-3′)
BCL2L11	Forward: CCGCCCTTATGATGAAGTGT
	Reverse: AAAGCCTGGAGTCAGCAAAA
CDH1	Forward: CTCAAAATCCTCCCTGTCCA
	Reverse: TGGAGAGACACTGCCAACTG
CDKN1C	Forward: ATGCCCATCTAGCTTGCAGT
	Reverse: GGTACAGACGGCTCAGGAAC
EZH2	Forward: TTCATGCAACACCCAACACT
	Reverse: CTCCCTCCAAATGCTGGTAA
B-ACTIN	Forward: ACCTTCTACAATGAGCTGCG
	Reverse: CCTGGATAGCAACGTACATGG
MDM2	Forward: CCGAATAAGGTTTGCCTGAA
	Reverse: CAAATTGCAAAAGGCACTGA
TP53	Forward: GGCCCACTTCACCGTACTAA
	Reverse: GTGGTTTCAAGGCCAGATGT
WDR4	Forward: TTGAAACTCAGCACGAATGC
	Reverse: CGTGCTCACACTTCCTGAAA

3 Supplementary Table 3. The average of relative expression of clinical and pathological parameters among patients with prostate cancer.

ID	miR-25-3p	miR-92a-1-5p	miR-92a-2-5p	miR-148a-3p
PSA				
≤ 4 ng/ul	0.52	0.31	-2.77	-0.81
4.1ng/ul – 10 ng/ul	0.67	1.95	-3.84	0.18
> 10.1 ng/ul	0.53	2.63	-3.24	0.59
Tumor Laterality (Biopsy)				
No	0.24	0.95	-3.38	-0.18
Yes	0.83	3.07	-3.54	0.63
ISUP (Prostatectomy)				
< 2	-0.52	2.55	-3.96	-0.11
≥ 3	0.82	3.37	-4.04	-0.44
Extracapsular extension				
No	0.51	1.59	-4.13	-0.20
Yes	0.64	4.71	-3.76	-0.48
Vesicle invasion				
No	0.69	2.66	-4.03	-0.10
Yes	-0.91	3.27	-3.23	-1.19
Perineural invasion				
No	0.62	2.57	-3.88	-0.19
Yes	-0.59	1.63	-6.07	-1.54
Tumor Laterality (prostatectomy)				
No	0.75	2.22	-3.62	-0.66
Yes	0.42	2.71	-4.26	-0.06
Lymph node invasion				
No	0.61	2.68	-4.16	0.43
Yes	-2.61	-3.08	-1.35	-4.53
Bone metastasis				
No	0.70	2.17	-3.52	0.17
Yes	-0.27	2.20	-2.74	0.70

5.2. CAPÍTULO 3: Circulating miR-21, miR-200c, miR-375 and miR-1290 as cell-free or incorporated into extracellular vesicles as diagnosis and prognosis biomarkers in prostate cancer

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ABSTRACT

Background: Liquid biopsies (LB) are easier to acquire patient-derived samples than conventional tissue biopsies, and their use enables real-time disease monitoring through continuous sampling after initial diagnosis, resulting in a paradigm shift to customized treatment according to the patients profile. Circulating miRNAs have emerged as promising minimally invasive biomarkers for early detection, prognosis and monitoring of cancer. They can exist in the extracellular space as cell-free miRNA (cf-miRNAs) either incorporated into extracellular vesicles (EV-miRNAs). This study aimed to evaluate the miR-21, miR-200c, miR-375 and miR-1290 in LB and compare the potential between cf-miRNAs and EV-miRNAs as non-invasive biomarkers.

Methods: Total RNA was isolated from whole plasma and plasma EV samples from 30 patients (20 patients with prostate cancer localized, 10 patients with metastatic prostate cancer and 15 healthy controls. MiRNAs with important roles in PCa were identified in the literature and quantified by RT-qPCR. The relative expressions of the miRNAs in the three groups were compared and were analyzed their association with clinicopathological parameters. The ROC curve was performed to evaluate the diagnostic potential of miRNAs.

Results: The EV-miRNAs showed higher expression than cf-miRNAs. All the EV-miRNAs analyzed showed a diagnostic potential. The EV-miR-21-5p, was upregulated in PCa and mPCa compared to controls. The EV-miR-200c-3p was upregulated in mPCa and patients with ISUP ≥ 3. miR-375-3p was upregulated in free cells and in exosomes from patients with ISUP ≥ 3. However, in patients with PCa and mPCa, only EV-miRNA-375-3p was overexpressed. EV-miRNA-1290-3p upregulated could distinguish PCa, mPCa patients, patients with ISUP ≥ 3, PSA ≥ 10 ng/mL and bone metastasis.

Conclusions: The exossomal miRNAs EV-miRNA-21-5p showed diagnostic potential for PCa, while EV-miRNA-200c-3p, EV-miRNA-375-3p and EV-miRNA-1290-3p showed diagnostic and prognostic potential for PCa and can be used as biomarkers both for early and late-stage of the disease.

Keywords: Liquid biopsy; exosomes, cell free; microRNA; biomarkers; plasma

INTRODUCTION

Prostate cancer (PCa) is an indolent and slow-growing disease and one of the most common forms of cancer among men, with approximately 1,414,259 new cases diagnosed and 375,304 deaths reported worldwide each year [1]. The lifetime risk of being diagnosed with PCa is estimated to be 1 in 9 men, while the risk of death is around 2% [2].

PCa has traditionally been diagnosed through a serum prostate-specific antigen (PSA) levels and digital rectal examination (DRE), followed by biopsy under the guidance of transrectal ultrasonography (TRUS) [3]. Due to the multifocal nature of most PCa, the clonal heterogeneity within each patient, and impossibility of sampling the entire tumor, the information from a single tissue biopsy is usually insufficient and not reflective of the tumor dynamics in the whole prostate [4]. Approximately 60 to 90% of PCa cases are multifocal with variable aggressiveness and independent progression. This heterogeneity limits the precision of genetic tests based on a single core to predict outcomes and guide treatment decisions. Because the tumor progresses, the static result from one biopsy will become insufficient and inaccurate to reflect tumor dynamics [5]. Despite the need for the sequential sampling of primary and metastatic lesions, this is not always feasible due to the invasive characteristic of the biopsies of metastatic lesions, logistical difficulties in accessing samples, and the inability to perform some genetic tests on formalin-fixed paraffin-embedded biopsy tissue [6–8].

The liquid biopsy (LB) is a minimally invasive procedure which facilitates tumor sampling, continuous monitoring by repeated sampling, devising personalized therapeutic regimens, and screening for therapeutic resistance. There is an unmet clinical need for non-invasive, easily performed diagnostic tests to assess whether a prostate biopsy is necessary [9]. The LB is a technique associated with both genomic and proteomic assessment of a wide array of tumor-derived such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating cell-free RNA (cf-miRNA), tumor-derived extracellular vesicles (EVs), and proteins [10]. In comparison to other molecules that can be analyzed, miRNAs present some inherent technical advantages, such as high resistance to RNase degradation, extending the detection time since sample collection [11].

Circulating miRNAs are a subclass of small non-coding RNAs that have been detected in almost all body fluids. Circulating miRNAs are involved in cell-to-cell communication and their main role is the up- or down-regulation of the expression of their target genes. miRNAs biogenesis occurs first in the nucleus and then in the cytoplasm; afterward, they are released in the circulation as cell-free miRNAs bound in specific RNA-binding proteins (cf-miRNAs) or

encapsulated in exosomes (EV-miRNAs) along with proteins, lipids, and nucleic acids. Exosomes are a subcategory of extracellular vesicles secreted by living cells in the extracellular space or in blood circulation; in cancer patients, exosomes are also secreted by tumor cells and it is now believed that they play important roles in the metastatic process [12].

This study aimed to analyze the cf-miRNAs and EV-miRNAs profile of four miRNAs (miR-21-5p, miR-200c-3p, miR-375-3p and miR-1290-3p) selected from literature between the most studied in PCa, providing information about the better miRNA source for non-invasive PCa biomarkers.

MATERIALS AND METHODS

Ethical Statement

The human subject study was approved by the Research Ethics Committee of the State University of Londrina (Londrina, PR- Brazil) (CAAE19769913.0.0000.5231). Data and samples from patients and controls were collected at Londrina Cancer Hospital (HCL) and Consórcio Intermunicipal do Paraná (CISMEPAR), respectively. The subjects provided their written informed consent and answered a modified questionnaire based on Carrano and Natarajan [13]. Demographic characteristics of all participants are shown in Table 1.

Study cohorts

A total of 15 controls cancer-free individuals without urinary disease symptoms and PSA levels ≤4.0 ng/mL were enrolled from CISMEPAR. As patients, 30 men were enrolled at the HCL (Londrina/PR, Brazil), during the period between 2014 to 2016 for all groups. We selected 30 patients, 20 with localized PCa and 10 with metastatic PCa at the diagnostic, according to the following criteria: abnormal findings in digital rectal examination (DRE) and or elevated PSA serum levels (≥4.0 ng/mL), therapy naïve or cancer, and histopathological confirmation of PCa for tissue biopsy, and metastatic cases with lymph node biopsy or bone scintigraphy positive. The clinicopathological information were obtained from medical and pathological reports (Table 2).

Sample collection

Peripheral blood samples were collected through BD Vacutainer® tubes containing 6% ethylenediaminetetraacetic acid (EDTA). Whole blood samples were centrifuged at 700 x

g for 10 minutes for plasma separation within 2 hours after collection. Next, the cell-free plasma was centrifugated at 2,000 x g for 10 minutes at 4ºC and stored at -80ºC until use.

Extracellular Vesicles isolation

A total of 300 µL of plasma was used for characterization, and 1 mL for screening. Extracellular vesicles isolation was performed using 1 mL of plasma and Total Exosome Precipitation Reagent (4484451, Invitrogen, Lithuania), according to the manufacturer's instructions. Briefly, 0.5 volume of 1× PBS and 0.05 volume of Proteinase K were added to the clarified plasma, and samples were incubated for 5 minutes at 37ºC. Subsequently, 0.2 volumes of exosome precipitation reagent were added to each tube and the samples were incubated at 4°C for 30 minutes. Precipitated extracellular vesicles were centrifuged at 10,000 × g for 10 minutes at room temperature, and the pellet was resuspended in 300 µL 1× PBS.

Characterization of extracellular vesicles enriched with exosomes

The isolated EVs from plasma samples from patients and controls were characterized using Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), and Western Blotting (WB) techniques, based on the minimum criteria recommended by the International Society of Extracellular Vesicles (ISEV) (THÉRY et al., 2018).

Nanoparticle Tracking Analysis (NTA)

NTA was used to quantify and characterize EV sizes present in plasma samples, using a Nano-Sight LM10 (Malvern Panalytical, United Kingdom). Two samples from each group (Control, PCa and metastatic PCa) were selected for the analysis. We characterized the EVs from the pellet as well as the supernatant. The supernatant was characterized for the purpose of confirming the successful isolation of exosomes. The EVs were diluted to 1:1,000 in filtered PBS. Additionally, for the analysis of supernatant we diluted 1:3 (v/v) of supernatant in filtered PBS. The analyzed particles size and quantity were captured in 3 videos of 60s for each sample by optical microscope from liquid suspension.

Transmission Electron Microscopy (TEM)

TEM was used to analyze the size and morphology of EVs. PBS-eluted EVs (7 µL) were fixed in 4% paraformaldehyde and added to a copper grid coated with Carbon/Formvar for one minute before adding the 2% uranyl sample. The EVs were examined using a JEOL 1200EX transmission electron microscope (JEOL, Japan).

Western Blotting (WB)

To further characterize the proteins of exosomes, EVs were quantified using the Bradford assay (Bio-Rad, USA), and 80 µg of protein from pellet and supernatant were loaded and ran under appropriate conditions (non-reducing conditions for CD9, CD63 and Calnexin markers; reducing conditions for TSG101). Primary antibodies specific for CD9 (10626D, Biolegend, USA), CD63 (10628D, Invitrogen, USA), TSG101 (Thermo Scientific, USA), and Calnexin (BD, USA) (1:4,000 dilution, except for CD9 – 1:2,000), and the Goat anti-Mouse horseradish peroxidase (HRP) conjugated secondary antibody (A16066, Invitrogen, USA) (1:2,000). Proteins were detected using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA) and captured with Amersham Hyperfilm ECL (GE Healthcare Life Science, USA).

miRNA isolation

miRNA was isolated from extracellular vesicles (EV-miRNA) and whole plasma (cf-miRNA) using the miRNeasy Mini kit (217004, Qiagen, Germany), according to the manufacturer's instructions with slight modifications. Briefly, 300 µL of the exosome suspension or whole plasma were divided in 3 tubes with 100 µL of the suspension. To each tube, 500 µL of Qiazol® Lysis Reagent was added (79306, Invitrogen, USA), samples were then vortexed for 1 minute and incubated 5 minutes at room temperature. Chloroform (100 µL) was added and mixed, by vortex, for 10 s, incubated 3 minutes at room temperature and samples centrifuged at 12,000 × g for 15 minutes at 4°C to separate the aqueous and the organic phase. The aqueous phase was collected and transferred to a new microtube with 1.25 volumes of 100% ethanol and the solution was transferred to RNeasy® Mini columns and centrifuged at 8,000 × g for 15 seconds, following washes with specific buffers. Samples were eluted with 25 µL of RNase-free water. Total RNA quantity and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Analysis of miRNA expression with RT-qPCR

For PCa and controls, the expression patterns of miRNAs (hsa-miR-21-5p [000397], hsa-miR-200c-3p [002300], hsa-miR-375-3p [00000564] and hsa-miR-1290-3p [002863]) were performed using 5 ng of total RNA and TaqMan miRNA Reverse Transcription kit (PN4427975, Applied Biosystems, USA), following the manufacturer's instruction. A 1:4-fold dilution of miRNA cDNA was used as input for the qPCR reaction. The qPCR reaction was performed using 5.5 µL of TaqMan 2X Universal PCR Master Mix (4440038, Applied

Biosystems, USA), 0.45 μL of miRNA-specific TaqMan Probe (PN4427975, Applied Biosystems, USA), and 7.0 μL of diluted miRNA cDNA on the Techne Quantum™ Real-Time PCR Cycler System (UK) at 50ºC for 2 min, 95ºC for 10 min and 50 cycles at 95ºC for 15 seconds and 60ºC for 1 minute. Two miRNAs (U6 snRNA [001973] and RNU48 [001006]) were used as endogenous controls for cell-free RNA, and a pool of samples was used as calibrator in every run.

Statistical analysis

The miRNA expression data were calculated by delta-delta Cycle threshold (ΔΔCT) to obtain the relative expression data [16]. Data were reported as mean values ± SD in duplicates and were analyzed by Student's t-test or two-way ANOVA using GraphPad Prism software, version 8.0.2 (GraphPad, USA). A value of $p< 0.05$ was considered statistically significant. The diagnostic values were evaluated by receiver operating characteristic (ROC) curves analysis (GraphPad, USA).

RESULTS

A total of 15 cancer-free controls and 30 patients with PCa were enrolled in this study. Of the 30 PCa patients, 20 had localized PCa and 10 were diagnosed with metastatic PCa (mPCa). The median age of cancer-free controls group was 66 years old, and the mean PSA level was 1.46 ng/mL. The median age of the patients in the PCa localized group was 69 years as well, and the mean PSA level was 70.91 ng/mL. The median age of patients in the mPCa group was 69 years, and the median PSA level was 22.97 ng/mL. Additionally, Table 1 shows demographic characteristics like ancestry, familial history of cancer, alcohol and tobacco habits.

In the samples analyzed in this cohort, 60% of patients in each group had ISUP 1. In the PCa group, we found ISUP 2 (20%), ISUP 3 (10%) and ISUP4 (10%). In the mPCa group, 10% of patients were ISUP 2, 10% ISUP 3, and 20% ISUP 4. None of the patients in the two analyzed groups presented ISUP 5, that is, a very high risk for PCa (Table 2).

1 Table 1 - Demographic characteristics of recruited participants

Characteristics		CTR (%) (n=15)	PCa (%) (n=20)	mPCa (%) (n=10)
Age [years]	Median (minimum–maximum)	66 (60-75)	69 (56-87)	69 (57-83)
PSA [ng/mL]	Median (minimum–maximum)	1.46 (0.6-3.7)	70.91 (4.5-933.9)	22.97 (3.7-100.0)
Ancestry	Afrodescendent	3 (20.0)	3 (15.0)	0 (0.0)
	Eurodescendent	12 (80.0)	17 (85.0)	10 (100.0)
Family history of cancer	No	7 (46.7)	6 (30.0)	3 (30.0)
	Yes	6 (40.0)	12 (60.0)	4 (40.0)
	Yes, PCa	2 (13.3)	2 (10.0)	3 (30.0)
Vasectomy	No	14 (93.3)	18 (90.0)	9 (90.0)
	Yes	1 (6.7)	2 (10.0)	1 (10.0)
Alcohol	No	6 (40.0)	13 (65.0)	8 (80.0)
	Yes	9 (60.0)	7 (35.0)	2 (20.0)
Tobacco	No	15 (0.0)	14 (70.0)	8 (80.0)
	Yes	0 (0.0)	6 (30.0)	2 (20.0)

CTR: Control; PCa: Prostate Cancer; mPCa: metastatic prostate cancer; PSA: prostate specific-antigen.

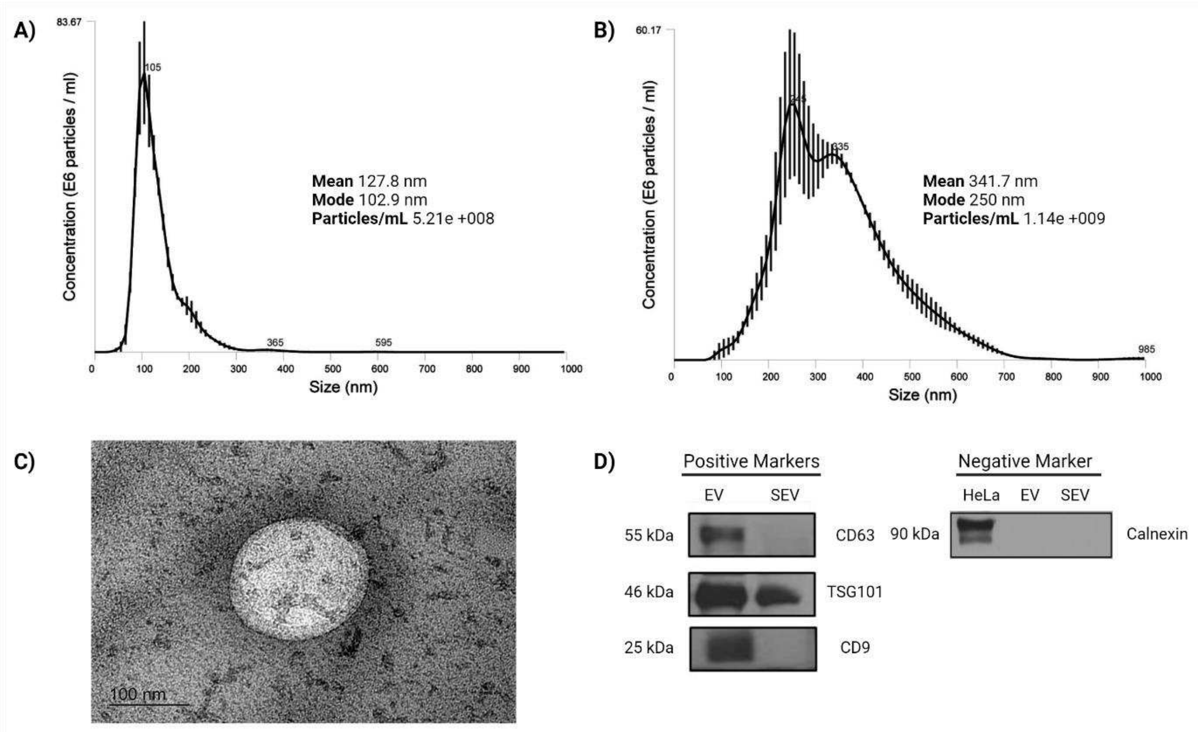
Table 2 – Clinicopathological characteristics of patients with prostate cancer

Characteristics		PCa n=20 (%)	mPCa n=10 (%)
ISUP- Grading (Risk group)	GS		
Group 1 (Low)	≤6	12 (60.0)	6 (60.0)
Group 2 (Intermediate Favorable)	7 (3 + 4)	4 (20.0)	1 (10.0)
Group 3 (Intermediate Unfavorable)	7 (4 + 3)	2 (10.0)	1 (10.0)
Group 4 (High)	8	2 (10.0)	2 (20.0)
Group 5 (Very High)	9–10	0 (0.0)	0 (0.0)
Treatment	Active surveillance	9 (45.0)	1 (10.0)
	Prostatectomy	10 (50.0)	9 (90.0)
	TURP	1 (5.0)	0 (0.0)
Bilaterality	No	5 (25.0)	3 (30.0)
	Yes	7 (35.0)	4 (40.0)
	NS	8 (40.0)	3 (30.0)
Seminal vesicle	No	12 (60.0)	6 (60.0)
	Yes	1 (5.0)	1 (10.0)
	NS	7 (35.0)	3 (30.0)
Perineural invasion	No	12 (60.0)	6 (60.0)
	Yes	0 (0.0)	1 (10.0)
	NS	8 (40.0)	3 (30.0)
Lymph-node status	No	11 (55.0)	5 (50.0)
	Yes	0 (0.0)	4 (40.0)
	NS	9 (45.0)	1 (10.0)

ISUP: International Society of Urological Pathology; GS: Gleason Score; PCa: Prostate Cancer; mPCa: metastatic prostate cancer; NS: Non-surgery.

Characterization of extracellular vesicles

The size distribution of the isolated population of exosome-enriched EVs and supernatant EVs were analyzed by NTA, revealing differences between them. The EVs and supernatant EVs had, respectively, an average size of 127 nm and 341nm; mode of size 103 nm and 250 nm and concentration of $5.21x10^8$ and $1.14x10^9$ as particles/mL (Figure 1A and B). TEM indicated that the EVs had a spherical shape with exosome characteristic with a diameter of approximately 150 nm (Figure 1C). WB analysis showed that the exosome-enriched EVs presented positive for typical markers D9, CD63, AGO2, TSG101, when compared to the EVs supernatant, and negative for the endoplasmic reticulum protein Calnexin (Figure 1D).

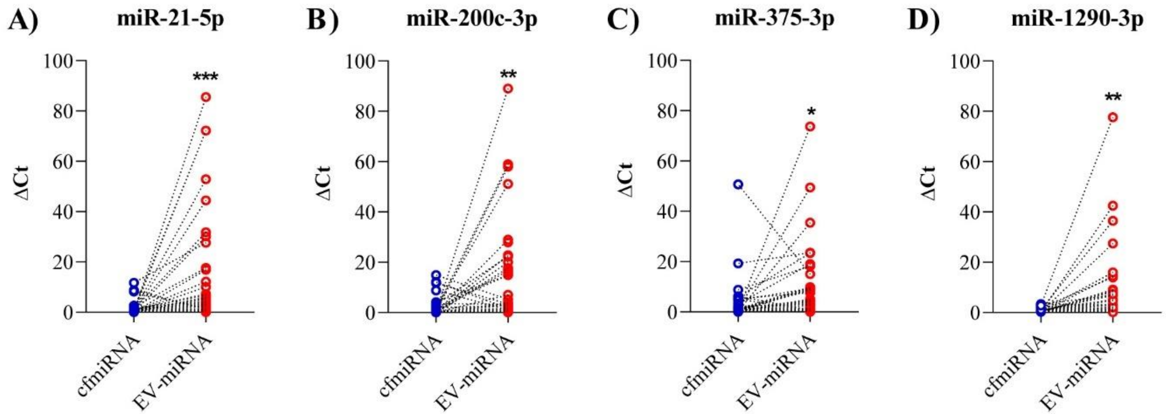


1 Figure 1 - Plasma-Isolated EVs characterization.

A and B) Histograms showing nanoparticle size distribution. On the right, parameters referring to the analyzed sample (individually), such as size distribution (mean and mode) and particles concentration. **A)** Plasma-isolated EVs; **B)** Supernatant-isolated EVs **C)** TEM images of EVs obtained showing spherical double-membrane vesicles with size below 150 nm. **D)** WB analysis for the positive exosomal markers CD9, TSG101, CD63, and AGO, as well as for the negative exosomal marker calnexin (used to identify cellular components co-isolated with the EVs) at plasma-isolated EVs (pellet) and the remaining SEV. EV: extracellular vesicle, SEV: supernatant extracellular vesicle. In opposite of EVs markers, HeLa whole cell lysate (80 µg) was used as intracellular control positive for calnexin.

MiRNAs expression in whole plasma and extracellular vesicles

To assess the relative abundance of cf-miRNAs and EV-miRNAs, a ratio between the ΔCt values was calculated (Figure 2). Mean ΔCt values for cf-miRNA and EV-miRNA were significantly different for all miRNAs evaluated in this study (Table 3).



2 Figure 2 - ΔCt values of cell-free miRNAs and extracellular vesicles miRNAs.

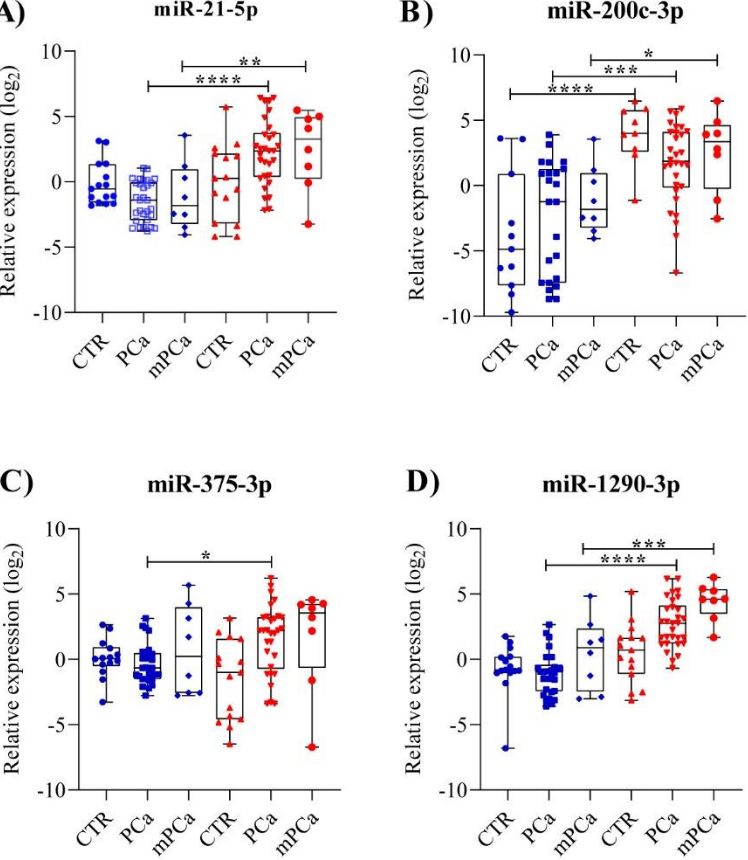
A paired dot plot shows the ranking of miRNAs according to ΔCt values in whole plasma and extracellular vesicles; lines connect samples from the same individual. **A)** miR-21-5p, **B)** miR-200c-3p, **C)** miR-375-3p and **D)** miR-1290-3p. Blue: cell-free miRNA (cf-miRNA); red: extracellular vesicles miRNAs (EV-miRNA); *** p <0.0009; ** p <0.001. * p <0.03.

2 Table 3 - ΔCt mean between cell free miRNAs and extracellular vesicles miRNAs.

miRNA	ΔCt mean		*P*
	cf-miRNA	EV-miRNA	
21-5p	1.26	11.92	0.002
200c-3p	1.90	11.72	0.0009
375-3p	2.97	8.07	0.03
1290-3p	0.88	8.75	0.0018

Leegnda tabela

A similar analysis was performed to evaluate the relative expression of CF-miRNAs and EV-miRNAs in control and patient groups. Within each group, EV-miRNAs were more abundant when compared to cell-free ones. In the groups of patients with localized and metastatic disease, miR-21-5p and miRNA-1290-3p were up-regulated. The EV-miRNA-200c-3p was up-regulated in controls, in patients with localized disease and metastatic PCa and the miR-375-3p only in the group of patients with localized disease (Figure 3).



3 Figure 3 - Relative expression between controls, PCa localized and metastatic PCa to assess cf-miRNA and EV-miRNA amount of miRNAs.

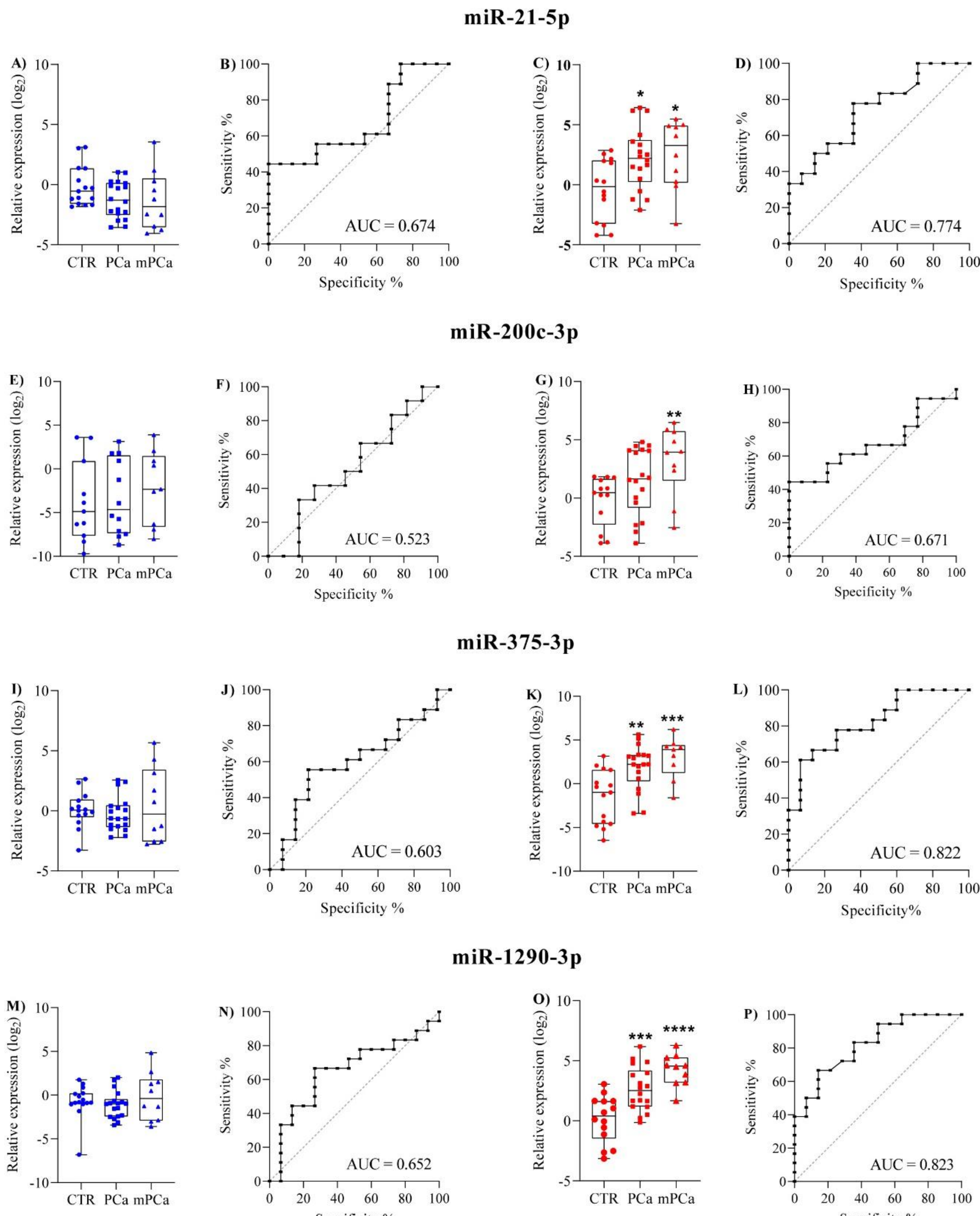
Box plot illustrating the change in plasma miRNAs between CTR and PCa and mPCa patients. **A)** miR-21-5p; **B)** miR-200c-3p; **C)** miR-375-3p; **D)** miR-1290-3p; Blue: cell-free miRNA; red: extracellular vesicles miRNAs; CTR: controls; PCa: prostate cancer; mPCa: metastatic prostate cancer *p= 0.01; **p= 0.0074; ***p ≤ 0.0005 **** p< 0.0001.

Diagnostic and prognostic potential of cf-miRNAs and EV-miRNAs

To assess the diagnostic potential of the selected miRNAs, their relative expression were evaluated in Controls (CTR), PCa localized (PCa) and metastatic PCa (mPCa) samples. An interesting finding of this study was a tendency of EV-miRNA levels to increase with disease aggressiveness, but cf-miRNA levels did not follow the same trend.

The EV-miR-21-5p was upregulated in PCa and mPCa with AUC 0.744 (95% CI: 0.574-0.914) compared to controls. The EV-miR-200c was upregulated in mPCa (p= 0.01) with AUC 0.671 (95% CI: 0.480 to 0.862), EV-miR-375 was upregulated in PCa (p= 0.01) and mPCa (p= 0.01) with AUC 0.822 (95% CI: 0.681-0.963) and EV-miR-1290 was upregulated in PCa

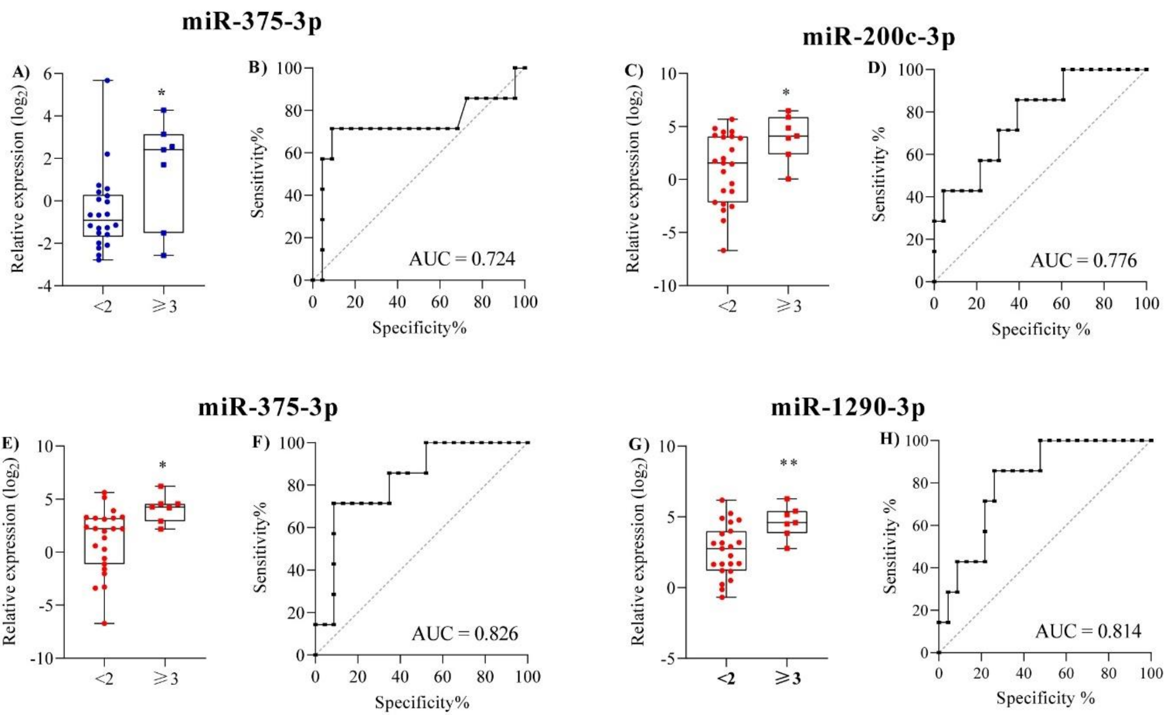
(p= 0.01) and mPCa (p= 0.01) with AUC 0.823 (95% CI: 0.681-0.966). However, as cell-free they could not classify the groups (Figure 4).



4 Figure 4 - Relative expression of cell-free and extracellular vesicles miRNAs to assess the diagnostic potential.

Box plot illustrating the expression of miRNAs between CTR and PCa or mPCa patients and their respective ROC curves. Blue: cell-free miRNA; red: extracellular vesicles miRNA; CTR: controls; PCa: prostate cancer; AUC: area under curve; * $p < 0.05$; ** $p \leq 0.001$; *** $p \leq 0.0001$.

When comparing the expression of miRNAs in PCa with controls, we observed three EV-miRNAs up-regulated: EV-miR-21-5p (Figure 4C), EV-miR-375 (Figure 4K), EV-miR-1290 (Figure 4O). In the mPCa group, EV-miR-21-5p (Figure 4C), EV-miR-200c (Figure 4G) and EV-miR-375 (Figure 4K) were upregulated compared to the control group. The Roc Curve values (AUC) obtained were 0.774 (95% CI: 0.574-0.914) to EV-miR-21-5p (Figure 4D); 0.671 (95% CI: 0.480 to 0.862) to EV-miR-200c (Figure 4H); 0.822 (95% CI: 0.681-0.963) to EV-miR-375 and 0.814 to EV-miR-1290 (95% CI: 0.654 to 0.973). The expressions of the same miRNAs in cell-free form did not distinguish the three analyzed groups (Figures 4A, E, I and M).

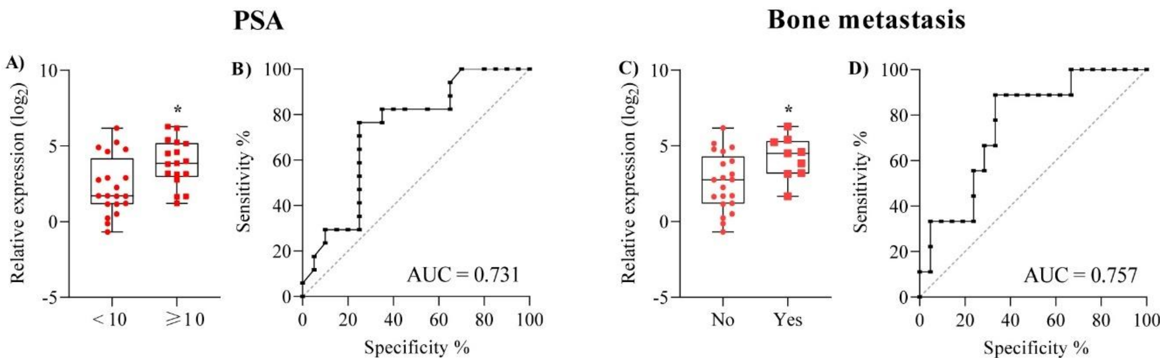


5 Figure 5 - Relative expression of cell-free and extracellular vesicles miRNAs to assess the prognostic power according to ISUP-grading.
Box plot illustrating the change in plasma miRNAs between ISUP <2 and ISUP ≥3 in PCa patients; Blue: cell-free miRNA; red: extracellular vesicles miRNA; *p ≤ 0.03; **p= 0.009.

To determine the prognostic potential of miRNAs, we performed associations between clinicopathological parameters and miRNAs. The results showed that cfmiR-375 with AUC 0.724 (95% CI: 0.447-1.00) (Figure 5B), EV-miRNA-200c with AUC 0.776 (95% CI: 0.587-0.966) (Figure 5D), EV-miRNA-375 with AUC 0.826 (95% CI: 0.660-0.992) (Figure 5F), and EV-miRNA-1290 AUC 0.814 (95% CI: 0.654-0.973) (Figure 5H), were all up-regulated in patients with ISUP ≥ 3 (Figures 5A, C, E and G). The other parameters such as tumor laterality,

seminal vesicle, perineural invasion and lymph-node status did not present any changes when associated with miRNAs (Supplementary Table 1)

Finally, the EV-miRNA-1290 was overexpressed related to other parameters of aggressiveness, such as PSA > 10 ng/mL and bone metastasis (Figure 6).



6 Figure 6 - Relative expression of miRNA to assess the aggressiveness of extracellular vesicles miR-1290-3p.

Box plot illustrating the change in plasma miRNAs in PCa patients PCa:; PSA: prostate specific-antigen; PSA <10 ng/mL; PSA ≥ 10 ng/mL * $p < 0.05$.

DISCUSSION

Circulating miRNAs in body fluid has now offered a new form of biomarker for disease diagnosis. Quantification of these miRNAs in blood from cancer patients may offer new opportunities for diagnosis and prognosis in a minimally invasive way. Dysregulated miRNAs can be released passively or actively from tumor cells. The cf-miRNAs can be associated with proteins (e.g., Ago2, lipoproteins) and or packaged within cellular structures (i.e., exosomes) as EV-miRNAs.

Several hypotheses have been proposed to justify the presence of circulating miRNA in body fluids. Despite evidence that miRNAs are actively secreted from cells, other studies suggest that release occurs passively, either because miRNAs are abundant within the cell or because necrotic and apoptotic cells have released miRNAs. Even with these contradictory hypotheses, circulating miRNAs have been shown to play an important role in tumor cell-environment communication [14]. Despite the fact that both sources of miRNAs are widely studied as biomarkers, few studies compare their expression, and the debate over which source would identify PCa-specific miRNAs is still in its infancy [14, 15].

Here, we performed a comparison of cf-miRNAs and EV-miRNAs isolated from the same plasma samples of controls versus patients with localized PCa or mPCa. Our results showed that the level of miR-21-5p, miR-200c-3p, miR-375-3p and miR-1290-3p in extracellular vesicles, in other words, EV-miRNAs, was higher than in cf-miRNAs in whole plasma. Previous findings [16, 17, 18], which showed that for some miRNAs, the exosome fractions contained a higher amount of the miRNA than the whole plasma.

miR-21-5p, one of the first miRNAs to be categorized as oncomiR, was subsequently evaluated for its potential use as a clinical biomarker in various cancers [19]. Concerning to PCa, miR-21-5p seems to be the most studied and had repeatedly identified in many studies as being overexpressed in PCa playing a fundamental role in tumor growth [19].

In the present study, we observed upregulation of miR-21-5p in extracellular vesicles of patients with localized PCa as well as in vesicles of mPCa patients, when compared with controls, corroborating Endzelinš et al. [15] and Malla et al. [20] who found miR-21-5p upregulated within extracellular vesicles. In a meta-analysis, Wang et al. (2019) cited association studies of exosomal miR-21 upregulation with PCa from urine samples from patients and controls and plasma from patients with PCa and BPH. These works together indicated EV-miRNA-21-5p as a diagnostic and risk classification marker for the disease [21].

Several studies have been conducted on cf-miRNA-21-5p, including [21-27] reported cf-miRNA-21-5p up-regulated in plasma or serum from patients with PCa. However, in our samples, we did not see a difference between cfmiRNA-21-5p in plasma compared controls and PCa or mPCa.

Aberrant expression of miR-200 family has been reported in different cancers [28]. In our study, we observed EV-miRNA-200c-3p up-regulated in mPCa and in patients with ISUP ≥3. Previous studies reported a similar result, showing that the expression of miR-200c increased in advanced spread disease compared to the localized PCa samples [29]. Khorasani and colleagues showed the increased expression of miR-200c in majority of tumor samples compared with their matched non-tumor tissue as well [30].

The upregulated EV-miR-21-5p and E-miR-200c-3p in the groups we studied shows a diagnostic and risk classification potential of these molecules, and it is in line with Endzeliņš et al. (2017), who compared EV-miRNAs and cf-miRNAs obtained from whole plasma, differentiating between PCa and BPH patients [15].

Bertoli et al. [14] did a meta-analysis approach and suggested 29 miRNAs with diagnostic properties in prostate cancer, among them, miR-21 and miR375, studied by us. Available data show that miR-375 defies simple classification as a tumor suppressor or

oncogenic miRNA. Andl et al. [32] conducted an extensive review and described contradictions in 24 publications about miR-375.

In our cohort, we detected EV-miRNA-375-3p up-regulated in PCa, mPCa, and patients with ISUP ≥ 3; however, no changes were detected in cf-miRNA-375-3p. These results are consistent with what was obtained by our group [31] in a larger and distinct cohort of patients. In line with what we observed in our sample, Bryant et al. [33] affirmed that this miRNA, when encapsulated in EVs, has the potential to be used as a prognostic biomarker, as it is upregulated in metastatic PCa patients compared with non-metastatic cases.

Despite the miR-1290-3p being one of the miRNAs less studied in our selection, it is the most promising, as it has been identified consistently by other groups as being overexpressed in PCa and playing a vital role in tumor growth. The expression level in our samples demonstrated a remarkable ability to distinguish patients and controls, and patients with worse prognoses such as metastasis at diagnosis, patients with PSA levels greater than 10 ng/mL, and patients with bone metastases. Furthermore, the miRNA profile analysis of PCa liquid biopsy revealed elevated levels of this miRNA within serum and urine samples [34]. As also, miR-1290 is considered to be the prognostic marker for castration-resistant PC [35]. Afterwards, Li et al. [36] confirmed that upregulation of miR-1290 observed in patients when occurring in PCa cell lines contributed to the malignant phenotype of PCa cells, confirming the presence of oncomiR in PCa [37].

CONCLUSION

The evaluation of the expression of cf-miRNAs and EV-miRNAs from samples of control individuals and patients with PCa and mPCa carried out in the present study indicated that in all groups analyzed the levels of expression of EV-miRNAs were higher than those of cf-miRNAs. This result allows us to suggest EV-miRNAs miR-21-5p, miR-200c-3p, miR-375-3p and miR-1290-3p as promising diagnostic biomarkers for PCa and the EV-miRNA-1290-3p as a potential prognostic marker of specific tumor characteristics associated with PCa.

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CONCLUSÃO GERAL

Os miRNAs desempenham um papel regulador em diferentes processos biológicos, e suas desregulações estão associadas com surgimento de várias doenças, entre elas o câncer. Devido à sua abundância e estabilidade em uma variedade de fluidos corporais, os miRNAs têm sido considerados uma das fontes biomarcadores minimamente invasivas e promissoras. A possibilidade de utilizar o perfil dos miRNAs no tratamento personalizado específico é promissora. No entanto, as aplicações clínicas dos miRNAs ainda são limitadas pelo conhecimento vigente.

Com o intuito de contribuir para o maior conhecimento sobre essas moléculas, e considerando que os miRNAs podem estar livres (cf-miRNAs) ou encapsulados por vesículas extracelulares (EV-miRNAs), investigamos por biópsia líquida o potencial de quatro cf-miRNAs e quatro EV-miRNAs, como potenciais biomarcadores para o CaP.

O miR-25-3p mostrou super expressão no plasma e, portanto, um potencial marcador diagnóstico e prognóstico para o CaP. Estudos funcionais realizados para entender os mecanismos de ação deste miRNA, mostraram que ele possui papel importante durante o processo de tumorigênese do CaP. A identificação do papel do miR-25-3p no CaP poderá, assim, auxiliar no desenvolvimento de novas terapias, melhorando a qualidade de vida dos pacientes.

A investigação dos padrões de expressão dos cf-miRNAs e EVmiRNAs: -21-5p, -200c-5p, -375-3p e -1290-5p derivados de plasma de indivíduos controles e de pacientes com a doença localizada ou metastática indicou que os EV-miRNAs -200c-5p, -375-3p e -1290-5p são potenciais biomarcadores prognósticos para CaP. É importate lembrar que esse tipo de investigação (avaliando a melhor fonte para se obter miRNAs a partir de plasma) utilizando biópsia líquida é pioneira no Brasil.

O presente estudo mostrou que, dos oito miRNAs avaliados, quatro (cf-miR-25-3p, e o EV-miR-200c-5p, EV-miR-375-3p e EV-miR-1290-5p) mostraram-se promissores como biomarcadores para o diagnóstico e prognóstico no CaP. O EV-miR-21-5p apresentou potecial para ser usado como biomarcador para o CaP. Desta forma, o artigo de revisão e os dois manuscritos em preparação apresentados neste estudo poderão contribuir para o uso futuro de miRNAs específicos como biomarcadores moleculares que possam ser identificados em amostras de biópsias líquidas de pacientes com CaP.

6. APÊNDICES

Durante o meu curso de doutorado tive a oportunidade de passar um ano no exterior (dois períodos de 6 meses, sob supervisão da Dra. Morag Park e co-supervisão da Dra. Hellen Kuasne, no Instituto Rosalind and Goodman, McGill University/Canadá, para realizar parte da minha pesquisa. O primeiro período foi financiado pela CAPES com a bolsa de PDSE (Setembro de 2021 a Março de 2022) e no segundo período (Agosto de 2022 – Fevereiro de 2023) fui contemplada com a bolsa “Emerging Leaders in the Americas”, concedida por Global Affairs Canada.

Entre as atividades realizadas com a bolsa PDSE destaco a análise funcional do miR-25-3p *in vitro* com o objetivo de compreender o papel deste miRNA em linhagem de células tumorais de próstata. Os dados obtidos foram inseridos no manuscrito: “Potential clinical application and functional roles of cell-free miRNA-25-3p in prostate cancer”. Neste período colaborei no projeto da doutoranda Gabrielle Brewer, realizando análise e validação dos genes alvos para envolvidos no perfil de fibroblastos associados ao câncer (CAFs do ingês, cancer associated fibroblasts). Esta colaboração gerou um manuscrito que está sob revisão na revista Nature Cancer (Apêndice 6.1).

No segundo período estive envolvida em dois projetos: Análise funcional do miR-34a, supressor tumoral, em linhagens celulares de mama triplo negativo para confirmar a alteração fenotípica nesta linhagem celular causada por este miRNA. Os dados preliminares estão apresentados no Apêndice 6.2. Durante este período também iniciei a análise de dados de miRNAs obtidos por meio de TaqMan® MicroRNA Arrays, de amostras de estroma e epitélio de pacientes com câncer de mama triplo negativo usando o Partek Software com o objetivo de determinar um painel de miRNAs relacionados ao microambiente imune tumoral. Os dados parciais estão apresentados no Apêndice 6.3. Além da participação nestes projetos, realizei a análise dos dados de cf-miRNAs e EV-miRNAs e redigi os manuscritos contidos no capítulo 2 e 3.

6.1 Apêndice - Manuscrito Submetido à revista Nature Cancer: Pro-invasive mechanoresponses of patient derived triple negative breast cancer CAFs are AhR-dependent and correlate with disease state

Gabrielle Brewer[1,2], Paul Savage[1,3], Anne-Marie Fortier[1], Hong Zhao[1] Alain Pacis[1] , Yu-Chang Wang[4,5], Dongmei Zuo[1], **Monyse de Nobrega[1,6]**, Annika Pederson[1,3], Camille Cassel de Camps[7], Margarita Souleimanova[1], Valentina Muñoz Ramos[1], Jiannis Ragoussis[4,5], Morag Park[1,2,3,8]* and Christopher Moraes[1,7,9]*

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Abstract

Cancer associated fibroblasts (CAFs) play a critical role in dynamically modulating the tumour microenvironment, by contributing to formation of fibrotic foci and facilitating tumour cell invasion. While the presence of collagen and CAF-dense fibrotic foci in the breast and corresponding changes in mechanical stiffness of tissue have been correlated with human breast cancer aggression, the impact of this evolving microenvironment on metastatic progression remains largely unknown. Here, by examining CAFs isolated from primary human triple-negative breast cancer (TNBC) tissue at surgery, we find that functional CAF invasive patterns and sensitivity to microenvironmental mechanics correlate with patient metastatic stage at surgery. We show these phenotypic changes reflect mechanosensitive differences in gene expression and reveal a functional role for the transcription factor, aryl hydrocarbon receptor (AhR), in facilitating CAF invasion. AhR protein levels vary with microenvironmental stiffness in CAFs that are mechanosensitive from tumours with no detectable lymph node metastasis, whereas proinvasive CAFs from TNBC with axillary lymph node metastasis have high AhR levels independent of stiffness. This work identifies a novel mechanobiological mediator for CAF invasion, providing new insight into AhR function, and suggests mechanisms responsible for clinical failure of drugs aimed at targeting CAF activation.

Keywords

- **Mechanobiology**
- **Stiffness**
- **Cancer-associated fibroblasts**
- **Triple-negative breast cancer**
- **Remodelling**
- **Contraction**
- **Invasion**
- **Fibrotic foci**
- **Aryl hydrocarbon receptor**

6.2 Apêndice - Manuscrito em Preparação: From stellate morphology to a round acinar structure: A deep analysis of a Triple Negative Breast Cancer cell lineage post-transcriptionally regulated with miR-34a.

Felipe Canto de Souza [1,2,] Hellen Kuasne [2], **Monyse de Nobrega** [2,3], Morag Park [2,4,5,7], Rodrigo Panepucci [1,8].

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Triple negative breast cancer (TNBC) is described as the most aggressive among the subtypes of breast cancer since it presents limitations to treatment, high recurrence rates and low survival of patients. The potential of TNBC to migrate and invade adjacent tissues may vary, however, on the expression of different markers such as adherent junction proteins, like transmembrane E-Cadherin, as well as mesenchymal/basal markers, like Vimentin, Twist and Zeb involved in the epithelium-mesenchymal transition (EMT), in which epithelial cells lose their attachment to other cells and acquire mesenchymal phenotype. To achieve success, however, those cells need to turn back into its original epithelial morphology by a process known as mesenchymal-epithelial transition (MET). It is of interest though to identify targets that reduce these invasiveness processes. The use of microRNAs (miRNAs) in the regulation of such stages is seen as a promising alternative to currently available drugs, since they post-transcriptionally regulate the expression of target messenger RNAs (mRNAs), leading to repression of translation and degradation of its complementary transcripts. In view of the involvement of miRNAs on EMT and MET processes, we performed with miR-34a, a known miRNA involved in reduction of breast tumor cell proliferation, time-lapse wound healing assays followed by

immunofluorescence image acquisitions. Briefly, MDA-MB-231 cells, previously cultured in DMEM medium 10% FBS were reversed transfected in a flat bottom 96 well plate. By that, 50nM of mimic-miR-34a were mixed with OptiMEM and complexed with 0,2% Lipofectamine 2000. After 20min incubation the wells were filled with cells on DMEM medium (10% FBS) ($8x10^3$ cells/well). After 48hr of incubation, the medium was removed, and cells were treated with 5µM Mitomycin C for 2hr to stop cell proliferation. Then, cells were scratched, and medium changed with another round of transfection. The assay was conducted in parallel transfection with a mimic miR-Ctr. Then, cells were incubated in a Incucyte microscope chamber (37°C, 5%CO_2, 85%RH) for Transmitted Light image acquisition. After 72hr of scratch, cells were fixed and Actin, Vimentin, E-Cadherin, Twist1 and Zeb1 immuno-stained with primary and secondary antibodies conjugated with fluorochromes. The image acquisition and quantification were performed on ImageXpress (Molecular Devices) and analyzed by softwares like MetaXpress, CellProfiler, ImageJ and Knime. After cell count and stained quantifications, the results showed that miR-34a expression reduced not only cell proliferation but also diminished the wound healed area. Interestingly, it also induced the treated cells to aggregate in an Acinar structure morphology that grew its size and perimeter as mores cells were aggregated. Indeed, we showed that as the cells were arranged in an acinar structure under miR-34a expression, also did the E-Cadherin levels on the edge of the aggregated cells increased. On the contrary, the Vimentin levels decreased as well as the area and perimeter of the aggregated cells. Our data show that miR-34a induced mesenchymal phenotype cells to its original epithelial morphology and led these aggregated grouped cells to a baso-apical polarity axis with more Actin on its lumen showing similarities to acinar morphologies of cultures from non-tumor breast cells.

6.3 Apêndice - Manuscrito em Preparação: Evaluating the modulatory role of microRNAs in the stroma and epithelial compartments of immune cold triple-negative breast cancers.

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Invasive breast carcinoma is a combination of heterogeneous diseases with distinct molecular and clinical features. Some subsets of breast cancer present major clinical challenges, including immune-cold triple-negative breast cancer (icTNBC), which are poor responders to neoadjuvant chemotherapy and not eligible to immunotherapy. microRNAs (miRNAs), are short noncoding RNA with crucial role in regulating gene expression. However, their role on TNBC microenvironment is not fully understood. We previously performed laser capture microdissection (LCM) of stroma and epithelial compartments of TNBC. By integrating spatial resolution of CD8+ T cells and LCM gene expression profiles we defined samples with distinct tumor immune microenvironment (TIME) (PMID: 30753167). TNBC varied from an immune-cold (IC) microenvironment with absence of tumoral CD8+ T cells to an immunoreactive microenvironment, exhibiting tumoral infiltration of CD8+ T cells (fully inflamed-FI). Here we evaluated a panel of 754 human microRNAs using a TaqMan Array Human MicroRNA Card Set (Thermo Fisher technology) in tumor stroma and tumor epithelial cells from the same set of TNBC samples that we previously investigated. We identified distinct signatures of miRNAs in TNBC epithelial and stroma compartments. We found a set of miRNAs that are overexpressed in the stroma of FI samples, including the hsa-miR-150, which is known to be highly expressed in immune cells. We also found several miRNAs that were overexpressed in the stroma of immune cold TNBC. Integration of mRNA and miRNA profiles in the epithelial and stroma compartments may provide a meaningful and comprehensive understanding of the

biological processes involved in TNBC and the crosstalk of cancer and microenvironment cells. Especially for the icTNBC, for which the therapeutic options are very limited, insights on the mRNA/miRNA profile may be essential for optimal cancer therapy.

6.4 Apêndice - Termo de consentimento livre e esclarecido para pacientes

Nós, Ilce Mara de Syllos Cólus e Marilesia Ferreira de Souza da Universidade Estadual de Londrina o convidamos para nossa pesquisa e solicitamos sua colaboração e o seu consentimento para incluí-lo em nosso projeto de pesquisa “Estudo comparativo do perfil transcricional e genotípico de genes relacionados ao CaP entre indivíduos sadios e portadores desta neoplasia para o desenvolvimento de assinaturas gênicas com fins diagnósticos, prognósticos e terapêuticos”. O objetivo deste estudo é avaliar alguns fatores genéticos que possam auxiliar no diagnóstico, prognóstico e na terapia de pacientes portadores de CaP. Assim, solicitamos a sua colaboração como voluntário neste projeto onde vamos avaliar e comparar as semelhanças e diferenças entre dois grupos de pessoas: sadias e com câncer. Portanto, solicitamos a sua autorização para que uma pequena quantidade de seu sangue (10 ml) seja coletada via punção venosa (picada na veia) com seringa e agulha descartáveis. Esclarecemos que não haverá desconforto físico adicional para a sua pessoa, além da picada da agulha.

Caso o senhor tenha que realizar prostatectomia (operação de retirada parcial ou total da próstata), solicitamos também sua permissão para que, depois de realizada a cirurgia e da amostra da sua próstata ter sido utilizada pelo laboratório do Hospital para diagnóstico, possamos coletar uma pequena amostra deste tecido que não foi utilizado pelo Hospital, mas que fica armazenado. Desta forma, a coleta do material para análise genética ocorrerá somente após a finalização do seu diagnóstico e não trará riscos adicionais ao seu tratamento.
Pedimos sua autorização para que moléculas (DNA, RNA ou proteínas) obtidas a partir da amostra de sangue e/ou tecido possam ser armazenadas para estudos futuros no Laboratório de Mutagênese e Oncogenética da UEL, quando será solicitada nova autorização do Comitê de Ética em Pesquisa com Seres Humanos da UEL para a realização das pesquisas posteriores. O material obtido ficará armazenado no Laboratório de Mutagênese e Oncogenética da UEL, sob responsabilidade dos pesquisadores responsáveis por esta pesquisa. Esclarecemos ainda que a autorização para manutenção destas amostras é por prazo indeterminado, podendo ser cancelada por aviso escrito à responsável pelo Laboratório de Mutagênese e Oncogenética da Universidade Estadual de Londrina.

Solicitamos também sua autorização para que possamos consultar seu prontuário médico (que fica no Hospital do Câncer de Londrina) e obter alguns dados clínicos. Solicitamos-lhe o preenchimento de um questionário sobre seu estilo de vida, histórico de exposição ocupacional, onde o senhor será identificado apenas por um código, preservando sua identidade. Este questionário ficará armazenado no laboratório de Mutagênese e

Oncogenética da Universidade Estadual de Londrina e somente poderão ter acesso a ele os pesquisadores responsáveis por esta pesquisa.

Sua identidade não será revelada e será mantido o caráter confidencial de todas as informações obtidas. Esclarecemos que o senhor a qualquer momento tem a liberdade de se recusar a contribuir com o estudo, sem ser prejudicado no seu tratamento e acompanhamento médico. Os resultados do estudo serão divulgados em congressos científicos e publicados em revistas especializadas, preservando sua identidade.

Esclarecemos que sua participação é voluntaria, não lhe trará nenhum gasto e que o senhor não terá quaisquer benefícios ou direitos financeiros sobre eventuais resultados desta pesquisa. Provavelmente os resultados desta pesquisa não trarão benefícios para a sua pessoa, mas poderão contribuir, no futuro, para uma melhora nos testes diagnósticos e prognósticos, assim como na conduta terapêutica para pacientes com CaP melhorando assim, a qualidade de vida destes pacientes.

No caso de autorizado, o senhor deverá assinar este Termo de Consentimento.

Os pesquisadores responsáveis por este estudo Ilce Mara de Syllos Cólus e Marilesia Ferreira de Souza, poderão ser contatados pelos telefones 3371-4608, 3371-4191, ou no endereço rodovia Celso Garcia Cid, Pr 445, Km 380, Campus Universitário, Centro de Ciências Biológicas, Bloco 11, Laboratório de Mutagenese e Oncogenética. Sempre que solicitados, estarão à sua disposição para esclarecimento de quaisquer questões relacionadas a esta pesquisa. O senhor também poderá entrar em contato com o Comitê de Ética em Pesquisa pelo telefone 3371-2490 ou pelo endereço Rua Robert Koch, 60 – Vila Operária ou pelo e-mail: cep268@uel.br.

Agradecemos-lhe a valiosa colaboração.

____
Prof^a^. Dr^a^. Ilce Mara de Syllos Cólus
Assinatura do pesquisador responsável

____
Marilesia Ferreira de Souza
Coletor/Entrevistador

6.5 Apêndice - Consentimento pós informado para pacientes

Eu,____, abaixo assinado, declaro que fui esclarecido sobre o objetivo do presente estudo sobre os eventuais desconfortos que poderei sofrer, assim como sobre os benefícios da pesquisa. Concordo, portanto, em participar na qualidade de voluntário, do referido Projeto de Pesquisa, sob livre e espontânea vontade, autorizando a coleta e o armazenamento de amostras de moléculas obtidas a partir dos meus fluídos (sangue) e tecidos para pesquisas futuras bem como os dados do questionário que respondi. Por ser expressão de verdade firmo o presente termo.

Nome do Voluntário:____

RG:____

Telefone: (_)_________-________________

Data ____ /____ /_______

Assinatura:____

6.6 Apêndice - Termo de consentimento livre e esclarecido para indivíduos controles.

Nós, Ilce Mara de Syllos Cólus e Marilesia Ferreira de Souza da Universidade Estadual de Londrina o convidamos para nossa pesquisa e solicitamos sua colaboração e o seu consentimento para incluí-lo em nosso projeto de pesquisa “Estudo comparativo do perfil transcricional e genotípico de genes relacionados ao CaP entre indivíduos sadios e portadores desta neoplasia para o desenvolvimento de assinaturas gênicas com fins diagnósticos, prognósticos e terapêuticos”. O objetivo deste estudo é avaliar alguns fatores genéticos que possam auxiliar no diagnóstico, prognóstico e na terapia de pacientes portadores de CaP.

O presente estudo terá dois grupos de indivíduos, um composto por pacientes com CaP e outro composto por pessoas sem histórico de câncer, denominados controles. Solicitamos a sua colaboração como voluntário neste projeto, como um dos membros do grupo denominado controles, ou seja, no grupo de pessoas livres de câncer.

Sua participação neste projeto é muito importante para nós, uma vez que necessitamos avaliar e comparar as semelhanças e diferenças entre estes dois grupos (pessoas sadias e pessoas com câncer). A partir desta comparação esperamos determinar quais são as características genéticas que predominam no grupo dos pacientes e quais são as mais frequentes no grupo dos indivíduos livres de câncer. Com isto, pretende-se contribuir para a melhora no prognóstico, diagnóstico e na conduta terapêutica para os pacientes com câncer.

Assim, solicitamos a sua autorização para que uma pequena quantidade de seu sangue (10 ml) seja coletada via punção venosa (picada na veia) com seringa e agulha descartáveis. Esclarecemos que não haverá desconforto físico adicional para a sua pessoa, além da picada da agulha.

Pedimos sua autorização para que moléculas (DNA, RNA ou proteínas) obtidas a partir da amostra de seu sangue possam ser armazenadas para estudos futuros no Laboratório de Mutagênese e Oncogenética da UEL, quando será solicitada nova autorização do Comitê de Ética em Pesquisa com Seres Humanos da UEL para a realização das pesquisas posteriores. O material obtido ficará armazenado no Laboratório de Mutagênese e Oncogenética da UEL, sob responsabilidade dos pesquisadores responsáveis por esta pesquisa. Esclarecemos ainda que a autorização para manutenção destas amostras é por prazo indeterminado, podendo ser cancelada por aviso escrito à responsável pelo Laboratório de Mutagênese e Oncogenética da Universidade Estadual de Londrina. Solicitamos-lhe o preenchimento de um questionário sobre seu estilo de vida, histórico de exposição ocupacional, onde o senhor será identificado apenas por um código, preservando sua identidade. Este questionário ficará armazenado no laboratório de Mutagênese e Oncogenética da Universidade Estadual de Londrina e somente poderão ter acesso a ele os pesquisadores responsáveis por esta pesquisa.

Sua identidade não será revelada e será mantido o caráter confidencial de todas as informações obtidas. Esclarecemos que o senhor a qualquer momento tem a liberdade de se recusar a contribuir com o estudo. Os resultados do estudo serão divulgados em congressos científicos e publicados em revistas especializadas, preservando sua identidade.

Esclarecemos que sua participação é voluntária e não lhe trará nenhum gasto e que o senhor não terá quaisquer benefícios ou direitos financeiros sobre eventuais resultados desta pesquisa. Provavelmente os resultados desta pesquisa não trarão benefícios para a sua pessoa, mas poderão contribuir, no futuro, para uma melhora nos testes diagnósticos e prognósticos, assim como na conduta terapêutica para pacientes com CaP, melhorando assim, a qualidade de vida destes pacientes.

Os pesquisadores responsáveis por este estudo Ilce Mara de Syllos Cólus e Marilesia Ferreira de Souza, poderão ser contatados pelos telefones 3371-4608, 3371-4191, 9648-1918 ou no endereço rodovia Celso Garcia Cid, Pr 445, Km 380, Campus Universitário, Centro de Ciências Biológicas, Bloco 11, Laboratório de Mutagenese e Oncogenética. Sempre que solicitados, estarão à sua disposição para esclarecimento de quaisquer questões relacionadas a esta pesquisa. O senhor também poderá entrar em contato com o Comitê de Ética em Pesquisa pelo telefone 3371-2490 ou pelo endereço Rua Robert Koch, 60 – Vila Operária ou pelo e-mail: cep268@uel.br.

No caso de autorizado, o senhor deverá assinar este Termo de Consentimento.

Agradecemos-lhe a valiosa colaboração.

______________________________	______________________________
Prof[a]. Dr[a]. Ilce Mara de Syllos Cólus	Marilesia Ferreira de Souza
Assinatura do pesquisador responsável	Coletor / Entrevistador

6.7 Apêndice - Consentimento pós-informado para indivíduos controles

Eu,

____, abaixo assinado, declaro que fui esclarecido sobre o objetivo do presente estudo sobre os eventuais desconfortos que poderei sofrer, assim como sobre os benefícios da pesquisa. Concordo, portanto, em participar na qualidade de voluntário, do referido Projeto de Pesquisa, sob livre e espontânea vontade, autorizando a coleta e o armazenamento de amostras de moléculas obtidas a partir dos meus fluídos (sangue) para pesquisas futuras bem como os dados do questionário que respondi. Por ser expressão de verdade firmo o presente termo.

Nome do Voluntário:

____

RG:____

Data ____ /____ /__________

Assinatura:____

6.8 Apêndice - Questionário pessoal

Por favor, leia as questões seguintes cuidadosamente e responda-as da forma mais completa e precisa possível.

A informação que você der não será associada a seu nome em nenhum documento público, e será conhecida somente dos principais pesquisadores deste estudo. As informações que você der podem ter influência direta na interpretação de nossos resultados, portanto, pedimos que coopere gentilmente, fornecendo informações corretas. Obrigado pelo interesse.

1- Nome: ____

último primeiro do meio

2- A ser preenchido pelo pesquisador:

Código:_________

Data: ___/___/___

Esta folha deve ser destacada do restante do questionário e preenchida pelo pesquisador. Somente o código será usado como identificação para as páginas subsequentes. Se for necessário espaço adicional para complementar a sua resposta, por favor escreva no verso da página e identifique a parte restante da questão com seu respectivo número.

Código n°____________

HISTÓRICO PESSOAL

1- Registro hospitalar: _______________________

2- Sexo: ( ) masculino ( ) feminino

3- Qual a cor da sua pele?

Negro ( ) Branco( ) Amarelo ( ) Outros ( )

4- Idade:________ Data de Nascimento:_____/_____/___________

5- Local de nascimento: Paraná ? ( ) SIM ( ) NÃO

Se NÃO: Que região brasileira ? Norte ( ) Sul ( ) Nordeste ( ) Centro-Oeste ( ) Sudeste ( )

6- Sua moradia é na zona rural ou urbana? ( ) Rural ( ) Urbana

7- Quanto tempo vive neste local? ______anos _______meses

8- Qual o seu grau de instrução?

( ) analfabeto ( ) 1° grau incompleto ( ) 1° grau completo ( ) 2° grau incompleto

( ) 2°grau completo ( ) técnico ( ) profissional ( ) superior

Histórico de exposição relacionado ou não ao trabalho

9- Você já se expôs a alguma destas substâncias abaixo em seu trabalho?

Se SIM, por quanto tempo e a quanto tempo foi isso:

Derivados de petróleo ( )sim ( )não

(querosene, gasolina, solventes,...)

Tintas/ corantes ( )sim ( )não

Indústrias têxteis ou tecelagem ( ) sim ( ) não

Praguicidas / Herbicidas ( )sim ( )não

Radiação ( )sim ( )não

Metais pesados (Pb, Ni, Cr,...) ( )sim ( )não

Processamento de madeira ( )sim ( )não

Papel ou celulose ( )sim ( )não

Mineração ( )sim ( ) não

Fábrica de sapatos ou curtume ( )sim ( )não

Metalúrgica ( )sim ( )não

Usina de açúcar ou álcool ( )sim ( )não

Plástico ou borracha ( )sim ( )não
Outras substâncias químicas ( )sim ( )não

10- Se SIM para a pergunta acima: Você utilizava equipamentos de proteção individual para trabalhar com essas substâncias químicas? (máscaras, luvas, óculos, etc.)
a) ( )sim b) ( )não

Histórico Tabagista

11- Você fuma atualmente? ( ) SIM ( ) NÃO

12- Se SIM, quanto você fuma por dia? ( ) menos de ½ maço
( ) de meio a 1 maço
( ) mais de um maço

13- Se SIM, há quanto tempo você fuma? ____________

14- Se NÃO, mas já fumou algum dia: há quanto tempo parou de fumar?
a) ( ) 0-5 anos b) ( ) 5-10 anos c) ( ) >10

15- Você convive/conviveu em seu trabalho ou em casa com pessoas que fumam?
a) ( ) SIM b) ( ) NÃO

Histórico de Etilismo

16- Você consome bebidas alcoólicas? ( ) SIM ( ) NÃO

17- Se SIM, que tipo de bebida alcoólica você costuma consumir?
a) ( )Destiladas b) ( )Não-Destiladas c) ( )Outra d) ( )Ambas

18-Quanto você costuma beber por semana?
( ) no máximo um copo ( ) de 2 a 5 copos ( ) de 6 a 10 ( ) mais de 10

19- Se já parou, há quanto tempo parou de consumir esta bebida?

a) ( ) 0-5 anos. b) ( ) 5 –10 anos. c) ( ) mais 10 anos.

20- Quanto você costumava beber por semana?

( ) no máximo um copo ( ) de 2 a 5 copos ( ) de 6 a 10 ( ) mais de 10

21- Durante a sua vida, já consumiu ou consome alguma bebida diariamente por mais de 6 meses continuamente?

( ) SIM ( ) NÃO

Histórico de Saúde

22- Nos últimos 12 anos você automedicou-se ou recebeu medicamentos?

( ) SIM ( ) NÃO ( )não sabe

Se SIM

( ) Hormônio

( ) Antinflamatório

( ) Analgésicos

( ) Antipertensivos

( ) Anabolizantes

( ) Outros

23- Você toma vitaminas ou tem tomado nos últimos seis meses?

( )SIM ( ) NÃO ( )não sabe

24 – Você já foi submetido a cirurgia nos testículos?

( ) SIM ( ) NÃO

Qual?____

25- Você foi tratado anteriormente de algum tipo de câncer? ( ) SIM ( ) NÃO

Qual? _________________________

26- Você tem antecedentes de câncer na sua família? ( ) SIM ( ) NÃO

27- Em casos de câncer na família, qual era o vínculo de parentesco?

( ) Pai ( ) Mãe ( ) Irmão ( ) Filho ( ) Tio ( ) Primo ( ) Outro

28- Qual foi a localização do tumor?

( ) Próstata ( ) Mama ( ) Bexiga ( ) Outro (qual?) ________________________________

29 – Você já teve alguma doença sexualmente transmissível?

( ) SIM ( ) NÃO

Se sim, qual?____________________________________

Histórico alimentar: (refira-se somente a hábitos frequentes)

30- Você segue uma dieta vegetariana? ( ) SIM ( )NÃO

31- Você come carne? ( ) SIM ( )NÃO

32- Se SIM, com que frequência você come estes alimentos:

Dias/Semana

	1-2	3-4	5-6	Diariamente
Carne de gado	( )	( )	( )	( )
Peixe	( )	( )	( )	( )
Frango	( )	( )	( )	( )
Porco	( )	( )	( )	( )
Outros	( )	( )	( )	( )

Histórico genético

33- Você possui algum irmão idêntico? ( ) SIM ( ) NÃO

7. ANEXO

7.1 Anexo – Aprovação pelo Comitê de Ética em Pesquisa com Seres Humanos da UEL

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	176/2013
CAAE:	19769913.0.0000.5231
Data da Relatoria:	19/09/2013
Pesquisador(a):	Ilce Mara de Syllos Cólus
Unidade/Órgão:	CCB - Departamento de Biologia Geral

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 466/12 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"Estudo comparativo do perfil transcricional e genotípico de genes relacionados ao câncer de próstata entre indivíduos sadios e portadores desta neoplasia para o desenvolvimento de assinaturas gênicas com fins diagnósticos, prognósticos e terapêuticos."

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL, via Plataforma Brasil, relatório final da pesquisa.

Londrina, 27 de setembro de 2013.

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina