

MiRNA downregulation dynamics
during acquisition of enzalutamide
resistance: Implications for early
detection and RNA-based
therapeutic approaches

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MIRNA DOWNREGULATION DYNAMICS DURING ACQUISITION OF ENZALUTAMIDE RESISTANCE: IMPLICATIONS FOR EARLY DETECTION AND RNA- BASED THERAPEUTIC APPROACH

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Abbreviations

#

2D – Two dimensional

3D - Three-dimensional

nm – nanometer

nM - nanomolar

μL - microliters

μM – micrometers

A

Abs - Absorbance

ADT - Androgen Deprivation Therapy

AGO2 - Argonaute 2

AKT - AKT serine/threonine kinase

AMBRA1 - Autophagy and Beclin 1
Regulator 1

AR - Androgen Receptor

AREs - Androgen response elements

ATCC - American Type Culture Collection

ATP - Adenosine Triphosphate

C

cDNA - complementary Deoxyribonucleic
Acid

CDH1 - Cadherin-1

CDK - cyclin-dependent kinase

CLL – Chronic Lymphocytic leukemia

c-MET – Mesenchymal-epithelial transition
factor

CO₂ - Dioxide Carbon

CRPC - Castration resistant prostate cancer

D

DBD - DNA-binding domain

DGCR8 - Double stranded RNA binding
proteinDiGeorge syndrome critical region

DHT - Dihydrotestosterone

DNA - Deoxyribonucleic Acid

DPBS - Dulbecco's Phosphate-Buffered
Saline

DROSHA - Double stranded RNase III
enzyme

E

EAU - European Association of Urology

EDTA - Ethylenediamine tetraacetic acid

EGFR - Epidermal Growth Factor Receptor

ELISA - Enzyme-Linked Immunosorbent
Assay

ENZ – Enzalutamide

ESCRT – Endosomal sorting complexes
required for transport

EVs – Extracellular vesicles

EMT – Epithelial-Mesenchymal Transition.

ERK – Extracellular signal-Regulated
Kinase

E2F1 - E2F Transcription Factor 1

F

FBS - Fetal Bovine Serum

FDA - Food and Drug Administration

FBXO31 - F-box only protein 31

G

GAPDH-Glyceraldehyde-3-Phosphate
Dehydrogenase

g – grams

H

HCN2 - Hyperpolarization Activated Cyclic
Nucleotide Gated Potassium and Sodium
Channel 2

HCN4 - Hyperpolarization Activated Cyclic
Nucleotide Gated Potassium Channel 4

HDI - Human Development Index

HMGA2 - High-mobility group AT-hook 2

HSP - heat shock protein

I

IARC - International Agency for Research
on Cancer

ISUP - International Society of Urological
Pathology

IL-8 – Interleukin-8

L

LBD - Ligand-binding domain

LH - Luteinizing hormone

LHRH – Luteinizing hormone-releasing
hormone

M

miRNA – microRNA

MAPK - Mitogen-Activated Protein Kinase

MISEV – Minimal information for studies
of extracellular vesicles

MKNK2 - MAPK Interacting
Serine/Threonine Kinase 2

mRNA - messenger Ribonucleic Acid

mTOR - mammalian Target of Rapamycin

MVBs - Multivesicular bodies

MFN2 - Mitofusin 2

N

NTA - Nanoparticle Tracking Analysis

NCDs – Noncommunicable diseases

NaCl - sodium chloride

NTD - N-terminal domain

O

O₂-Oxygen

OFR – Open Reading frame

P

PCa - Prostate cancer

PCR – Polymerase Chain Reaction

PIN - Prostatic intraepithelial neoplasia

PMPMEase - Polyisoprenylated methylated protein methyl esterase

Pre-miRNA - precursor-microRNA

Pri-miRNA - primary-microRNA

PSA - Prostate-specific antigen

PTEN - Phosphatase and Tensin Homolog

PI3K - Phosphoinositide 3-kinase

Q

qPCR - quantitative Polymerase Chain Reaction

R

RISC - RNA-induced silencing complex

RNA - Ribonucleic Acid RPMI - Roswell Park Memorial Institute

S

SMAD7 - Suppressor of Mothers against Decapentaplegic 7

T

T - Testosterone

TEM - Transmission Electron Microscopy

TME- Tumor Microenvironment

TRBP - Transactivation Responsive RNA-Binding Protein

TWF1 - Twinfilin Actin Binding Protein 1

TGF- β - Transforming Growth Factor-beta

TGFBR2 - Transforming Growth Factor Beta Receptor Type 2

U

UTR - Untranslated Region

V

VEGFA - Vascular Endothelial Growth Factor A

W

WHO – World Health Organization

X

XPO5 - Exportin 5

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Abstract

Recently, extracellular vesicles (EVs) have gained significant attention in oncology due to their role as essential vehicles in the cellular communication network established within the tumor microenvironment (TME). These small lipid membrane vesicles can promote the acquisition of therapy resistance and enhance the formation of pre-metastatic niches, which are crucial for subsequent tumor dissemination. It is now accepted that all cells can produce EVs, which may contain various biomolecules (DNA, RNA, microRNAs, etc.) that can be released when in contact with other cells, altering them through the transfer of information, whether under physiological or pathological conditions, such as in prostate cancer (PCa). Furthermore, the structure of EVs makes them stable in circulation, increasing their potential for analysis via liquid biopsy.

Androgen deprivation therapy (ADT) is frequently used in advanced and metastatic PCa due to its high dependency on androgens. However, the majority of patients eventually develop resistance to this form of treatment, progressing to castration-resistant prostate cancer (CRPC). Enzalutamide (ENZ) was developed to overcome this limitation, however, a significant percentage of patients also develop resistance to this new drug, and 15-20% of patients exhibit primary resistance, with no effective therapeutic option currently available for ENZ-resistant PCa. Therefore, it is crucial to identify predictive biomarkers for the acquisition of ENZ resistance. In this study, we aimed to identify EV-derived miRNAs associated with the acquisition of ENZ resistance, with the goal of identifying resistance biomarkers that could be applied during patient follow-up, facilitating the early detection of clinical resistance acquisition and thus promoting the translation of basic research into clinical practice. Furthermore, this work could open new opportunities for the development of innovative RNA-based therapies, focusing on restoring normal miRNA levels.

In the past, an *in vitro* model of ENZ resistance, named LNCaPER, was developed through the continuous and gradual exposure of LNCaP cells to increasing concentrations of ENZ (2.5 μ M, 5 μ M, 10 μ M, 20 μ M, and 30 μ M). The miRNA expression profile of LNCaP cells versus LNCaPER cells was compared revealing the overexpression of 12 miRNAs and the under expression of 13 miRNAs in LNCaPER cells. Among the under expressed miRNAs, the 6 with the highest number of validated targets were selected for the present study (hsa-miR-15b-5p, hsa-miR-19a-3p, hsa-miR-20a-5p + hsa-miR-20b-5p, hsa-miR-25-3p, hsa-miR-93-5p) to understand their deregulation dynamics throughout the development of ENZ resistance up to 10 μ M.

The resistance phenotype was monitored by assessing metabolic activity and relative quantification of the mRNA expression levels of the androgen receptor (AR) and prostate-

specific antigen (PSA), showing that this cell line shows an independent AR mechanism in establishing ENZ resistance. *In silico* analysis revealed that *VEGFA* and *SMAD7* are common targets of several miRNAs in this study, implicating them in the underlying mechanisms of ENZ resistance acquisition. Interestingly, the expression dynamics of these target genes varied significantly between 2D and 3D culture models, possibly due to the distinct physical and structural conditions of each culture type.

In fact, since 3D models more faithfully mimic the TME, they provide more accurate evidence of the mechanisms associated with the acquisition of therapy resistance, including ENZ. Therefore, we established 3D spheroid *in vitro* models for LNCaP cells and ENZ-resistant LNCaP cells. Regarding the quantification of selected miRNA expression, we observed that the expression patterns of these miRNAs differed significantly between 2D and 3D models, with certain miRNAs exhibiting altered regulatory behavior in 3D models. This highlights the importance of 3D models in revealing the subtle behavior of miRNAs in a more physiologically representative tumor environment. Additionally, in 3D models, we also observed an increasing trend in intracellular miRNA expression associated with the lowest concentration of ENZ, suggesting an acute effect of the drug.

Among the studied miRNAs, EV-derived hsa-miR-20a-5p (LNCaP 2.5 μ M: fold-decrease 0.26, $p < 0.021$, LNCaP 5 μ M: fold-decrease 0.12, $p < 0.002$, LNCaP 10 μ M: fold-decrease 0.14, $p = 0.001$), hsa-miR-20b-5p (LNCaP 10 μ M: fold-decrease 0.04, $p < 0.001$), and hsa-miR-25-3p (LNCaP 2.5 μ M: fold-decrease 0.22, $p = 0.017$) stood out by showing consistently downregulated profiles in ENZ-resistant cells.

Based on these results, hsa-miR-20a-5p and hsa-miR-25-3p were selected for transfection assays. Transfection was successful, as evidenced by increased miRNA expression levels and decreased expression of their target genes, *VEGFA* and *SMAD7*, after administration of their mimics. Interestingly, we observed a significant reduction in spheroid diameter and decreased metabolic activity compared to the negative control, after transfection, which suggests that the increased expression of these miRNAs, induced by the mimics, led to reduced cell proliferation, which may be associated to a reversion of the aggressive phenotype associated with ENZ resistance.

The results suggest that hsa-miR-20a-5p and hsa-miR-25-3p are involved in the development of ENZ resistance through their incorporation into EVs, which may influence the modification of the TME. Therefore, these miRNAs demonstrate potential as liquid biopsy biomarkers for predicting ENZ resistance acquisition.

Moreover, hsa-miR-20a-5p and hsa-miR-25-3p emerge as promising therapeutic targets, as restoring their expression levels significantly reduced pro-tumorigenic properties. The

exploration of new therapeutic approaches, such as miRNA mimic administration, is a crucial need in the fight against CRPC, offering the possibility of improving patient survival and quality of life.

In the future, it would be important to establish 3D spheroid *in vitro* models with the remaining ENZ-resistant concentrations model (LNCaP 20 μ M, LNCaP 30 μ M) and analyze the expression dynamics of miRNAs and VEGFA and SMAD7 molecules to enhance the predictive capacity of the biomarkers. Moreover, different targets common to the most promising miRNAs should also be quantified to better understand which mechanisms are behind the establishment of ENZ resistance. Additionally, it would be relevant to study the miRNA profile in CRPC patients undergoing ENZ treatment until resistance acquisition and explore the co-transfection of hsa-miR-20a-5p, hsa-miR-20b-5p, and hsa-miR-25-3p, which have shown great potential as predictive biomarkers for ENZ resistance acquisition. Advanced phenotypic and molecular changes after transfection are needed to better understand a potential reversal of enzalutamide resistance.

Resumo

Recentemente as vesículas extracelulares (VEs) têm ganho grande destaque em oncologia dado serem veículos essenciais na rede de comunicação celular estabelecida no microambiente tumoral (MT). Estas pequenas vesículas de membrana lipídica podem promover a aquisição de resistência à terapia e potenciar a formação de nichos pré-metastáticos essenciais para a posterior disseminação tumoral. Atualmente admite-se que todas as células podem produzir VEs, podendo estas conter no seu interior diferentes biomoléculas (DNA, RNA, microRNAs...) que podem ser libertadas quando em contacto com outras células, modificando-as, ocorrendo esta transferência de informação, quer em condições fisiológicas ou patológicas, como é o caso do cancro da próstata (CP). Para além disso, a estrutura das VEs torna-as estáveis em circulação, aumentando o seu potencial de análise através de biópsia líquida. A terapia de bloqueio androgénico (TBA) é frequentemente utilizada no CP avançado e metastático, dada a sua alta dependência dos androgénios. Contudo, a grande maioria dos doentes acaba por desenvolver resistência a esta forma de tratamento, progredindo para CP resistente a castração (CPRC). A enzalutamida (ENZ) foi desenvolvida de forma a ultrapassar esta limitação, no entanto, uma elevada percentagem de doentes acaba também por desenvolver resistência a este novo fármaco e 15-20% dos doentes apresenta resistência primária, não existindo uma opção terapêutica efetiva para o CP resistente à ENZ. Assim, é crucial a identificação de biomarcadores preditivos de aquisição de resistência à ENZ. Deste modo, neste estudo, pretendemos identificar miRNAs derivados de VEs associados à aquisição de resistência à ENZ, com o objetivo de identificar biomarcadores de resistência que possam ser aplicados durante o follow-up dos doentes, facilitando a identificação precoce de aquisição de resistência clínica, potenciando assim uma translação da investigação básica para a prática clínica. Além disso, este trabalho poderá abrir novas oportunidades para o desenvolvimento de terapias inovadoras baseadas em RNA, com foco na restauração dos níveis normais de miRNAs.

Anteriormente, um modelo *in vitro* da resistência à ENZ, denominado LNCaPER, foi desenvolvido através da exposição contínua e gradual de células LNCaP a concentrações crescentes de ENZ (2.5 μ M, 5 μ M, 10 μ M, 20 μ M e 30 μ M). O perfil de expressão de miRNAs das células LNCaP versus LNCaPER foi comparado, tendo-se verificado que a linha celular LNCaPER apresentava a sobreexpressão de 12 miRNAs e a subexpressão de 13 miRNAs. Dos miRNAs subexpressos, os 6 com o maior número de alvos validados foram selecionados para o presente estudo (hsa-miR-15b-5p, hsa-miR-19a-3p, hsa-miR-20a-5p+hsa-miR-20b-5p, hsa-miR-25-3p, hsa-miR-93-5p) para perceber a sua dinâmica de desregulação ao longo do processo de desenvolvimento de resistência à ENZ até 10 μ M.

A monitorização do fenótipo de resistência foi efetuada por avaliação da atividade metabólica e quantificação relativa dos níveis de expressão de mRNA do recetor de androgénios (RA) e do antigénio específico da próstata (PSA), demonstrando que esta linha celular apresenta um mecanismo independente do AR no estabelecimento da resistência à ENZ.

A análise *in silico* revelou que o VEGFA e o SMAD7 são alvos comuns a um maior número de miRNAs em estudo, implicando-os nos mecanismos subjacentes à aquisição de resistência à ENZ. Interessantemente, a dinâmica de expressão destes genes alvo variou significativamente entre os modelos de cultura 2D e 3D, possivelmente devido às condições físicas e estruturais distintas de cada tipo de cultura.

De facto, uma vez que os modelos 3D mimetizam mais fielmente o MT, eles fornecem evidências mais precisas dos mecanismos associados à aquisição de resistência a terapias, incluindo a ENZ. Assim sendo, estabelecemos modelos *in vitro* 3D do tipo esferoide para as células LNCaP e para as células LNCaP ENZ-resistentes.

Relativamente à quantificação da expressão dos miRNAs selecionados observamos que os padrões de expressão desses miRNAs diferiram significativamente entre os modelos 2D e 3D, com certos miRNAs a exibir um comportamento regulatório alterado nos modelos 3D. Isto destaca a importância dos modelos 3D em revelar o comportamento subtil dos miRNAs num ambiente tumoral mais representativo do ponto de vista fisiológico. Adicionalmente, em modelos 3D, foi ainda possível observar, associado à concentração mais baixa de ENZ, uma tendência de aumento da expressão dos níveis intracelulares do perfil de miRNAs, o que sugere um efeito agudo do fármaco.

Entre os miRNAs estudados, os hsa-miR-20a-5p (LNCaP 2.5 μ M: fold-decrease 0.26, $p < 0.021$, LNCaP 5 μ M: fold-decrease 0.12, $p < 0.002$, LNCaP 10 μ M: fold-decrease 0.14, $p = 0.001$), hsa-miR-20b-5p (LNCaP 10 μ M: fold-decrease 0.04, $p < 0.001$) e hsa-miR-25-3p (LNCaP 2.5 μ M: fold-decrease 0.22, $p = 0.017$) derivados de VEs destacaram-se por apresentarem perfis consistentemente subexpressos em células ENZ-resistentes.

Com base nos resultados mencionados, o hsa-miR-20a-5p e o hsa-miR-25-3p foram os miRNAs selecionados para o ensaio de transfeção. A transfeção foi bem-sucedida, como evidenciado pelo aumento dos níveis de expressão dos miRNAs, juntamente com a diminuição da expressão dos seus genes alvo, VEGFA e SMAD7, após a administração dos seus mímicos. Interessantemente, observamos uma redução significativa no diâmetro dos esferoides e uma diminuição da atividade metabólica em comparação com o controlo negativo, após a transfeção, o que sugere que o aumento da expressão desses miRNAs,

induzido pela administração de mímicos, levou à redução da proliferação celular, o que pode estar associado a uma reversão do fenótipo agressivo associado à resistência à ENZ.

Os resultados obtidos sugerem que o hsa-miR-20a-5p e o hsa-miR-25-3p estão envolvidos no processo de desenvolvimento de resistência à ENZ, através da sua incorporação em VEs, o que pode influenciar a modificação do MT. Assim sendo, estes miRNAs demonstram potencial enquanto biomarcadores de biópsia líquida preditivos de aquisição de resistência à ENZ.

Os miRNAs hsa-miR-20a-5p e hsa-miR-25-3p emergem como potenciais alvos terapêuticos promissores, uma vez que a restauração dos seus níveis de expressão demonstrou uma redução significativa nas propriedades pró-tumorigênicas. A exploração de novas abordagens terapêuticas, como a administração de mímicos de miRNA, revela-se uma necessidade crucial no combate ao CRPC, oferecendo a possibilidade de melhorar a sobrevivência e a qualidade de vida dos pacientes.

Futuramente seria importante estabelecerem-se modelos *in vitro* 3D do tipo esferoide com as concentrações restantes de resistência à ENZ (LNCaP 20 μ M, LNCaP 30 μ M) e analisar a dinâmica de expressão dos miRNAs e das moléculas VEGFA e SMAD7 de forma a aprimorar a capacidade preditiva dos biomarcadores. Também seria útil quantificar diferentes targets comuns aos miRNAs mais promissores de forma a melhor entender os mecanismos responsáveis pela aquisição de resistência à ENZ. Adicionalmente, seria relevante estudar o perfil dos miRNAs em pacientes com CRPC em tratamento com ENZ e explorar a co-transfecção de hsa-miR-20a-5p, hsa-miR-20b-5p e hsa-miR-25-3p, que mostraram grande potencial enquanto biomarcadores preditivos de aquisição de resistência à ENZ. São ainda necessários realizar estudos fenotípicos e moleculares avançados após a transfecção, para compreender melhor uma possível reversão da resistência à ENZ.

Introduction

1. Introduction

1.1. Cancer: general concepts

Cancer is a major social, public health and economic issue in the 21st century, accounting for nearly one in six deaths and one in four deaths from noncommunicable diseases (NCDs) worldwide [1].

In 2022, there were an estimated 20 million new cancer cases (Figure 1A) and 9.7 million deaths (Figure 1B) worldwide [2]. Over the course of this century, cancer is expected to become the global leading cause of premature death and the main obstacle to increasing average life expectancy [3]. However, the impact of different types of cancer is unevenly distributed across the global population. Disparities in incidence and mortality rates are largely influenced by socioeconomic status, ethnicity and lifestyle factors [3, 4].

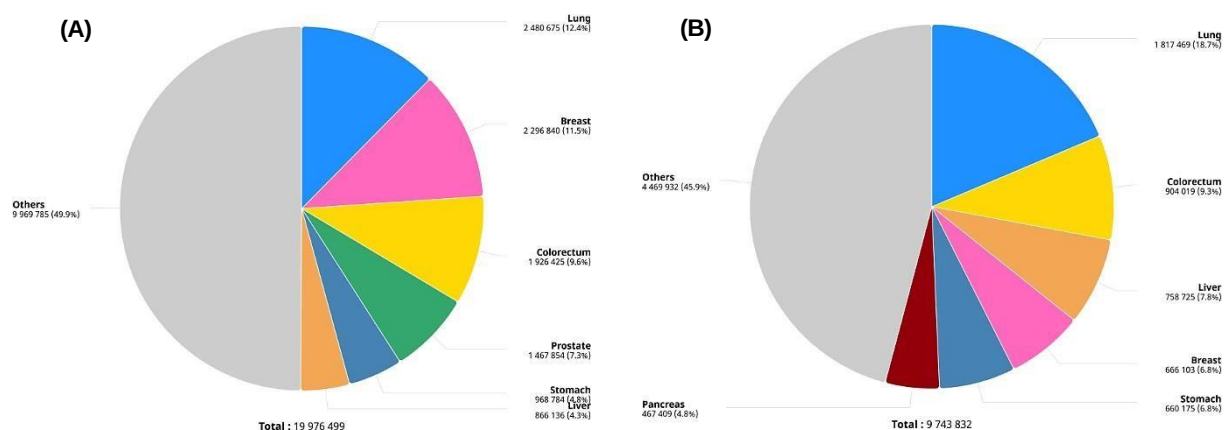


Figure 1- Cancer incidence **(A)** and cancer mortality **(B)** worldwide in both sexes and considering all ages (adapted from GLOBOCAN 2022, IARC) [2].

As the Human Development Index (HDI) rises, incidence and mortality rates for most cancers also tend to rise. This can be explained by the easy access to health care, which allows for screening programs and early detection, such as the prostate specific antigen (PSA) test, which allows for an early diagnosis of prostate cancer (PCa), and in some cases, it could remain undetectable due to the absence of symptoms [5]. The prevalence of certain risk factors, such as obesity, alcohol and tobacco consumption and an increase in average life expectancy, may also be associated with the large annual increase in the number of new cases [6-8].

Asia, home to approximately 60% of the world's population, accounts for nearly half of all new cases and the majority of cancer deaths in 2022 [2]. The burden of cancer mortality in

Africa and Asia is disproportionately higher than the incidence rates. This is partly due to the distribution of certain types of cancer in these regions, such as lung and breast cancer coupled with higher death rates, often due to late diagnosis [2]. Europe, with less than 10% of the world's population, has a relatively higher burden, accounting for 22.4% of global cancer cases and 20.4% of cancer deaths [2].

Although the absolute differences in the projected cancer burden in 2050 are greatest in high and very high HDI countries, the largest relative increases will occur in low HDI countries, largely due to factors such as population growth, aging and the increasing prevalence of cancer risk factors such as smoking and poor diet, as well as limited access to health care and preventive measures. [9]. The widespread impact of cancer and the diversity of cancer types across regions and levels of development highlight the urgent need for global cancer control efforts. By investing in prevention and addressing key risk factors, we can avoid millions of future cancer cases and save lives, resulting in significant economic and social benefits for countries in the future.

Cancer is a genetic disease that occurs due to the accumulation of genetic and epigenetic alterations that lead to the deregulation of pathways involved in the processes of cell growth, division, differentiation and metabolism, giving altered cells a selective advantage [10, 11]. Most genetic and epigenetic alterations occur in the genome of somatic cells, and their accumulation leads to the development of cancer, forming the basis of its progression [12, 13]. Genetic alterations are induced by ageing, mutagenic chemicals and ultraviolet light, among others [13]. On the other hand, epigenetic changes are mainly induced by ageing and chronic inflammation [13]. Cancer can also be defined as a group of diseases characterized by the abnormal and uncontrolled proliferation of genetically altered cells [12]. Moreover, it is a heterogeneous disease with multiple etiologies and development patterns [13].

The process of cancer development, known as carcinogenesis, is multifactorial and multiphasic and can be divided into 3 main stages: initiation, promotion and progression [14]. The initiation phase results from alterations in the cells genome, resulting from the action of one or a combination of chemical, physical or biological agents that damage the cell's DNA. If this damage remains unrepaired, it can result in permanent genetic changes in the cell's DNA [14]. The promotion phase does not involve molecular alterations to the DNA structure, it involves epigenetic alterations [16]. At this stage, the genetically altered cells suffer the effects of oncopromoters, which enhance their proliferative capacities. These cells then slowly and gradually start to manifest the characteristics of malignant cells [14]. The third and final stage, progression, is characterized by the uncontrolled and irreversible

multiplication of genetically altered cells [14]. This leads to the development of a malignant tumor composed of different malignant cell subclones, each defined by distinct genetic and chromosomal mutation patterns. These cells are genetically unstable and become increasingly aggressive as genetic and epigenetic alterations accumulate [15].

Two main classes of genes are involved in cancer development: oncogenes and tumor suppressor genes [12]. Oncogenes, which results from alterations such as chromosomal translocations, amplifications, and mutations in proto-oncogenes, are associated with the stimulation of cell growth, division, and survival [16]. In neoplastic situations, specific mutations in proto-oncogenes cause them to become constitutively active, leading to excessive cell proliferation [12]. It is only necessary that these alterations occur in one of the alleles, to change the cellular behavior [17]. On the other hand, tumor suppressor genes encode proteins that regulate the cell cycle and inhibit cell proliferation [12]. In neoplastic conditions, the activity of these genes is reduced [18]. Unlike oncogenes, for a change in cellular behavior to occur, both alleles must be mutated in tumor suppressor genes [12]. Mutations in oncogenes and tumor suppressor genes have a similar biological effect: they promote the neoplastic process by increasing the number of cancerous cells, stimulating cell proliferation, or inhibiting apoptosis [18].

During the process of carcinogenesis, neoplastic cells acquire new phenotypic traits that give them a selective advantage over normal cells. In 2000, Hanahan and Weinberg proposed a set of functional capabilities acquired by tumor cells, which they termed the "hallmarks of cancer" [19]. These hallmarks include: self-sufficiency in growth signals, evasion of apoptosis, unlimited replicative potential, promotion of angiogenesis, the ability to invade surrounding tissues and metastasize [20].

Later, in 2011, the researchers added four new hallmarks, considering the significant role of the tumor microenvironment (TME) in tumor progression, as well as the metabolic and genetic alterations undergone by cells during carcinogenesis: genomic instability, promotion of inflammation, immune system evasion, and metabolic reprogramming [21].

Very recently, in 2022, Hanahan and Weinberg proposed new hallmarks of cancer. The authors believed that the previously proposed hallmarks alone could not fully explain the precise molecular and cellular mechanisms that enable initiated cells to develop and acquire these aberrant phenotypic capabilities throughout tumor development and malignant progression [19]. Thus, Hanahan and Weinberg proposed four new hallmarks: phenotypic plasticity unlock, polymorphic microbiome, non-mutant epigenetic reprogramming, and cellular senescence (Figure 2) [19].

The crucial role of the TME in tumor development and progression has been recognized by the authors as it was integrated into the hallmarks of cancer. Tumors grow within a challenging and complex tumor microenvironment, where interactions between cells happen through various signaling networks that are crucial for tumor progression and metastasis [22].

Indeed, significant advances have been made in recent years in our understanding of the components of the TME and their interactions. Recent findings indicate that extracellular vesicles (EVs) are crucial in reshaping the TME. EVs from tumor cells facilitate communication at both local and distant locations, influencing the outcome of the tumor and the effectiveness of antitumor therapies [23].

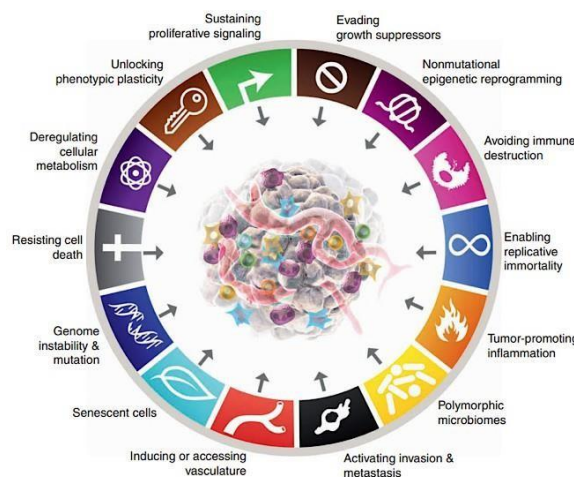


Figure 2 - Hallmarks of cancer: new additions (adapted from 'Hallmarks of Cancer: New Dimensions' [19]).

1.2. Extracellular Vesicles: Key Mediators of Intercellular Communication in the Tumor Microenvironment

The last few decades have shown that the malignant properties and progression of tumors are not determined only by cancer cells, being the TME an important ecosystems to support cancer progression [24]. The area surrounding the tumor, known as the TME, consists of various non-malignant cell types, including fibroblasts, lymphocytes, inflammatory cells, endothelial cells, and adipose tissue with the ability to interact with each other [24]. In the early stages of tumorigenesis, the microenvironment exhibits anti-tumor immunity and controls tumor growth [25]. However, as the tumor progresses, the microenvironment's role shifts to supporting tumor development [25]. These cells within the TME and cancer cells interact, forming intrinsic communication networks that influence several cancer hallmarks, as described by Hanahan and Weinberg [21]. Numerous studies have shown that

intercellular communication is regulated by several humoral factors, including cytokines, chemokines and growth factors [25]. Furthermore, recent advances in cancer biology have highlighted that extracellular vesicles (EVs) also play a key role in regulating this communication [26].

EVs are highly heterogeneous membrane-enclosed vesicles secreted by nearly all cell types [23]. Different cell types or even a single cell type can produce different populations of EVs under normal conditions or different stimulations [27]. The similar appearance, overlapping size distributions and complicated cargoes of EV subpopulations complicate current efforts to identify specific markers of EV subtypes and to establish an accurate EV nomenclature [27].

Typically, EVs are classified into exosomes, microvesicles (or ectosomes), and apoptotic bodies [28] (Figure 3). These vesicles originate from a variety of subcellular sources [28]. Exosomes, small membrane-bound vesicles measuring 50 to 150 nm in diameter, are formed by exocytosis of multivesicular bodies (MVBs) containing intraluminal vesicles [29]. Their production and release is controlled by the endosomal sorting complexes required for transport (ESCRT) machinery in a ceramide-dependent pathway [29]. In EVs biology, researchers have identified various exosome markers, such as tetraspanins like CD63, membrane transporters, heat shock proteins, and proteins involved in MVB synthesis, as well fusion proteins [30]. Microvesicles, which are larger (100 -1000 nm), are produced directly from the plasma membrane by a budding process and are characterized by an enrichment in certain lipids and phosphatidylserine [31]. The formation of microvesicles is driven by the redistribution of phospholipids and the contraction of cytoskeletal structures [32]. On the other hand, apoptotic bodies, which are the largest (500-4000 nm), are produced during apoptosis and contain cellular organelles, nuclear fragments, DNA and RNA [33].

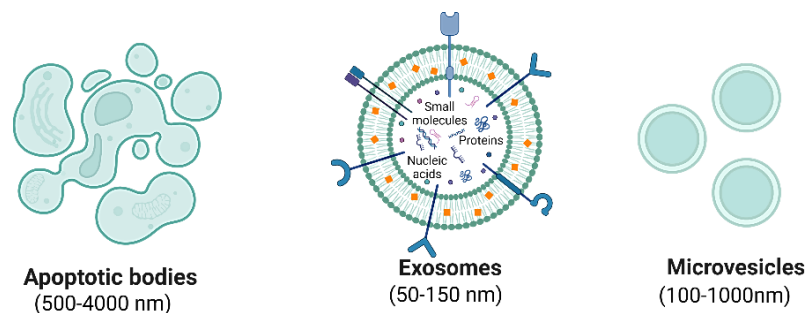


Figure 3 - Illustration of the main classes of extracellular vesicles (created with BioRender, according to Minimal information for studies of extracellular vesicles (MISEV) [34]).

EVs transport bioactive molecular cargoes such as proteins, lipids and nucleic acids (including DNA, mRNA fragments, miRNA, small nucleolar RNA, Y RNA, mitochondrial RNA and other non-coding RNAs) that reflect the phenotype of their parental cells [34]. These

cargoes can modulate the functions and characteristics of recipient cells by altering gene expression through *de novo* protein synthesis, post-translational modification of target mRNAs, or by triggering various signaling pathways [35]. EVs and their cargo can be transferred to recipient cells by a variety of mechanisms. They can attach to the plasma membrane of the target cell, fuse directly with it or be internalized by processes such as phagocytosis, macropinocytosis and different forms of endocytosis [35, 36]. Once inside, EVs can be degraded in lysosomes or fuse with endocytic membranes, releasing their contents into the cytosol of recipient cells [35, 36].

As potent mediators of local and systemic intercellular communication in the TME, EVs have gained significant attention, particularly in cancer research [37]. There is increasing evidence that EVs play a critical role in tumor dissemination, including in the establishment of a pre-metastatic niche, in the promotion of angiogenesis, in the disruption of the peritoneum or blood-brain barrier, and in the induction of drug resistance, which is a huge hurdle in the treatment of cancer [24, 38-41]. As a result, intercellular communication mediated by EVs in the TME is now considered a promising therapeutic target [42].

Furthermore, EVs are found in various human body fluids, including blood, urine, saliva and breast milk [43-46]. They are known for their inherent properties such as biocompatibility, ability to cross biological barriers, low toxicity and minimal immunogenicity [47]. Due to these unique characteristics, EVs are considered promising clinical biomarkers and nanocarriers with potential for non-invasive early diagnosis, disease monitoring and treatment [23]. Importantly, the microRNAs (miRNAs) carried by EVs have been recognized as key biomarkers due to their stability in body fluids and their ability to reflect the pathological conditions of their cells of origin [48]. This makes miRNAs within EVs particularly valuable for early disease detection, prognostic assessment and monitoring of therapeutic response, further reinforcing the critical role of EVs in advancing precision medicine [48].

1.3 MicroRNAs

More than 30 years ago, the first microRNA (miRNA) was discovered in *Caenorhabditis elegans* with the developmental regulator *lin-4*, which did not encode a protein but a 22-nucleotide regulatory RNA [49, 50]. Subsequently, the discovery of *let-7*, a miRNA conserved in several organisms, highlighted the importance of these RNAs [51]. To date, more than 30,000 miRNAs have been identified in 206 species, of which 2,588 are present in the human genome and regulate approximately one third of human genes [52].

MiRNAs are short, single-stranded RNA molecules, typically 18 to 25 nucleotides in length. They act as key regulators of gene expression by controlling post-transcriptional gene regulation in various organisms [53]. Each miRNA can control the expression of numerous target genes, highlighting the importance of the miRNA pathway in gene regulation [54]. The level of complementarity between a miRNA and its target gene determines the type of gene suppression. Full complementarity typically results in mRNA cleavage, whereas partial complementarity results in translational inhibition [55].

MiRNA production involves a series of steps beginning with transcription in the nucleus and ending with processing and maturation in the cytoplasm [56, 57]. RNA polymerases II or III first transcribe primary miRNA transcripts (pri-miRNAs), which have a hairpin-like structure containing the sequence for the mature miRNA (Figure 4 (1)) [56, 57]. The microprocessor complex, which includes the RNase III enzyme Drosha and the DGCR8 protein, identifies and cleaves these pri-miRNAs, producing precursor miRNAs (pre-miRNAs) with a hairpin shape (Figure 4 (2)) [56, 57]. These pre-miRNAs are then transported to the cytoplasm by exportin 5 (XPO5) (Figure 4 (3)) [56, 57].

In the cytoplasm, the RNase III endonuclease Dicer further processes the pre-miRNAs by recognizing specific features at their 5' end and the 3' overhang near the terminal loop, resulting in a short double-stranded RNA duplex (Figure 4 (4)) [56, 57]. This duplex consists of the mature miRNA strand (the guide strand) and its complementary strand (miRNA*) [56, 57]. Dicer, assisted by the transactivation-responsive RNA-binding protein (TRBP), enhances its cleavage activity through helicase interaction [56, 57]. TRBP also facilitates the binding of Dicer to Argonaute proteins (AGO2), which promotes the formation of the miRNA-induced silencing complex (RISC) (Figure 4 (5)).

Within the RISC complex, the leader strand is retained while the passenger strand is typically degraded [56, 57]. The mature miRNA within the RISC then binds to target mRNA molecules via complementary base pairing (Figure 4 (6)) [56, 57]. Depending on how well the miRNA matches its target mRNA, it can either cause mRNA degradation or inhibit its translation (Figure 4 (7)). High complementarity generally leads to mRNA degradation, whereas partial complementarity leads to translational repression without mRNA degradation [56, 57] (Figure 4).

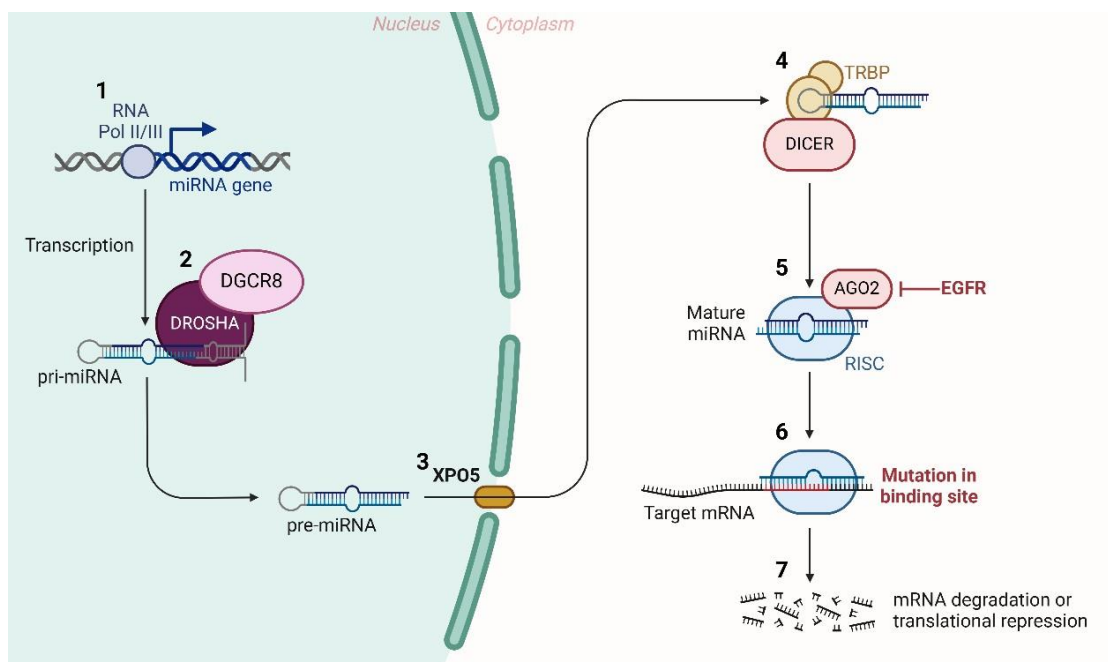


Figure 4 - MiRNA biogenesis and mRNA translation repression (Adapted from Biorender).

MiRNAs are known to interact with different regions of mRNA, including the open reading frame (ORF), the 5' untranslated region (UTR) and gene promoters [58]. They play critical roles in a wide range of cellular processes including development, differentiation, metabolism, proliferation, invasion, migration, apoptosis and maintenance of genomic stability [54, 59]. Regulation occurs through specific base pairing with target transcripts and involves both transcriptional and post-transcriptional mechanisms [58]. MicroRNAs play essential roles in a wide variety of cellular processes, ensuring that gene expression is carefully modulated to maintain cellular homeostasis [60]. Given their profound influence on gene regulation, it is not surprising that deregulation of miRNA expression or function can have significant consequences. Indeed, over the last decades, deregulation of miRNAs expression has been observed in diverse human diseases such as metabolic diseases and cancer [61, 62].

1.3.1 Deregulated miRNAs in cancer

The deregulation of miRNAs in cancer can be due to epigenetic changes, alterations in miRNA biogenesis, polymorphisms or mutations in the sequences that encode for these miRNAs or chromosomal abnormalities. These factors can also occur together, leading to aberrant expression of specific miRNAs in cancer cells [63, 64]. The first identification of a cancer linked to miRNA was documented in 2002 [65]. Calin and colleagues discovered

that the miR-15 and miR-16 coding sequences, which are often deleted or downregulated in chronic lymphocytic leukemia (CLL), are in a critical 13q14 chromosomal region [65]. This loss contributes to CLL by increasing levels of Bcl-2 protein, an anti-apoptotic factor targeted by these miRNAs, thereby promoting cancer cell survival [65]. Since this discovery, numerous studies have revealed the aberrant expression of miRNAs in various tumor types [66-68]. Indeed, the observed aberrant miRNA expression in different tumor models has been shown to influence one or several of the cancer hallmarks proposed by Hanahan and Weinberg, thereby contributing to tumor initiation and progression [69].

In fact, miRNAs can be classified as tumor suppressor miRNAs or oncomiRNAs regarding their function and expression deregulation in cancer [70]. Tumor suppressor miRNAs are known to inhibit cancer progression. Reduced expression of these molecules plays a critical role in the processes of cancer development and proliferation [64]. For example, the Let-7 miRNA family acts as a tumor suppressor by targeting key oncogenes such as *Myc* and *HMGA2*, and its ectopic expression induces cell death in lung cancer cells [71-73]. Similarly, the miR-29 family plays an important role in regulating thousands of genes in several cancers, including lung adenocarcinoma and neuroblastoma, and its deregulation is associated with tumor progression and invasion [74-77].

In contrast, oncomiRNAs, which are overexpressed in tumors, promote cancer progression by suppressing tumor suppressor mRNAs [70]. For example, miR-21 is highly expressed in several cancers, including pancreatic, prostate and ovarian cancer, and its inhibition reduces tumor proliferation and reverses drug resistance [78-84]. Similarly, miR-221/222 is upregulated in cancers such as liver and breast, where it promotes tumor growth by targeting the CDK inhibitor p27 and affecting key signaling pathways [85, 86].

The potential of miRNAs as biomarkers in oncology has attracted considerable interest due to their role in the molecular characterization of tumors, which improves diagnosis, patient follow-up and therapy monitoring [87]. However, traditional tissue biopsies are invasive and often insufficient for comprehensive tumor profiling, especially when considering tumor heterogeneity and metastasis [88]. To address these limitations, liquid biopsies have emerged, allowing the analysis of tumor-derived material in fluids such as blood, urine and cerebrospinal fluid [89]. This minimally invasive technique allows repeated sampling and more effective monitoring of treatment response and recurrence early detection [90].

miRNAs have unique properties that make them strong candidates as biomarkers by liquid biopsy. They can circulate in body fluids, either protected by protein complexes such as the RISC (involving binding to AGO 2) or enclosed in EVs [91]. These mechanisms protect miRNAs from RNase degradation and ensure their stability even under harsh conditions,

increasing their potential as reliable biomarkers. Additionally, miRNAs are relatively easy to quantify due to their stability and the availability of sensitive detection methods [92]. Their expression is highly conserved within species, further supporting their reliability in diagnostic applications. [92] Notably, different miRNA deregulations are associated with different stages of tumor development, suggesting that their analysis could provide insight into disease progression and help determine the stage of cancer [93]. Their frequent deregulation in cancer underlines their critical relevance in oncology [91].

Deregulation of miRNAs is common in cancer, resulting in unique expression profiles that can assist in early diagnosis. For example, miR-1290 has been identified as a particularly promising biomarker in PCa, showing better diagnostic performance than the traditional marker CA19-9, and it also effectively distinguishes early-stage pancreatic cancer from healthy states [94, 95].

Moreover, various studies have pointed out the prognostic and predictive importance of circulating miRNAs associated with cancer, since as cancer progresses to a more invasive phenotype, miRNAs undergo changes, serving as molecular markers of tumor cells, observable from tumorigenesis to subsequent stages of progression [96, 97].

For example, serum miR-200c levels are significantly elevated during colorectal cancer metastasis formation and correlate with lymph node and distant metastasis, making it a potential marker for predicting metastasis [98]. In addition, miR-375 and miR-200b are significantly increased in the serum of patients with metastatic PCa compared to those with localized disease, further highlighting the role of circulating miRNAs as valuable biomarkers for monitoring cancer progression [99]. Notably, miR-21 promotes tumor invasiveness and induces a castration-resistant phenotype in PCa, highlighting its critical role in enhancing tumor aggressiveness and resistance to therapy [100].

Circulating miRNAs show great potential as cancer biomarkers, yet many studies focus only on changes in miRNA levels in patient plasma or serum patients [98, 99, 101]. The lack of standardized methods for sample preparation, RNA isolation, and internal controls complicates the comparison of findings across different laboratories [102]. To advance research in this area, it is essential to develop standardized protocols for miRNA quantification. Furthermore, there is a notable shortage of large-scale validation studies in extensive cohorts, as well as limited research on using miRNA panels to improve specificity and sensitivity. Additionally, understanding how circulating miRNAs relate to established cancer markers is vital for translating these findings into clinical practice. Understanding the origin of circulating miRNAs in both healthy individuals and cancer patients, as well as the factors that influence their levels and roles in cancer pathology, requires in-depth research.

This knowledge not only establishes circulating miRNAs as reliable cancer biomarkers, but also paves the way for new therapeutic strategies.

1.3.2 miRNAs: Promising Approaches for Cancer Therapy

Of the many treatments available for cancer, none have been able to completely eradicate cancer cells, often with worrying side effects. However, advances in our understanding of the molecular mechanisms of cancer have made it possible to target cells at their origin. Aberrant expression of certain miRNAs involved in carcinogenesis occurs during tumor initiation and progression, suggesting that manipulation of miRNA expression may be a promising approach to cancer therapy [103, 104]. Recent strategies in miRNA-based therapy have focused on two main approaches: enhancing or restoring endogenous miRNAs that act as tumor suppressors and reducing or inhibiting miRNAs that promote tumor growth [105]. This dual approach is a promising way to develop effective therapies that target the deregulated miRNA patterns observed in cancer cells.

Tumor suppressor miRNAs are often downregulated in cancer, resulting in the increased expression of their target oncogenes [70]. To overcome this, the administration of exogenous tumor suppressor miRNAs can help restore their reduced levels and disrupt the pathways that lead to cancer development. miRNA mimics, which are small double-stranded RNA molecules modified with 2'-O'-methoxy groups to resemble natural mature miRNAs, are a key method for re-establishing the function of these tumor suppressors [106] (Figure 5B). For instance, miR-34, a well-known tumor suppressor miRNA involved in pathways like p53, is typically downregulated in several cancers, such as lung and colon carcinoma [107]. Research has demonstrated that reintroducing miR-34 using mimetics can effectively inhibit tumor growth and cancer progression, as demonstrated *in vivo* in lung cancer models [107].

In contrast, oncogenic miRNAs, which are often overexpressed in tumors, need to be reduced to normal levels to reactivate tumor suppressor genes and inhibit cancer growth [70]. Anti-miRNAs, which are synthesized to target endogenous miRNAs, typically consist of single-stranded oligonucleotides with complementarity to the endogenous miRNA sequence [108]. Several therapeutic strategies target these miRNAs, including anti-miRNA oligonucleotides, miRNA sponges, small molecule inhibitors and miRNA masking (Figure 5B).

Anti-miRNA oligonucleotides are short, chemically modified strands of nucleic acids, typically 17-22 nucleotides in length [105]. These molecules are designed to specifically

bind to target miRNAs, blocking their interaction with target genes and thus disrupting their regulatory role in gene expression [105]. To increase their stability, these anti-miRNAs are often chemically modified. For example, in *C. elegans*, anti-miRNAs oligonucleotides with 2'O-methyl modifications have been used to target the let-7 miRNA, resulting in the loss of let-7 function [109]. MiRNA masking involves the creation of sequences that are fully complementary to the miRNA binding sites on the mRNA, forming strong duplexes that prevent the miRNA from binding to its target [110]. This effectively prevents the miRNA from exerting its regulatory effects. For instance, in one study, miRNA masks were designed to target the miR-1 and miR-133 binding sites in the 3' UTR of the HCN2 and HCN4 genes [110]. This masking led to increased expression of HCN2 and HCN4, highlighting the potential of miRNA masking as a therapeutic approach to regulating gene expression [110]. MiRNA sponges work as competitive inhibitors by containing multiple binding sites for specific endogenous miRNAs, effectively blocking their interaction with target mRNAs [111]. These sponges are as effective as anti-miRNA oligonucleotides in suppressing target miRNAs. They can also be designed with complementary heptamer seeds to target an entire miRNA family [111]. For example, research by Ma et al. used miRNA sponges to inhibit miR-9 in breast cancer cells, which led to a significant reduction in metastasis by upregulating CDH1 [112]. The growing need for miRNA-based inhibition therapies has led to the development of small molecules that can either prevent miRNA biogenesis or interfere with miRNA-target interactions [108]. For example, a study by Hei et al. introduced fluoroquinolone derivatives as potential miR-21 inhibitors [113]. However, further evaluation is needed to determine the stability and efficacy of these small molecule inhibitors.

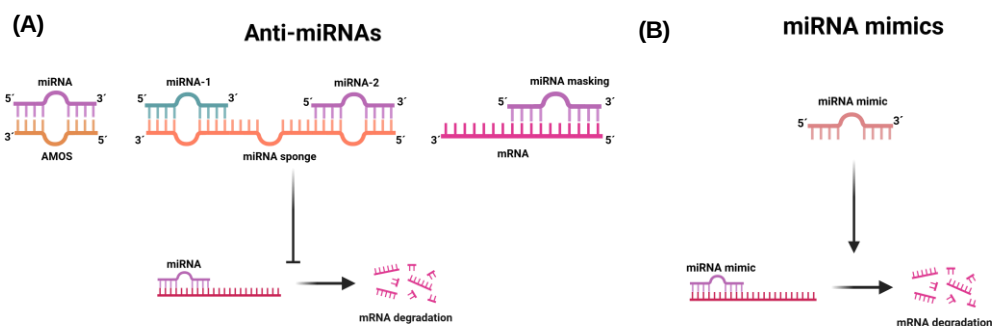


Figure 5- miRNA-based therapies; (A) Different types of anti-microRNAs; (B) miRNA mimics. (Adapted from Ferreira M. 2024. Submitted manuscript).

A major hurdle in bringing miRNA therapies to the clinic is the lack of a reliable delivery system. Despite their small size, miRNAs cannot easily cross lipid membranes, and their limited biological stability further complicates delivery [114]. An ideal miRNA delivery system

would offer high loading capacity, stability, prolonged circulation time, slow degradation of the miRNA and low toxicity [114]. Currently, both non-viral and viral vectors are being explored as potential solutions for efficient miRNA delivery (Figure 6) [115, 116].

Genetically engineered viruses provide a flexible method of delivering specific oligonucleotides, allowing either the upregulation of miRNA expression or the delivery of anti-miRNAs to suppress target miRNAs [117]. Various viral vectors such as lentiviruses, retroviruses, adenoviruses and adeno-associated viruses are commonly used in these viral-based miRNA delivery system [116].

An effective non-viral delivery system is characterized by its ability to transport miRNAs or vectors expressing miRNAs while protecting them from degradation by nucleases [116]. These delivery methods can be physical, such as electroporation, gene guns or ultrasound, or chemical, using organic, inorganic or polymer-based carriers [116]. Non-viral systems typically have lower toxicity and immunogenicity compared to viral methods, although they may have slightly reduced transfection efficiency [116]. Organic-based approaches, particularly those using liposomes, are the most popular and involve combining lipids with cationic head groups and helper lipids to enhance delivery [116]. Inorganic options such as gold, silica-based and Fe_3O_4 -based nanoparticles are also used [118-120]. Polymer-based delivery systems utilize both natural and synthetic polymers, offering versatility and high transfection efficiency [121]. In addition, EVs have emerged as a means of encapsulating and delivering miRNAs, either by transfecting exosome-producing cells with plasmids or by introducing synthetic oligonucleotides into mature EVs by electroporation [122]. EVs offer several advantages over traditional biomarkers: they are released from almost all cell types, can be isolated from any body fluid, reflect disease state and influence treatment efficacy [123]. Additionally, they protect their cargo during storage and improve the stability of disease biomarkers [123]. These innovative miRNA therapeutic systems present a promising avenue for novel therapeutics, offering a unique approach to cancer treatment by modulating entire biological pathways.

Advances in the understanding of miRNAs, together with improved anti-miRNA and miRNA mimic strategies, are gradually moving miRNA research towards clinical application [124]. Although some RNA-based drugs have received FDA approval and mRNA therapies are expanding in clinical trials, miRNA-based therapeutics are still in early development [125]. Few miRNA therapies for cancer have entered clinical trials, none have reached phase III or FDA approval, and many have been terminated due to toxicity concerns. Key challenges such as vector encapsulation, bioavailability, delivery efficiency and off-target effects must be overcome to make miRNA-based treatments viable [126] [127]. Despite these obstacles,

the potential of miRNA therapies, particularly in cancer, continues to attract significant scientific interest.

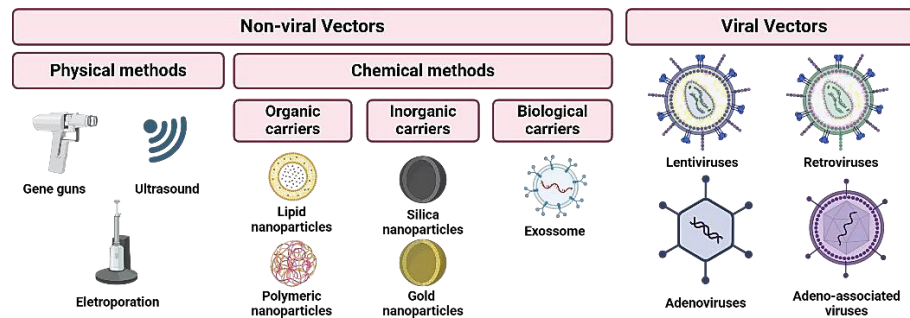


Figure 6 - Different types of miRNA delivery system. (Adapted from Ferreira M. 2024. Submitted manuscript).

1.4 Prostate Cancer

Prostate cancer (PCa) is a heterogeneous disease that mostly affects middle-aged men between the ages of 45 and 60 being one of the most incident cancers in the men worldwide [128]. With an estimated 1.5 million new cases and 397,000 deaths worldwide, PCa is the world's second most frequent cancer and the fifth leading cause of cancer death among men in 2022 [2]. It is estimated that 1 in every 25 men globally will receive a PCa diagnosis during their lifetime [129]. In Portugal, it is the most frequent neoplasia in men, accounting for 20% of new cases and standing as the third cause of cancer-related death in men [130].

The incidence of PCa is particularly high in countries with a high HDI [131]. The incidence varies considerably by region, with the highest rates observed in Northern Europe, Australia/New Zealand, the Caribbean and North America, and the lowest rates in several Asian and African regions [2]. This higher incidence is largely due to the widespread use of the PSA test, which allows early diagnosis of the disease, even in cases that might otherwise go undetected due to a lack of symptoms [2]. However, the widespread use of this test may also contribute to overdiagnosis and overtreatment of indolent cases [132]. Furthermore, these countries tend to have longer life expectancies and larger elderly populations, resulting in a higher proportion of men in the age group most affected by prostate cancer [133, 134].

Although several behavioral risk factors have been associated with an increased risk of prostate cancer, only three non-modifiable risk factors have been firmly established: age, ethnicity and family history [135]. However, several modifiable factors, such as diet, obesity and physical activity, also appear to influence the development of this type of cancer [135].

PCa develops from the accumulation of genetic alterations that lead to increased cell proliferation relative to cell death, giving cells the ability to proliferate, invade and metastasize to distant sites [136]. Histological changes can be found in the prostate of men in their 20s, but diagnosis typically occurs 3 to 4 decades later, suggesting that the disease develops slowly through multiple stages [12, 136, 137]. In the early stages there are no associated symptoms. However, in more advanced stages, symptoms may include weak urine flow, difficulty starting or stopping the urine, frequent urination (especially at night), blood in the urine, and pain or burning when urinating. In more advanced stages, the cancer may spread to the bones, causing pain [6].

The process of malignant transformation in the prostate begins as prostatic intraepithelial neoplasia (PIN), characterized by the presence of cytologically atypical or dysplastic epithelial cells within benign acini [136]. PIN is classified into low-grade and high-grade PIN [136]. In cases of high-grade PIN, this pre-malignant stage can progress to localized PCa due to the subsequent loss of the basal cell layer surrounding the prostate glands and the development of anaplastic cell morphology with nuclear pleomorphism and prominent nucleoli [136]. This localized prostate neoplasia may then advance to an advanced prostatic adenocarcinoma and, ultimately, to metastatic PCa [136, 138].

PCa has different degrees of aggressiveness. The stage and degree of neoplasia is determined by analyzing the histological characteristics of the tumor and the degree of cellular differentiation. Similar to other cancers, the less differentiated the cancer cells are, and therefore the less they resemble normal cells, the more aggressive the cancer will be, resulting in faster and more severe progression [136].

The widely accepted histological classification for this cancer is the Gleason Classification, developed by Dr Donald Gleason [139]. This system grades cancer cells based on their cellular and histological characteristics from 1 to 5, with grade 1 indicating well-differentiated, less aggressive cells and grade 5 indicating poorly differentiated, more aggressive cells [136, 139, 140]. Due to the intra tumoral heterogeneity of prostate adenocarcinoma, the Gleason score, which ranges from 2 to 10, was created by combining the two most common cell types in the cancer [140]. Although widely used, this system has been modified over the years [138]. In 2014, the International Society of Urological Pathology (ISUP) proposed a new grading system, the ISUP grading from I to V, which was incorporated into the World Health Organization (WHO) classification in 2016 [141, 142]. In this system, Gleason scores ≤ 6 were combined into ISUP grade 1, Gleason scores 9 and 10 were combined into ISUP grade 5, and Gleason score 7 was divided into ISUP grades

2 and 3 [143, 144]. This updated grading method has also been adopted by the European Association of Urology (EAU) Prostate Cancer Guidelines Committee [143, 144].

Most men are diagnosed with localized or locally advanced PCa, which generally has a favorable prognosis [145]. Treatment strategies vary depending on the stage of the disease at diagnosis, which is determined by the extent of the cancer, serum PSA levels and Gleason score [142]. For low-risk patients, active surveillance is often chosen, which involves regular PSA tests, physical examinations and prostate biopsies to monitor for progression [142]. Intermediate risk patients, particularly those with higher PSA levels, typically undergo radical prostatectomy, radiotherapy or brachytherapy [142]. For high-risk, advanced or metastatic PCa, androgen deprivation therapy (ADT) or hormone therapy, is the key therapeutic strategy, along with radiotherapy and, in cases of high-risk disease, pelvic lymphadenectomy [146]. This treatment affects the androgen pathway since both androgens and androgen receptors are important regulators in the proliferation and development of PCa [147].

PCa is recognized as an androgen-dependent disease for its progression and proliferation. Consequently, the androgen receptor (AR) was identified early on as a clear target for the development of effective therapies [148]. Testosterone (T) is mainly produced in the testicles and adrenal glands. Its derivative, dihydrotestosterone (DHT), is formed by the action of the enzyme 5 α -reductase on free testosterone that enters the prostate cells. In healthy men, both hormones are essential for prostate cell survival and function [149]. However, in PCa, tumor cells demonstrate excessive activation of the androgen signaling pathway, leading to uncontrolled proliferation [150]. The function of these hormones is mediated by their binding to the AR.

The AR is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily and is composed of several key domains: the N-terminal domain (NTD), which is essential for initiating transcription and recruiting coactivators and corepressors; the central DNA-binding domain (DBD), which enables the AR to bind to specific DNA sequences known as androgen response elements (AREs) located in the promoter regions of target genes; and the C-terminal ligand-binding domain (LBD), which binds to androgens and plays a role in regulating AR activation [151].

In the prostate, it is expressed in the epithelial compartment, where it stimulates differentiation, and in the stroma, where it promotes proliferation [152]. In the absence of ligand binding, when inactive, the AR is located in the cytoplasm bound to heat shock proteins (HSPs) [153]. Binding of androgens, particularly DHT, which has a higher affinity for the AR, induces a conformational change in the receptor that dissociates it from the

HSPs [153]. The DHT-AR complex is then translocated to the nucleus to promote transcription of genes encoding metabolic enzymes, growth factors and cell cycle regulatory proteins [148]. Thus, the central role of this pathway in prostate cancer biology provided the basis for ADT [148].

ADT aims to control the progression of the disease by reducing circulating androgen levels or by preventing androgens from interacting with the AR in prostate cells [148]. Various ADT strategies can be employed. Originally, bilateral orchiectomy was the form of ADT used, but although it is still utilized worldwide, this surgical procedure has been largely replaced by other medical options. Currently, ADT includes luteinizing hormone-releasing hormone (LHRH) agonists and antagonists, as well as anti-androgens [148]. LHRH agonists target the LHRH receptor in the anterior pituitary gland. Continuous levels of LHRH agonists stimulate the receptor, causing a temporary increase in LH and T release, followed by receptor downregulation, resulting in decreased LH levels and subsequent suppression of T production [148]. Conversely, LHRH antagonists bind competitively and reversibly to LHRH receptors, blocking them and inhibiting LH release and T production [148]. Anti-androgens, such as bicalutamide and nilutamide, are drugs that compete with androgens for binding to the AR [154].

ADT has shown great results, with a remission of disease in about 90% of patients [155]. However, in 2-3 years, disease progresses to the most advanced form of the disease, the Castrate-Resistant Prostate Cancer (CRPC), which is associated with a poor prognosis and impaired quality of life. [155]. Thus, the need to develop new strategies emerged [151].

Before the development of second-generation antiandrogens, such as enzalutamide (ENZ), apalutamide and darolutamide, the most effective treatment strategy for CRPC was the taxane chemotherapy agent docetaxel, followed by cabazitaxel [154, 156]. The introduction and FDA approval of second-generation antiandrogen therapies has significantly changed the treatment landscape for prostate cancer, particularly CRPC [157]. These next-generation antiandrogens have greater specificity for the androgen receptor, act with higher affinity than their precursors, and act exclusively as antagonists, avoiding androgen withdrawal flare [156, 157]. As a result, these treatments have significantly improved patient outcomes by increasing life expectancy, improving metastasis-free survival, reducing circulating and intra tumoral androgens, and lowering serum PSA levels [154]. However, despite these advances, CRPC remains a challenging disease.

ENZ was the first second-generation antiandrogen to be characterized and approved by the FDA in 2012 for the treatment of men with metastatic CRPC [154]. This multi-targeted AR inhibitor blocks AR nuclear translocation, testosterone binding to AR, co-activator

recruitment and AR binding to DNA (figure 7) [158]. Clinical trials showed that ENZ significantly improved overall survival compared to placebo, extending survival from 13.6 to 18.4 months in patients who had relapsed on chemotherapy [159]. It also increased radiographic progression-free survival in chemotherapy-naïve patients [160]. In head-to-head comparisons with the first-generation antiandrogen bicalutamide, ENZ significantly reduced the risk of PCa progression and death in both metastatic and non-metastatic CRPC [161]. Later, ENZ was also approved for the treatment of non-metastatic CRPC and metastatic castration-sensitive PCa [162].

Although second-generation antiandrogens significantly improve overall survival in CRPC patients, many develop primary resistance or secondary resistance over time, leading to disease progression and ultimately death [151]. This highlights the critical need to understand the mechanisms underlying antiandrogen resistance to enable early detection and improve treatment strategies for CRPC, which remains incurable.

While not fully understood, several key deregulated pathways and mechanisms of resistance to ENZ have been identified [163]. AR-dependent mechanisms include AR amplification, AR overexpression, AR somatic point mutations, constitutively active AR splice variants and altered intra tumoral androgen biosynthesis [154]. Although AR pathways are the most discussed in relation to CRPC and ENZ-resistant PCa, several AR-independent mechanisms also contribute to resistance, including activation of the glucocorticoid receptor, impairment of DNA repair pathways, hyperactivation of the PI3K/AKT/mTOR and Wnt/ β -catenin pathways, aberrant activation of the EGFR pathway, and epithelial plasticity with neuroendocrine differentiation [164]. Interestingly, the transfer of miRNAs by EVs, although much less studied than the resistance mechanisms associated with AR, has been implicated in the acquisition of resistance to drugs, particularly ENZ [164].

Identifying new early and precise biomarkers for ENZ resistance is crucial for clinicians to track disease progression and personalize patient care since current biomarkers, such as the PSA test, often lack the specificity needed for accurate prognosis and therapy response assessment [165].

Emerging research underscores the role of EVs and their associated miRNAs in tumor progression and the development of therapy resistance [166]. Investigating miRNAs from EVs related to ENZ response could provide insights into the molecular mechanisms of resistance, potentially revealing new therapeutic targets [165, 166]. These miRNAs could help stratify patients based on their likelihood of benefiting from ENZ treatment, allowing for more personalized therapy approaches. Additionally, this could facilitate the early detection of resistance and improve treatment efficacy while minimizing unnecessary side effects

[165, 166]. Therefore, analyzing EVs in liquid biopsies is a promising avenue for identifying biomarkers of ENZ resistance, especially considering ENZ's widespread use as a powerful antiandrogen therapy [166]. Furthermore, this approach may also lead the way to the development of new therapeutic strategies by providing critical insights into resistance mechanisms and potential targets for new therapies.

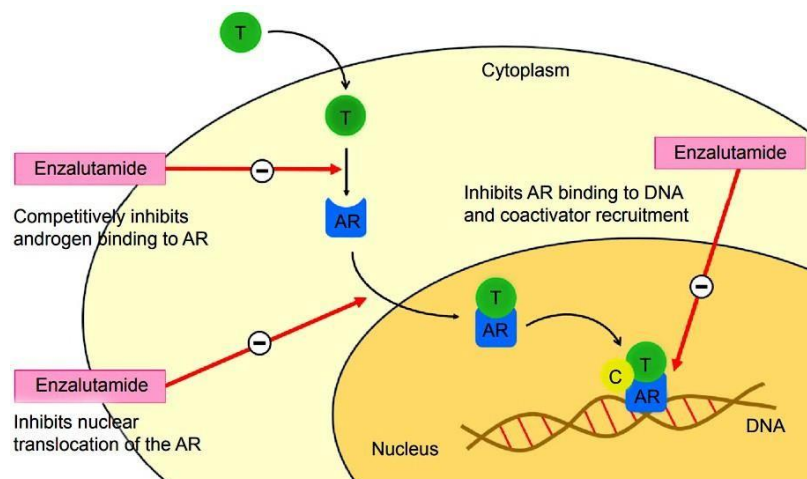


Figure 7 - Mechanism of action of enzalutamide (adapted from 'Enzalutamide for the treatment of metastatic castration-resistant prostate cancer'[167]).

1.4.1 Deregulated microRNAs in prostate cancer context

Several deregulated miRNAs are involved in PCa progression, CRPC development, metastasis formation and treatment resistance [100].

In the context of tumor progression, miR-1 and miR-99b are examples of noteworthy miRNAs. miR-1 is downregulated in PCa cells and has been shown to reduce cell viability and proliferation by targeting the c-Met/AKT/mTOR signaling pathway [167]. Similarly, miR-99b, another tumor suppressor miRNA, is frequently downregulated and affects differentiation, proliferation, invasion, and migration in PCa [168]. In the development of CRPC, miR-221-3p is significantly downregulated, particularly in bone metastases, which contributes to disease progression and increased metastatic potential [169]. Additionally, miR-23c and miR-4328 are downregulated during metastasis, suggesting a loss of their tumor suppressive effects [170]. Conversely, certain miRNAs such as miR-20b-5p and the miR-183-96-182 cluster are upregulated and associated with aggressive PCa phenotypes and poor prognosis [171].

Recent research has highlighted the role of specific miRNAs in ENZ resistance. [172]. For example, hsa-miR-361, which is found at lower levels in patients with recurrent PCa,

increases ENZ sensitivity by targeting the ARv7 and MKNK2 oncogenes, which drive PCa progression through their roles in cell growth, proliferation and invasion [173]. Conversely, hsa-miR-198, which is upregulated in ENZ-resistant cells, inhibits autophagic cell death by directly targeting AMBRA1 [174].

Given the central role of the AR pathway in resistance, certain miRNAs that affect this pathway have been shown to influence sensitivity and resistance to therapy. In particular, overexpression of miRNAs such as hsa-miR-30c-1-3p, hsa-miR-103a-2-5p, hsa-miR-124, hsa-miR-181c-5p and hsa-miR-8080 can effectively reduce the expression of ARv7, a prominent AR variant associated with advanced and ENZ-resistant PCa [175-177].

Understanding the specific miRNAs involved in ENZ resistance and their targets may provide information about the underlying molecular mechanisms and potentially identify new therapeutic targets. Furthermore, the identification of crucial miRNA profiles linked to ENZ resistance presents an opportunity for the advancement of RNA-based therapies. Emerging evidence suggests that a promising potential approach to treat PCa involves targeting overexpressed miRNAs through anti-miRNA therapies, while simultaneously increasing the expression of under expressed miRNAs through administration of mimics [178].

Indeed, the value of miRNAs as therapeutic agents in the context of cancer has been extensively studied. However, little is known about the use of these molecules as therapeutic agents in CRPC and the consequences of restoring their expression levels, as well as their target mRNAs, on disease progression.

It is noteworthy that many therapeutic agents identified through 2D models often fail when advanced to *in vivo* studies and subsequent clinical trials due to the limitations of these models in replicating the complex tissue architecture and microenvironment. To overcome these limitations, 3D models have emerged as a more reliable approach, offering a more accurate simulation of the *in vivo* environment, thereby improving the predictive validity of therapeutic efficacy and safety assessments.

1.5 *In vitro* study models: 3D spheroids

Cell cultures emerged in the 19th century with the aim of addressing problems associated with *in vivo* models [179]. *In vitro* techniques allow cells to grow outside their natural environment under highly controlled and reproducible conditions such as temperature, pH, osmotic pressure, oxygen (O₂), and carbon dioxide (CO₂) levels [180, 181]. These assays have become the primary focus of biopharmaceutical research due to their few limitations, such as bacterial and fungal contaminations [182]. Moreover, cell cultures enable the simple

manipulation of numerous experimental variables to mimic *in vivo* conditions [183]. Thus, *in vitro* cell culture models circumvent the ethical, legal, and economic issues associated with animal experimentation and manipulation [183].

Currently, 2D cell culture, which involves the growth of cells in a monolayer, is the main procedure used to evaluate anticancer agents. This approach allows the assessment of different therapeutic combinations in a controlled, reproducible, cost-effective, and easy-to-handle manner [184]. However, cell growth on flat surfaces does not fully represent cell-to-cell and cell-to-extracellular matrix interactions that occur in tumors, nor the proliferation, survival, migration, or invasion capacities exhibited by cancer cells [185]. To overcome these limitations, 3D cell culture models have been developed, capable of mimicking tumor properties [185].

3D cell culture can be divided into spheroid and organoid models and there are several scaffold or scaffold-free 3D cell culturing techniques [186, 187]. One of the most widely used *in vitro* 3D models is spheroids [188]. This technique consists of a small, tightly bound cellular aggregate that tends to form when cells are grown on non-adherent surfaces [188, 189]. Spheroids have been extensively used in cancer research because the 3D architecture and cell interactions provided by spheroid growth better mimic the *in vivo* cellular environment [189]. This technique is also considered essential for studying solid tumors, such as PCa, as several similarities have been found between them. These similarities include growth kinetics, gene expression levels, and cellular layer organization (including proliferative, quiescent, and necrotic strata) [189, 190]. These 3D cellular aggregates also exhibit nutrient, gas (O₂ and CO₂), and pH gradients, as well as therapy resistance, in a manner similar to solid tumors [190].

Several studies emphasize the significance of 3D cultures in cancer research, particularly for studying EVs [191-193]. Recent findings indicate that the cargo of EVs is highly dependent on culture conditions, with the TME significantly influencing EV biogenesis and cargo loading [191-193]. Culturing cancer cells in 3D provides a more realistic tumor-like context, allowing for a better understanding of EV release. Since the processes involved in EV biogenesis and loading are often absent in 2D cultures, EVs derived from 3D models closely resemble those found in patients [191-193].

Analyzing the specific cargo of EVs in 3D settings offers valuable insights into their roles in cancer progression, metastasis, and drug resistance [191-193]. Adopting 3D culture models can address limitations of 2D systems, aiding in the identification of molecular biomarkers and resistance mechanisms, particularly those related to ENZ resistance.

Objective

2. Objective

The main aim of this study is to determine the miRNAs downregulation profile dynamics during the acquisition of ENZ resistance. In the future, these findings may aid in the identification of novel biomarkers that could facilitate early detection of resistance phenotypes, enabling early intervention and personalized monitoring of patients, thereby anticipating disease progression. Additionally, this opens avenues for the development of innovative RNA-based therapies aimed at restoring normal miRNA levels.

2.1 Specific objectives:

- Establish 3D spheroid-like models using the LNCaP-ER cell line;
- Analyze the dynamic changes of the downregulated EVs derived-miRNAs defined during the induction of ENZ resistance both in 2D and 3D *in vitro* models;
- Identify the targets of our selected downregulated miRNAs through an *in silico* analysis;
- Restoring the expression levels of the downregulated miRNA profile and evaluating its functional effect on resistance to ENZ reversion.

Material and Methods

3. Material and Methods:

Previously, our research team developed an *in vitro* cellular model to study ENZ resistance by establishing an ENZ-resistant cell line called LNCaPER. This cell line was established by exposing LNCaP cells to increasing concentrations of ENZ (2.5 μ M, 5 μ M, 10 μ M, 20 μ M and 30 μ M) over time [194].

To profile the miRNAs associated with ENZ resistance, miRNAs were extracted from LNCaPER cells resistant to the highest concentration of ENZ and from the original LNCaP cells. The miRNA expression was quantified using the *NanoString® nCounter human miRNA* expression assay. A total of 799 miRNAs were analyzed and 12 upregulated and 13 downregulated miRNAs showed significantly different expressions in the LNCaP-ER cells compared to the original LNCaP cells as shown in figure 8 and table 1 [194].

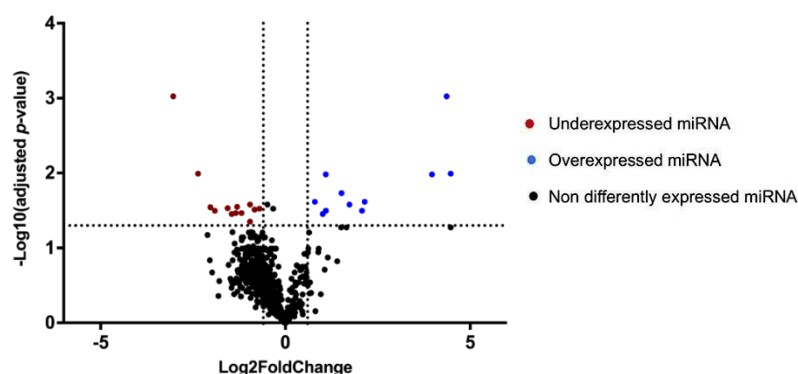


Figure 8 - Volcano plot of miRNAs quantified in LNCaP versus LNCaP-ER cells. miRNAs significantly upregulated (Log2 fold change > 0.6) are shown as blue dots, while significantly downregulated miRNAs (Log2 fold change < -0.6) are depicted as red dots. Only miRNAs with an adjusted p-value of Log10 > 1.3 are highlighted [194]

Table 1 - MiRNAs significantly downregulated and upregulated in LNCaP-ER in comparison to LNCaP cells.

Downregulated microRNAs	Upregulated microRNAs
hsa-miR-7-5p	hsa-miR-22-3p
hsa-miR-18a-5p	hsa-miR-30d-5p
hsa-miR-1973	hsa-miR-1269b
hsa-135a-5p	hsa-miR-200a-3p
hsa-miR-19a-3p	hsa-miR-30e-5p
hsa-miR-15b-5p	hsa-miR-21-5p
hsa-miR-20a-5p+hsa-miR-20b-5p	hsa-miR-30a-5p
hsa-miR-93-5p	hsa-miR-221-5p
hsa-miR-421	hsa-miR-15a-5p
hsa-miR-940	hsa-miR-221-3p
hsa-miR-25-3p	hsa-miR-196b-5p
hsa-miR-570-3p	hsa-miR-222-3p
hsa-miR-328-3p	

From the determined profile, 6 downregulated miRNAs with the highest number of validated targets were selected (hsa-miR-15b-5p, hsa-miR-19a-3p, hsa-miR-20a-5p+hsa-miR-20b-5p, hsa-miR-25-3p, hsa-miR-93-5p). These miRNAs were used to assess the dynamics of their downregulation profile during the development of ENZ resistance up to the LNCaP-ER 10 μ M ENZ.

3.1. Characterization and Maintenance of Cell Line

The LNCaP cell line used in this study was obtained from the American Type Culture Collection (ATCC) biobank and was derived from a left supraclavicular lymph node biopsy of a 50-year-old Caucasian man diagnosed with metastatic prostate cancer [195]. These cells express the AR and are responsive to 5-alpha-dihydrotestosterone [195].

Cells were maintained in RPMI 1640 medium (*PAN-Biotech*[™]) supplemented with 10% Fetal Bovine Serum (FBS) (*Gibco*®) and 1% Antibiotic-Antimycotic (*Gibco*®) and incubated at 37°C in a humidified atmosphere with 5% CO₂. The medium was refreshed, and the designated concentration of ENZ was administered every 48 hours.

When cells reached 80-90% confluence, they were trypsinized using trypsin-EDTA (1X) (*Gibco*®), resuspended in fresh growth medium, and the concentration of viable cells in the suspension was determined using trypan blue solution (*VWR*[™]) and the *EVE*[™] Automated Cell Counter (*NanoEnTek*®). This allowed for accurate plating and preparation of cell pellets with the desired number of cells.

Additionally, samples of the cultured medium were collected weekly and tested for mycoplasma infection to ensure culture integrity and purity.

3.2. Assessment of Metabolic Capacity

For confirmation of the resistance phenotype, metabolic capacity was assessed using the resazurin assay. A 96-well plate was used to culture 100 μ L of a 200,000 cells/mL suspension per well. For each experiment, cells were seeded in 8 wells with ENZ at each specified concentration and 8 wells with untreated LNCaP cells serving as controls. After 48 hours of incubation, Resazurin (*ACROS Organics*®) was added to each well at a 10% (V/V) ratio. Absorbance (Abs) readings were taken at 570 nm and 595 nm using the

Varioskan™ LUX multimode microplate reader (*Thermo Fisher Scientific®*) to determine the percentage of metabolized resazurin.

The calculations were made according to the equation:

$$\% \text{ Metabolized Resazurin} = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

O1 - Extinction coefficient of oxidized Alamar Blue at 570 nm (80586)

A1 - Abs 570 nm sample

O2 - Extinction coefficient of oxidized Alamar Blue at 600 nm (117216)

A2 - Abs 595 nm sample

R1 - E from reduced Alamar Blue at 570 nm (101088)

N1 - Abs 570 nm blank

R2 - E from reduced Alamar Blue at 595 nm (9514)

N2 - Abs 595 nm blank

3.3. Relative Quantification of AR and PSA mRNA Levels by Real-Time qPCR

To quantify *AR* and *PSA* mRNA expression levels, RNA was extracted using the MagMAX™ mirVana™ Total RNA Isolation Kit (*Thermo Fisher Scientific®*). in accordance with the manufacturer's protocol. After extraction, the RNA concentration and purity of each sample was assessed by measuring the Abs ratio at 260 nm and 280 nm using the NanoDrop® Lite spectrophotometer (*Thermo Scientific™*).

The extracted mRNA was used as a template for the synthesis of cDNA for subsequent amplification by real-time quantitative PCR (Polymerase Chain Reaction) using StepOne™ PCR (*Applied Biosystems®*). The synthesis of cDNA from mRNA was carried out using the commercial High-Capacity cDNA Reverse Transcription Kit (*Applied Biosystems®*). The thermal conditions used in the thermocycler were as follows: 10 minutes at 25°C, followed by 120 minutes at 37°C and finally 5 minutes at 85°C. lastly, 5 minutes at 85°C.

For mRNA quantification, 1X Master Mix (*Applied Biosystems®*), 1X TaqMan® specific mRNA Expression Assays (*AR*: Hs00171172_m1; *PSA*: Hs02576345_m1 (*Applied Biosystems®*)), and a cDNA sample. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase: Hs99999905_m1; *Applied Biosystems®*) was used as an endogenous control to normalize the results.

All the samples were quantified in duplicate and relative expression was quantified using the $2^{-\Delta\Delta CT}$ method. A negative control was always included on each plate. The analysis of the results was carried out using StepOne™ Software v2.3 (*Applied Biosystems®*).

3.4. In silico analysis

The target mRNAs of the selected miRNAs were identified using the miRTarbase v9.0 database, which compiles experimentally validated miRNA-mRNA interactions from peer-reviewed literature published up to 2021 and manually curated. Only interactions supported by robust evidence methods, such as Western blot, qPCR, and luciferase assays, were considered. Subsequently, a specialized platform (<https://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/>) was utilized to construct a Venn diagram, illustrating the convergence of target genes shared among the selected miRNAs, highlighting their overlapping regulatory networks.

3.5. Assembly and Characterization of Spheroids

3.5.1. Spheroids formation

To prepare agarose molds, 1g of pure agarose powder (*SeaKem™ LE Agarose, Lonza™*) was weighed and autoclaved on a dry cycle. A 0.9% (v/v) NaCl solution was then prepared and filtered under sterile conditions. The autoclaved agarose was dissolved in 50 mL of the sterile saline solution. Under aseptic conditions, 550µL of the melted agarose was pipetted into pre-autoclaved #12-81 micro-molds (*MicroTissues®*). Once the agarose solidified, the agarose counter-mold (*3D Petri Dish®*) was carefully removed and placed in a 12-well plate.

Before cell seeding, the agarose molds were equilibrated with RPMI 1640 culture medium supplemented with 1% Antibiotic-Antimycotic. Trypsinized LNCaP and ENZ-treated LNCaP cells were then resuspended in RPMI 1640, supplemented with 10% exosome-depleted FBS (*Gibco®*) and 1% Antibiotic-Antimycotic (*Gibco®*). Lastly, the cells were seeded into each agarose mold at densities of 20,000 cells per spheroid.

3.5.2. Spheroids morphology and metabolic activity

The morphology of the spheroids was evaluated at each time point using microscopy (Olympus IX51) and their diameters were measured using ImageJ software (National Institutes of Health).

Concurrently, the metabolic activity of the 3D-cultured cells was assessed at each timepoint using the *CellTiter-Glo® 3D Cell Viability Assay* which quantifies ATP production of cells, correlating its metabolic capacity with viability. Five to six spheroids were collected from each mold and pipetted into an opaque 96-well plate containing up to 100 µL of medium.

The plate was shaken vigorously for 5 minutes in the dark, followed by incubation at room temperature for a further 25 minutes. Luminescence was measured using the Varioskan™ LUX multimode microplate reader (*Thermo Fisher Scientific®*). Each condition was tested in triplicate for both cell lines. To graph the metabolic activity of LNCaP and ENZ treated spheroids over time, luminescence values at 72 hours and 7 days were normalized by dividing by the corresponding value at 72h hours (fold change).

3.6. Isolation of extracellular vesicles

EVs were precipitated from the culture media of the cell spheroids at 72 hours and 7 days post establishment. Isolation of EVs followed a protocol adapted from a previously optimized method developed by the research group for EV extraction from 2D cell cultures [196]. Spheroid culture medium was collected and centrifuged at 3500 rpm for 30 minutes at 4°C to remove cells and debris. The supernatant was then filtered through a 0.22 µm filter (*GE Healthcare Whatman™*). The filtered medium was then mixed with Total Exosome Isolation Reagent (*Invitrogen™*) in a 1:2 ratio and incubated overnight at 4°C. After incubation, the mix was centrifuged at 10,000g for 1 hour. The resulting pellet containing pre-enriched EVs was resuspended in filtered DPBS and stored at -80°C until further analysis.

3.7. Characterization of extracellular vesicles

EV samples were analyzed for particle concentration and size distribution using the NS300 Nanoparticle Tracking Analysis (NTA) system (*NanoSight—Malvern Panalytical, Malvern, UK*). To achieve optimal concentration for NTA measurements, the samples were pre-diluted in filtered DPBS at a 1:100 ratio. Video recordings were made with a camera level of 16 and a threshold setting of 5. Particle concentration per mL and size distribution were analyzed using NanoSight NTA software v3.2 (*Malvern Panalytical*).

For further characterization, including morphology, size distribution, and identification of aggregates and non-vesicular particles, the EV samples were also examined using transmission electron microscopy (TEM) at the I3S Histology and Electron Microscopy facility using a Jeol JEM 1400 transmission electronic microscope (Tokyo, Japan) and images were acquired.

To confirm the presence of EVs in our samples, we performed EV-CD63 quantification using the Human CD63 ELISA Kit (*ThermoFisher™, Waltham, MA, USA*). CD63, a glycoprotein

commonly found on EV surfaces, serves as a reliable EV biomarker. Quantification was based on a solid phase sandwich ELISA where EV-CD63 was detected and measured. Absorbance at 450 nm was recorded using the Varioskan™ LUX multimode microplate reader and CD63 concentration was determined by comparison with a standard curve, providing insight into the quantity and integrity of EVs.

3.8. Relative quantification of the expression levels of deregulated miRNAs in cells and in the respective EVs, in 2D and 3D, by Real-Time qPCR

To quantify the expression levels of our miRNAs of interest, both in the cells and in their secreted EVs, RNA was first extracted using the *MagMAX™ mirVana™ Total RNA Isolation Kit (Thermo Fisher Scientific®)* in accordance with the manufacturer's protocol. After extraction, the RNA concentration and purity of each sample was assessed by measuring the absorbance ratio at 260 nm and 280 nm using the *NanoDrop® Lite spectrophotometer (Thermo Scientific™)*.

The extracted miRNA was used as a template for the synthesis of cDNA for subsequent amplification by real-time quantitative PCAR (Polymerase Chain Reaction) using *StepOne™ PCAR (Applied Biosystems®)*. The synthesis of cDNA from miRNA was carried out using the commercial *TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems®)*. The thermal conditions used were as follows 16°C for 30 minutes, followed by 60 minutes at 42°C and 85°C for 10 minutes.

For miRNA quantification, 1X Master Mix (*Applied Biosystems®*), 1X *Taqman® microRNA Expression Assays (Applied Biosystems®)* (hsa-miR-15b-5p: TM000390, hsa-miR-19a-3p: TM000395, hsa-miR-20a-5p: TM000580, hsa-miR-20b-5p: TM001014, hsa-miR-25-3p: TM000403, hsa-miR-93-5p: TM001090). RNU44 (TM001094; *Applied Biosystems®*) was used as an endogenous control to normalize the results, both in cells and EVs.

All the samples were quantified in duplicate and relative expression was quantified using the $2^{-\Delta\Delta CT}$ method. A negative control was always included on each plate. The analysis of the results was carried out using *StepOne™ Software v2.3 (Applied Biosystems®)*.

3.9. Real-Time qPCR Analysis of Target Gene Expression Levels of Deregulated miRNAs in 2D and 3D Cultures by Real-Time qPCR

The targets of the deregulated miRNAs under study were quantified in cells. The RNA extraction was performed in the automatic extractor *KingFisher™ Duo Prime Purification*

System (Thermo Scientific™) using MagMAX™ mirVana™ Total RNA Isolation Kit (Thermo Scientific™) in accordance with the manufacturer's protocol. After extraction, the RNA concentration and purity of each sample was assessed by measuring the absorbance ratio at 260 nm and 280 nm using the NanoDrop® Lite spectrophotometer (Thermo Scientific™).

The extracted mRNA was used as a template for the synthesis of cDNA for subsequent amplification by real-time quantitative PCR (Polymerase Chain Reaction) using StepOne™ PCR (Applied Biosystems®).

For mRNA quantification, 1X Master Mix (Applied Biosystems®), 1X TaqMan® specific mRNA Expression Assays (VEGFA: Hs00900055; SMAD7: Hs00998193_m1 (Applied Biosystems®)), and a cDNA sample. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase: Hs99999905_m1; Applied Biosystems®) was used as an endogenous control to normalize the results.

All the samples were quantified in duplicate and relative expression was quantified using the $2^{-\Delta\Delta CT}$ method. A negative control was always included on each plate. The analysis of the results was carried out using StepOne™ Software v2.3 (Applied Biosystems®).

3.10. Transfection miRNA mimics

Transfection was performed on 3D models of LNCaP 2.5 μ M cells, 72 hours after spheroid seeding. First, transfection mix, each one containing 12.5 nM of each miRNA mimic (mirVana® miRNA hsa-miR-20a-5p mimic: #4464066, Invitrogen™; mirVana® miRNA hsa-miR-25-3p mimic: #4464066, Invitrogen™) were prepared in combination with 15 μ L Lipofectamine™ RNAiMAX. At 72 hours post spheroid formation, the transfection mix were added to the spheroids in a final volume of 200 μ L per mold using Opti-MEM supplemented with 10% exosome-free FBS and incubated for 48 hours. After incubation, cells were collected for RNA extraction and metabolic activity assays. The pre-miR™ hsa-miR-1 miRNA Precursor Positive Control (#4464062, Invitrogen™) was used as a positive control for transfection and Taqman® microRNA Expression Assay (Applied Biosystems®) (hsa-miR-1: TM002222) was used for miRNA quantification. To enable interpretation of the results, the relative expression of TWF1 (Twinstin Actin Binding Protein 1: Hs00702289_s1; Applied Biosystems®), a target of the positive control, was also quantified by real-time PCR. The experiment was repeated twice for each condition.

3.11. Statistical Analysis

Statistical analysis of differences in metabolic activity assays and spheroid diameters was conducted using GraphPad Prism 8. To assess data distribution, the Shapiro-Wilk normality test was performed.

For datasets with a normal distribution, one-way ANOVA followed by Tukey's multiple comparisons test was used to determine significant differences between groups.

In cases where data did not follow a normal distribution, the Kruskal-Wallis test and Dunn's multiple comparisons test were applied. The analysis of miRNA and mRNA expression level differences was conducted using *IBM® SPSS® Statistics*, employing t-test analysis and the Livak method ($2^{-\Delta\Delta C_t}$) for relative quantification.

Results

4. Results

4.1. Monitoring the resistance phenotype of the LNCaP-ER cell line

Figure 9 presents the results of the metabolic capacity tests performed for each ENZ concentration studied. The purpose of this assay was to evaluate the resistance phenotype of ENZ-treated cells.

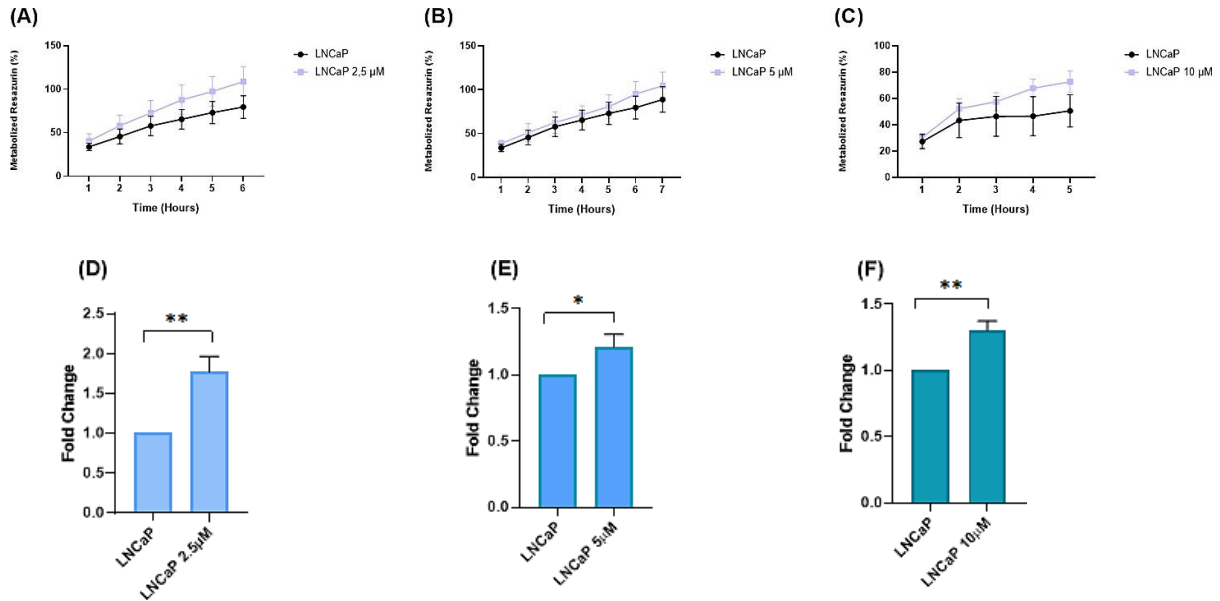


Figure 9 - Graphical representation of the metabolic capacity of (A) LNCaP cells treated with 2.5 μ M, (B) 5 μ M and (C) 10 μ M of ENZ in 2D cell culture; and of the metabolic activity in 3D spheroids for the same concentrations of ENZ: (D) 2.5 μ M, (E) 5 μ M and (F) 10 μ M, compared to LNCaP (fold change) (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

To facilitate this evaluation, the metabolic capacity of ENZ-treated LNCaP cells was compared to that of untreated LNCaP cells, which served as a 100% baseline.

In 2D cell culture, a resistance phenotype was confirmed when the metabolic capacity of ENZ-treated cells at any concentration was within 25% of that of untreated LNCaP cells, based on the amount of resazurin metabolized. The results indicated that all ENZ-treated cells exhibited a higher metabolic capacity compared to untreated LNCaP cells.

The results obtained in 3D cell culture were similar, with all concentrations studied showing a higher metabolic capacity compared to LNCaP (LNCaP 2.5 μ M $p = 0.003$; LNCaP 5 μ M $p = 0.02$; LNCaP 10 μ M $p = 0.002$).

In addition, for evaluation of the resistance phenotype, PSA and AR mRNA levels were quantified at all studied concentrations in both cell culture models (2D and 3D) (Figure 10).

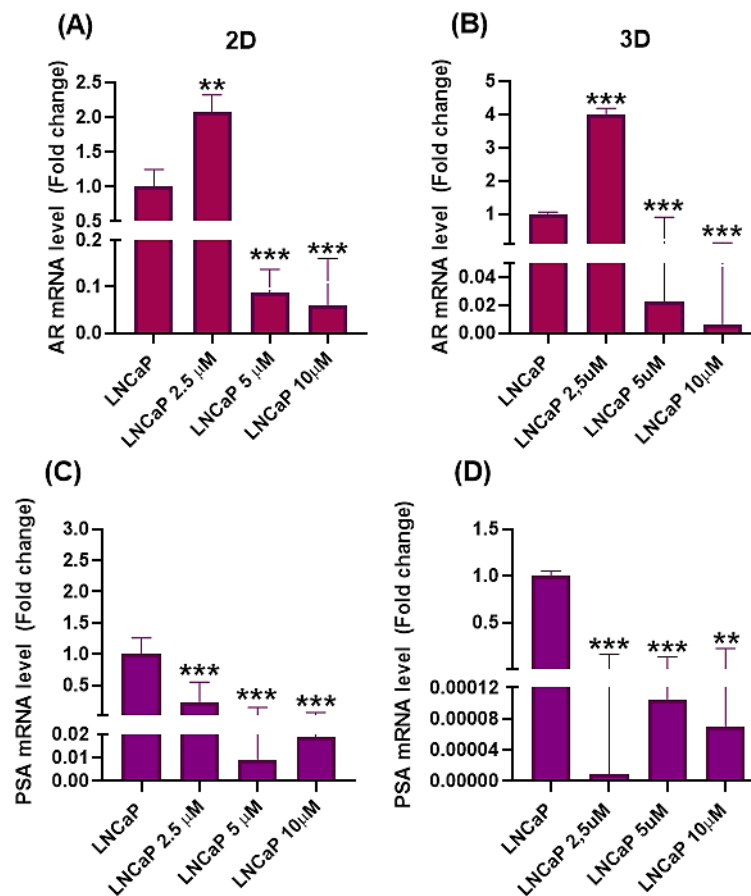


Figure 10- Graphical representation of the relative quantification of the expression levels of **(A)** AR mRNA in 2D, **(B)** AR mRNA in 3D, **(C)** PSA mRNA in 2D and **(D)** PSA mRNA in 3D by real-time qPCR in LNCaP and cells resistant to each of the established concentrations of ENZ (**: $p < 0.01$; ***: $p < 0.001$).

According to the results, AR mRNA expression decreased significantly during the resistance establishment process both in 2D and 3D models compared to LNCaP cells, except in LNCaP 2.5 μM cells, where it increased.

In 2D cell cultures, LNCaP 2.5 μM cells exhibited a 100% increase in AR mRNA levels ($2^{-\Delta\Delta Ct} = 2.07$, $p = 0.009$, Figure 10A). Conversely, LNCaP 5 μM and 10 μM cells showed decreases of 91% ($2^{-\Delta\Delta Ct} = 0.09$, $p < 0.001$, Figure 10A) and 94% ($2^{-\Delta\Delta Ct} = 0.06$, $p = 0.001$, Figure 10A), respectively. A similar pattern was observed in 3D, with LNCaP 2.5 μM cells showing a 4 fold-increase in AR mRNA expression ($2^{-\Delta\Delta Ct} = 4.00$, $p < 0.001$, Figure 10B), while LNCaP 5 μM cells showed a 98% decrease ($2^{-\Delta\Delta Ct} = 0.023$, $p < 0.001$, Figure 10B) and LNCaP 10 μM cells demonstrated a 99% decrease ($2^{-\Delta\Delta Ct} = 0.006$, $p < 0.001$, Figure 10B).

Similar to *AR*, *PSA* mRNA expression levels also decreased significantly during resistance establishment compared to LNCaP cells in both 2D and 3D models.

In 2D, LNCaP 2.5 μ M cells showed a 77% decrease ($2^{-\Delta\Delta Ct} = 0.23$, $p < 0.001$, Figure 10C), LNCaP 5 μ M cells showed a 99% decrease ($2^{-\Delta\Delta Ct} = 0.009$, $p < 0.001$, Figure 10C), and LNCaP 10 μ M cells showed a 98% reduction ($2^{-\Delta\Delta Ct} = 0.019$, $p < 0.001$, Figure 10C) in *PSA* mRNA expression levels. In 3D, a significant reduction of 99.9% was observed in LNCaP 2.5 μ M cells ($2^{-\Delta\Delta Ct} = 0.000009$, $p < 0.001$, Figure 10D), LNCaP 5 μ M cells ($2^{-\Delta\Delta Ct} = 0.0001$, $p < 0.001$, Figure 10D) and LNCaP 10 μ M cells ($2^{-\Delta\Delta Ct} = 0.00007$, $p < 0.001$, Figure 10D).

4.2. *In silico* analysis

The miRTarBase database was used to identify the mRNA targets of the selected miRNAs. According to miRTarBase, there are a total of 4923 targets for the 6 miRNAs selected for the present study. However, we focused only on the 188 targets whose miRNA: mRNA interaction was validated by strong validation methods such as luciferase reporter assay, Western blot and qPCR (Supplementary Table 1).

Among the 188 validated target genes, *PTEN*, *VEGFA* and *SMAD7* were found to be the common targets of the largest number of miRNAs investigated (Figure 11). To further select which targets would be studied regarding their role in ENZ resistance acquisition, we focused on their potential biological role. Since the selected miRNAs seem to function as tumor suppressors, our focus will be exclusively on targets that may act as oncogenes, and only the levels of *VEGFA* and *SMAD7* will be further evaluated.

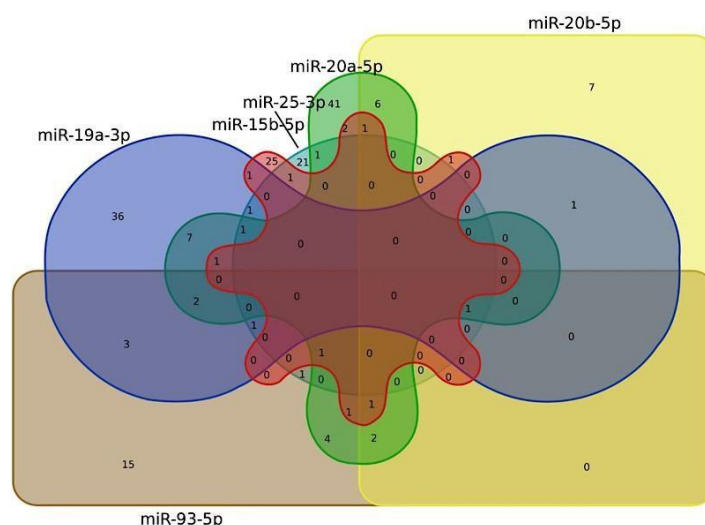


Figure 11 - Venn diagram representing the common targets of the deregulated miRNAs under study.

4.3. Expression dynamics of the selected miRNAs profile

4.3.1. Assembly and Characterization of ENZ-treated LNCaP Spheroids

Figure 12A shows bright field microscopy images of spheroids after 72 hours and 7 days in culture. Over time, spheroids formed by ENZ-resistant cells, like those formed by LNCaP cells, showed increased compaction and density, as evidenced by the darkening and reduction in spheroid size visible in the images. This pattern was consistent across all ENZ concentrations tested (Figure 12A).

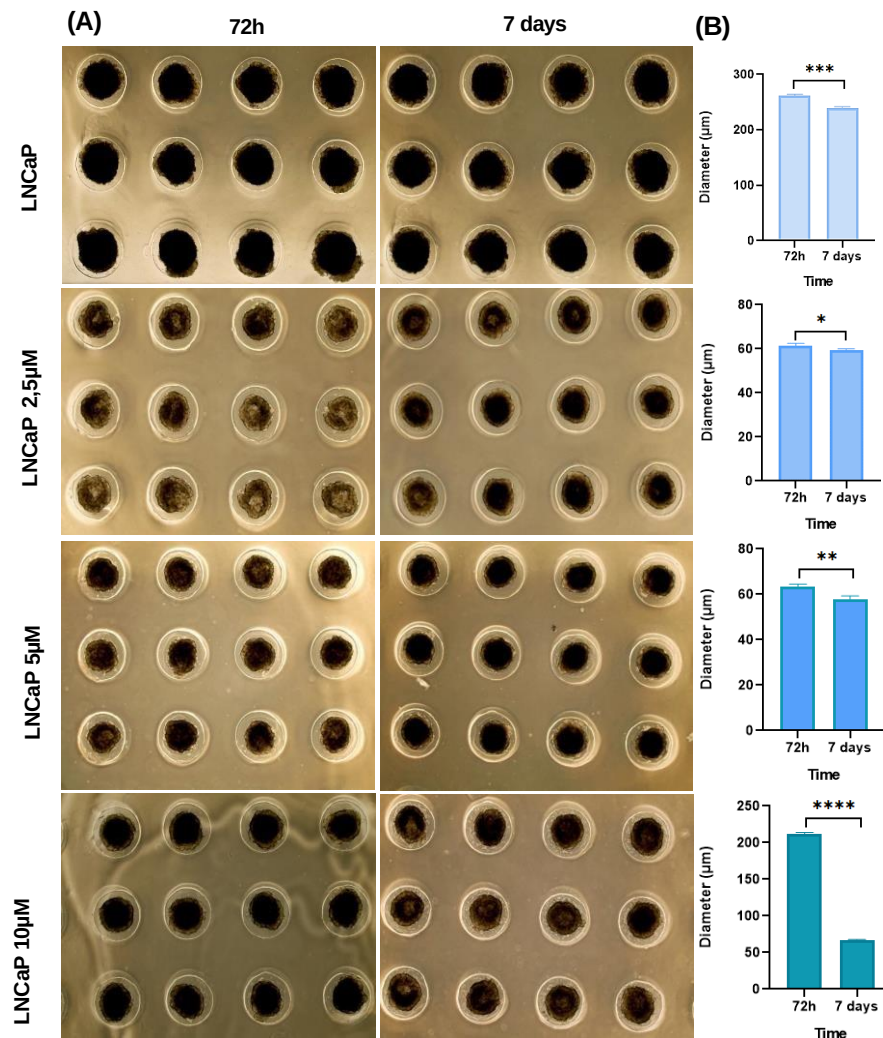


Figure 12 - (A) Microscopic images of LNCaP and ENZ-resistant LNCaP cells over time obtained with EVOS™ XL Core Imaging system (4x). **(B)** Spheroid diameter measurements (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

Both LNCaP cell-derived spheroids and those derived from ENZ-resistant LNCaP cells showed a marked decrease in diameter over time (Figure 12B). Significant reductions were observed between the 72h and 7 days timepoints for all conditions: LNCaP ($p = 0.0008$),

LNCaP with 2.5 μM ENZ ($p = 0.0455$), LNCaP with 5 μM ENZ ($p = 0.0063$) and LNCaP with 10 μM ENZ ($p < 0.0001$) (Figure 12B).

Figure 13 shows the metabolic activity of spheroids formed by LNCaP cells and ENZ-resistant LNCaP cells at different concentrations after 72 hours and 7 days, expressed as a fold change relative to the metabolic activity measured at 72 hours. The results suggest that the metabolic activity of spheroids derived from both LNCaP and ENZ-resistant LNCaP cells generally increases over 7 days compared to 72 hours. This trend is observed at most concentrations tested, including LNCaP ($p < 0.0001$), LNCaP 5 μM ($p < 0.0001$) and LNCaP 10 μM ($p = 0.0008$). However, no significant variations on metabolic activity were registered in LNCaP 2,5 μM spheroids ($p=0.2428$).

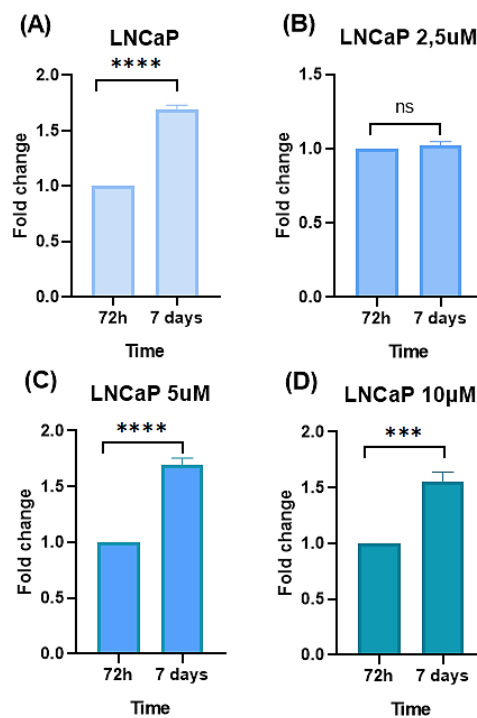


Figure 13 - Metabolic activity for **(A)** LNCaP; **(B)** LNCaP 2.5 μM ; **(C)** LNCaP 5 μM ; **(D)** LNCaP 10 μM over time in relation to 72h (fold change) (***: $p < 0.001$; ****: $p < 0.0001$).

4.3.2. Characterization of extracellular vesicles

The NTA analysis indicated that the isolated EVs from LNCaP, LNCaP 2.5 μ M, LNCaP 5 μ M and LNCaP 10 μ M presented a size range between 50 and 150 nm, which is consistent with the size of exosomes (Figure 14). The TEM showed rounded structures with a predominantly white appearance and size range in nanometers (Figure 14). This morphology and size are indicative of the EVs.

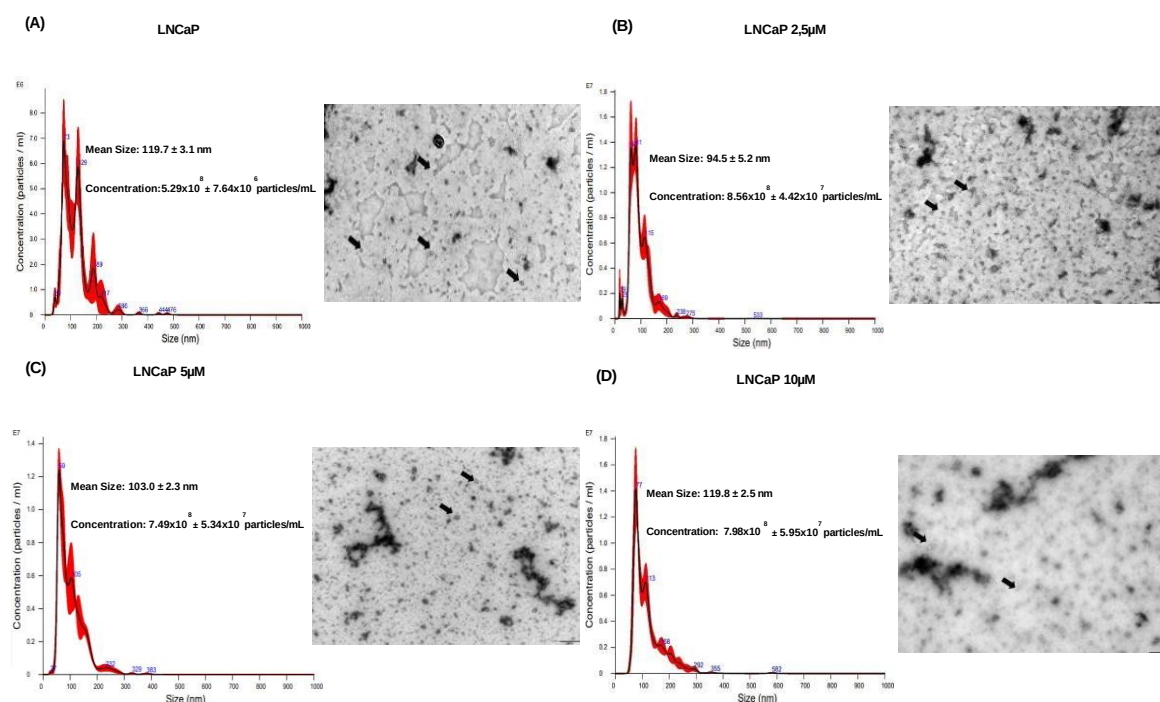


Figure 14 - Nanoparticle tracking analysis (NTA) and Transmission electron microscopy (TEM) (50000x) of EVs derived from **(A)** LNCaP, **(B)** LNCaP 2.5 μ M, **(C)** LNCaP 5 μ M and **(D)** LNCaP 10 μ M spheroids culture medium after 7 days of culture.

Furthermore, CD63 was also successfully detected in the EV samples using a Human CD63 ELISA assay (Table 2).

Table 2 - EV-CD63 concentration of LNCaP, LNCaP 2.5 μ M, LNCaP 5 μ M and LNCaP 10 μ M.

	concentration (pg/uL)
LNCaP	88,89
LNCaP 2.5 μ M	30,27
LNCaP 5 μ M	57,15
LNCaP 10 μ M	18,59

4.3.3. Relative quantification of the selected miRNAs profile performed both intracellularly and extracellularly during the progression of ENZ resistance

Figure 15 shows the variation of miRNA levels in 2D cell culture. When analyzing intracellular miRNA levels, cells resistant to increasing concentrations of ENZ consistently showed reduced expression of all miRNAs compared to LNCaP cells. For example, hsa-miR-15b-3p showed significant decreases in all ENZ-resistant cells (LNCaP 2.5 μ M: fold decrease 0.0003, $p < 0.001$; LNCaP 5 μ M: fold decrease 0.0004, $p < 0.001$; LNCaP 10 μ M: fold decrease 0.0004, $p = 0.001$ Figure 15A). Similar reductions were observed for hsa-miR-19a-3p (LNCaP 2.5 μ M: fold-decrease 0.0005, $p < 0.001$, LNCaP 5 μ M: fold-decrease 0.0005, $p < 0.001$, LNCaP 10 μ M: fold-decrease 0.0003, $p = 0.001$ Figure 15B), hsa-miR-20a-5p (LNCaP 2.5 μ M: fold-decrease 0.00001, $p < 0.001$, LNCaP 5 μ M: fold-decrease 0.00002, $p < 0.001$, LNCaP 10 μ M: fold-decrease 0.00002, $p = 0.001$ Figure 15C), hsa-miR-20b-5p (LNCaP 2.5 μ M: fold-decrease 0.00001, $p < 0.001$, LNCaP 5 μ M: 0.000009, $p < 0.001$, LNCaP 10 μ M: fold decrease: 5.46e-007, $p = 0.001$ Figure 15D), hsa-miR-25-3p (LNCaP 2.5 μ M: fold decrease: 0.0003, $p < 0.001$, LNCaP 5 μ M: fold decrease: 0.0004, $p < 0.001$, LNCaP 10 μ M: fold decrease: 0.00009, $p < 0.001$ Figure 15E) and hsa-miR-93-5p (LNCaP 2.5 μ M: fold decrease: 0.00001, $p < 0.001$, LNCaP 5 μ M: fold-decrease 0.00002, $p < 0.001$, LNCaP 10 μ M: fold-decrease 0.000006, $p = 0.001$ Figure 15-F) as ENZ resistance increased.

For EVs-derived miRNA levels, the results generally showed decreased expression in ENZ-resistant cells compared to LNCaP cells. For example, hsa-miR-20a-5p showed significant decreases in all ENZ-resistant cells (LNCaP 2.5 μ M: fold decrease 0.21, $p < 0.022$, LNCaP 5 μ M: fold decrease 0.04, $p < 0.001$, LNCaP 10 μ M: fold decrease 0.06, $p = 0.001$ Figure 15C). A similar pattern was observed for hsa-miR-20b-5p (LNCaP 2.5 μ M: fold decrease 0.16, $p = 0.005$, LNCaP 5 μ M: fold decrease 0.02, $p < 0.001$, LNCaP 10 μ M: fold decrease 0.01, $p < 0.001$ Figure 15D), hsa-miR-25-3p (LNCaP 2.5 μ M: fold decrease: 0.23, $p < 0.001$, LNCaP 5 μ M: fold decrease: 0.05, $p < 0.001$, LNCaP 10 μ M: fold decrease: 0.14, $p < 0.001$ Figure 15E) and hsa-miR-93-5p (LNCaP 2.5 μ M: fold decrease: 0.20, $p = 0.005$, LNCaP 5 μ M: fold decrease: 0.05, $p < 0.001$, LNCaP 10 μ M: fold decrease: 0.09, $p = 0.001$ Figure 15F). In addition, hsa-miR-15b-3p showed a significant reduction in LNCaP 5 μ M (fold decrease 0.09, $p = 0.001$ Figure 15A). Hsa-miR-19a-3p showed a significant increase in LNCaP 2.5 μ M compared to LNCaP cells (fold-increase: 1.94, $p < 0.001$ Figure 15B) and a downward trend in the expression levels of this miRNA in the other cells as ENZ resistance increased (LNCaP 5 μ M: fold-decrease 0.04, $p < 0.001$, LNCaP 10 μ M: fold-decrease 0.03, $p < 0.001$ Figure 15B).

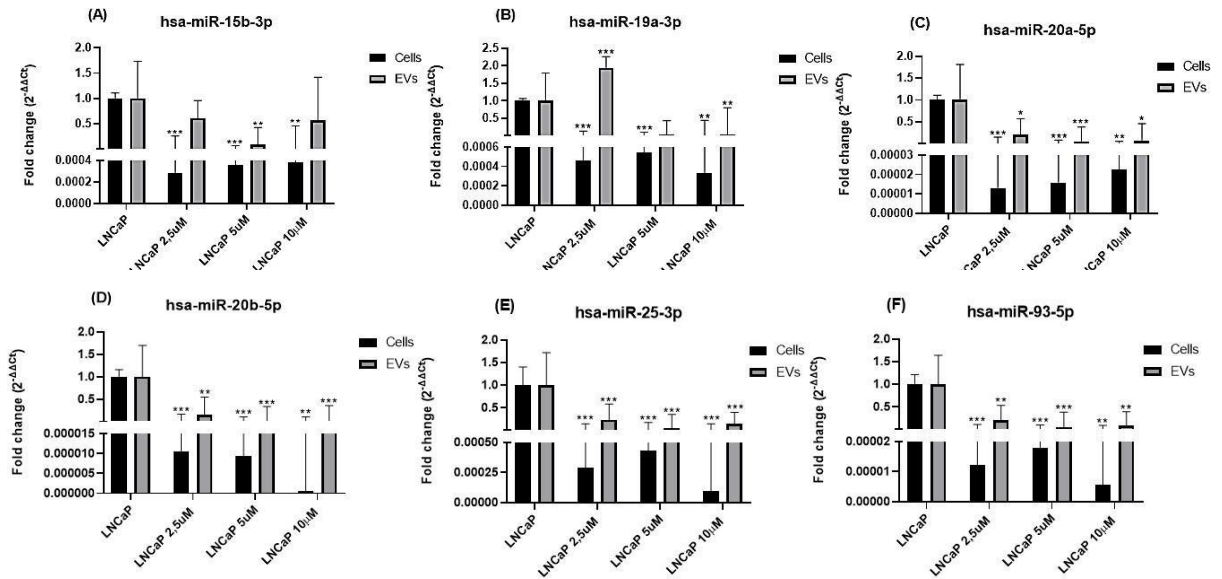


Figure 15 - Variation of the intracellular and EVs levels of **(A)** hsa-miR-15b-3p, **(B)** hsa-miR-19a-3p, **(C)** hsa-miR-20a-5p, **(D)** hsa-miR-20b-5p, **(E)** hsa-miR-25-3p and **(F)** hsa-miR-93-5p in 2D cell culture (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

Figure 16 shows the variation of miRNA levels in 3D cell culture, 7 days after spheroid seeding.

Interestingly, at the intracellular level, a different trend was observed in 3D culture, where several miRNAs showed increased expression. For example, hsa-miR-15b-3p and hsa-miR-19a-3p were significantly upregulated at lower ENZ concentrations (LNCaP 2.5 μM: fold-increase 14.17 and 12.59, respectively, $p < 0.001$ Figure 16A,B), although hsa-miR-19a-3p was downregulated at the highest ENZ concentration (LNCaP 10 μM: fold-decrease 0.44, $p < 0.001$ Figure 16B). Additionally, hsa-miR-20a-5p followed a similar pattern with increased expression at lower ENZ concentrations (LNCaP 2.5 μM: fold-increase: 8.69, $p < 0.001$ Figure 16C) and decreased expression at the highest concentration (LNCaP 10 μM fold-decrease: 0.54, $p = 0.002$ Figure 16C). Hsa-miR-20b-5p showed decreased expression with increasing ENZ resistance (LNCaP 5 μM: fold decrease: 0.58, $p < 0.001$, LNCaP 10 μM: fold decrease: 0.12, $p < 0.001$ Figure 16D). However, although not statistically significant, we observed that hsa-miR-20b-5p levels also showed a tendency to increase at the concentration of 2.5 μM ($p = 0.11$ Figure 16D). Interestingly, hsa-miR-93-5p in 3D showed mixed results, with a significant increase in LNCaP 5 μM (fold-increase: 39.40, $p < 0.001$ Figure 16F), but only a slight decrease at the highest concentration LNCaP 10 μM (fold-decrease: 0.88, $p = 0.001$ Figure 16F) compared to LNCaP cells. LNCaP 2.5 μM also showed an increase in the expression levels of this miRNA in 3D, but this increase was not statistically significant ($p = 0.339$ Figure 16F).

However, when compared to 2D culture, in 3D culture EV-derived miRNAs showed a more complex behavior. Hsa-miR-15b-3p showed a significant increase in LNCaP 5 μ M (fold increase:14.89, $p<0.001$ Figure 16A). The same was observed for hsa-miR-93-5p (fold increase: 5.18, $p=0.013$ Figure 16F). Hsa-miR-15b-3p (fold-decrease: 0.43, $p=0.045$ Figure 16A), hsa-miR-19a-3p (fold-decrease: 0.42, $p<0.001$ Figure 16B), hsa-miR-20b-5p (fold-decrease: 0.04, $p<0.001$ Figure 16D) and hsa-miR-93-5p (fold-decrease: 0.32, $p=0.024$ Figure 16F) showed a significant reduction in LNCaP 5 μ M. Hsa-miR-19a-3p (fold decrease 0.24, $p=0.016$ Figure 16B), hsa-miR-25-3p (fold decrease: 0.22, $p=0.017$ Figure 16E) and has-miR-93-5p (fold decrease: 0.21, $p=0.005$ Figure 16F) showed a significant reduction in LNCaP 2.5 μ M. Interestingly, has-miR-20a-5p shows in 3D the same trend as in 2D, with all cells resistant to different concentrations of ENZ showing a decrease in expression of this miRNA compared to LNCaP cells (LNCaP 2.5 μ M: fold-decrease 0.26, $p<0.021$, LNCaP 5 μ M: fold-decrease 0.12, $p<0.002$, LNCaP 10 μ M: fold-decrease 0.14, $p=0.001$ Figure 16C).

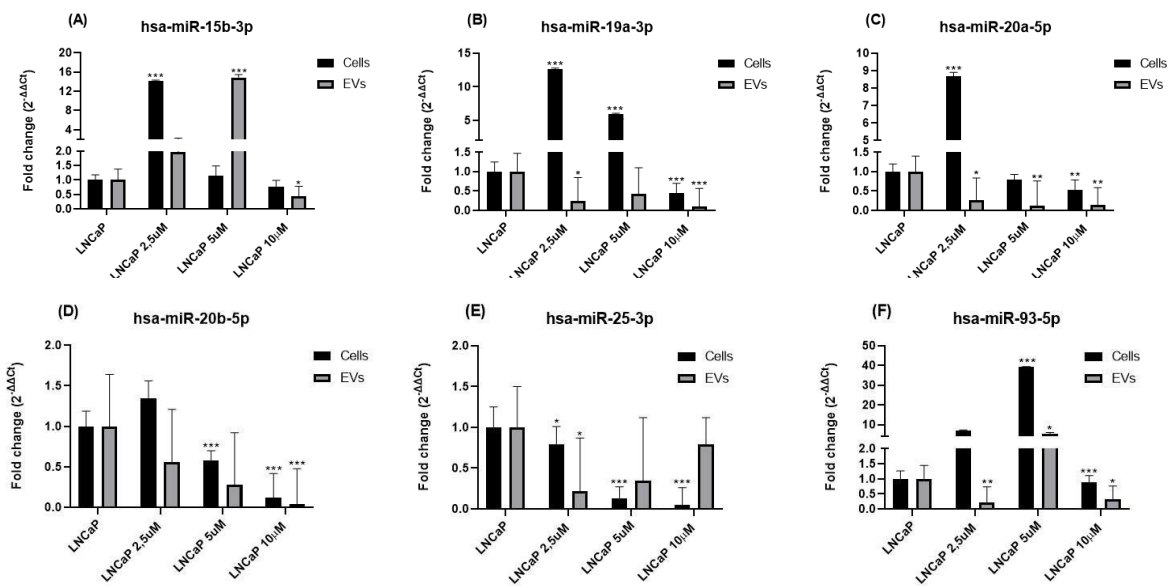


Figure 16 - Variation of the intracellular and EVs levels of (A) hsa-miR-15b-3p, (B) hsa-miR-19a-3p, (C) hsa-miR20a-5p, (D) hsa-miR-20b-5p, (E) hsa-miR-25-3p and (F) hsa-miR-93-5p in 3D cell culture (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$).

4.3.4. Relative quantification of VEGFA and SMAD7 mRNA target genes

VEGFA and SMAD7, identified as common targets of most of the miRNAs analyzed, were quantified in both 2D and 3D cultures of LNCaP cells and ENZ-resistant LNCaP cells.

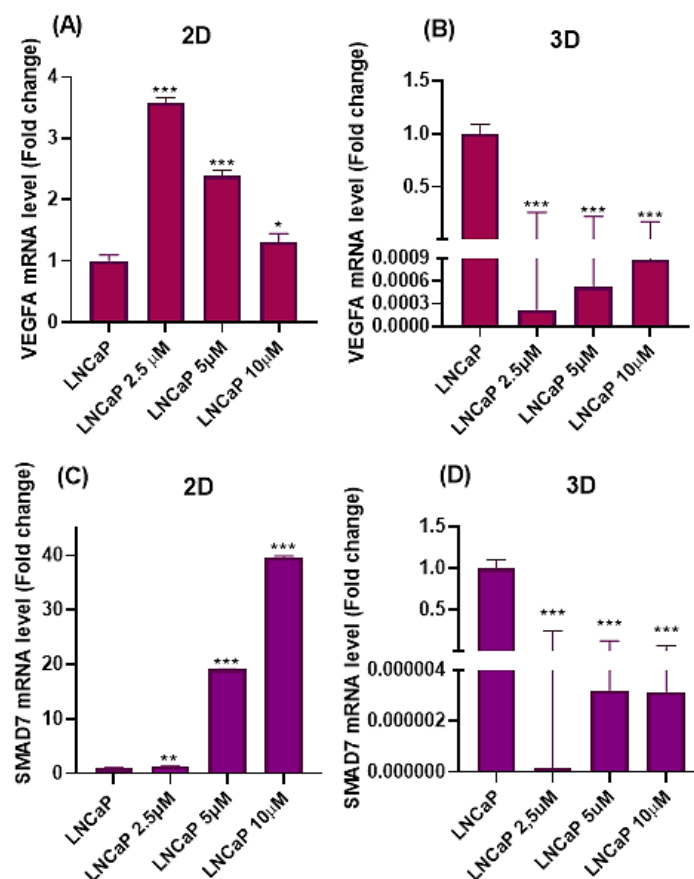


Figure 17 - Graphical representation of the relative quantification of the expression levels of **(A)** VEGFA mRNA in 2D, **(B)** VEGFA mRNA in 3D, **(C)** SMAD7 mRNA in 2D and **(D)** SMAD7 mRNA in 3D by qPCR in LNCaP and cells resistant to each of the established concentrations of ENZ (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

The results show that in 2D cell culture, VEGFA mRNA expression increased at all levels of ENZ resistance compared to LNCaP cells. Specifically, LNCaP 2.5 μM cells showed a 3 fold increase in VEGFA mRNA levels ($2^{-\Delta\Delta C_t} = 3.57$, $p < 0.001$ Figure 17A), while LNCaP 5 μM and 10 μM cells showed increases of 2 fold increase ($2^{-\Delta\Delta C_t} = 2.38$, $p < 0.001$ Figure 17A) and 1 fold increase ($2^{-\Delta\Delta C_t} = 1.31$, $p = 0.037$ Figure 17A), respectively. In contrast, VEGFA mRNA expression was significantly decreased in 3D cell culture at all levels of ENZ resistance. LNCaP 2.5 μM cells showed a reduction of 99.98% ($2^{-\Delta\Delta C_t} = 0.0002$, $p < 0.001$ Figure 17B), while LNCaP 5 μM and 10 μM cells showed a reduction of 99.95% ($2^{-\Delta\Delta C_t} = 0.0005$, $p < 0.001$ Figure 17B) and 99.91% ($2^{-\Delta\Delta C_t} = 0.0009$, $p < 0.001$ Figure 17B), respectively.

Similarly, *SMAD7* mRNA expression in 2D cell culture increased significantly as ENZ resistance developed, with LNCaP 2.5 μ M cells showing an increase ($2^{-\Delta\Delta Ct} = 1.28$, $p=0.007$ Figure 17C). LNCaP 5 μ M and 10 μ M cells showed even greater increases of 19 fold increase ($2^{-\Delta\Delta Ct} = 19.07$, $p<0.001$ Figure 16C) and 40 fold increase ($2^{-\Delta\Delta Ct} = 39.68$, $p=0.001$ Figure 17C), respectively. However, in 3D cultures, *SMAD7* mRNA expression decreased at all levels of ENZ resistance. LNCaP 2.5 μ M cells showed a 99.99% reduction ($2^{-\Delta\Delta Ct} = 1.52e-007$, $p<0.001$ Figure 17D), with LNCaP 5 μ M and 10 μ M cells also showing a 99.99% reduction ($2^{-\Delta\Delta Ct} = 0.000003$ and $2^{-\Delta\Delta Ct} = 0.000003$, $p<0.001$ Figure 17D).

4.4.Effect on expression levels of hsa-miR-20a-5p, hsa-miR-25-3p and their targets in 3D culture by transfection of miRNA mimics

Based on results mentioned above, hsa-miR-20a-5p and hsa-miR-25-3p were selected for the transfection assay once their 3D cell culture behavior was the most consistently downregulated among different concentrations, both in cells and in extracellular vesicles, reinforcing their potential as reliable candidates for further investigation. In an effort to restore hsa-miR-20a-5p and hsa-miR-25-3p expression levels and potentially reverse the ENZ resistance phenotype, a chemical method through an organic carrier (Lipofectamine™ RNAiMAX) and mimic technology was used. Although limited by an insufficient number of replicates, which precluded comprehensive statistical analysis, the results presented in Figure 18 indicate a successful transfection.

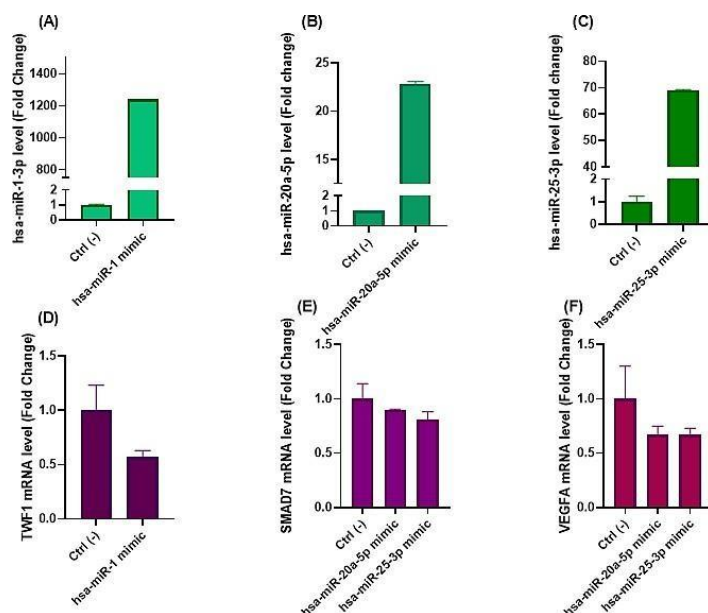


Figure 18 - Graphical representation of the relative quantification of the expression levels of (A) hsa-miR-1, (B) hsa-miR-20a-5p, (C) hsa-miR-25-3p, (D) TWF1, (E) SMAD7 and (F) VEGFA after transfection, when compared to negative control.

The transfection of hsa-miR-1, used as a positive control, resulted in a marked upregulation of the expression levels of this miRNA following the introduction of its mimic (Figure 18A). At the same time, a pronounced reduction in the expression of its known target, TWF1, was observed, confirming the efficiency of the transfection process (Figure 18D).

Similar results were obtained with the transfection of hsa-miR-20a-5p and hsa-miR-25-3p mimics (Figure 18B, C). After transfection, both microRNAs showed a considerable increase in expression levels, which was coupled with a corresponding downregulation of their target genes, *VEGFA* and *SMAD7* (Figure 18E, F). These results highlight the effective transfection and functional impact of the introduced microRNA mimics on their target pathways.

Figure 19A shows bright field microscopy images of the spheroids used in the transfection assay taken at two timepoints: immediately post-transfection (0h) and 48h after transfection.

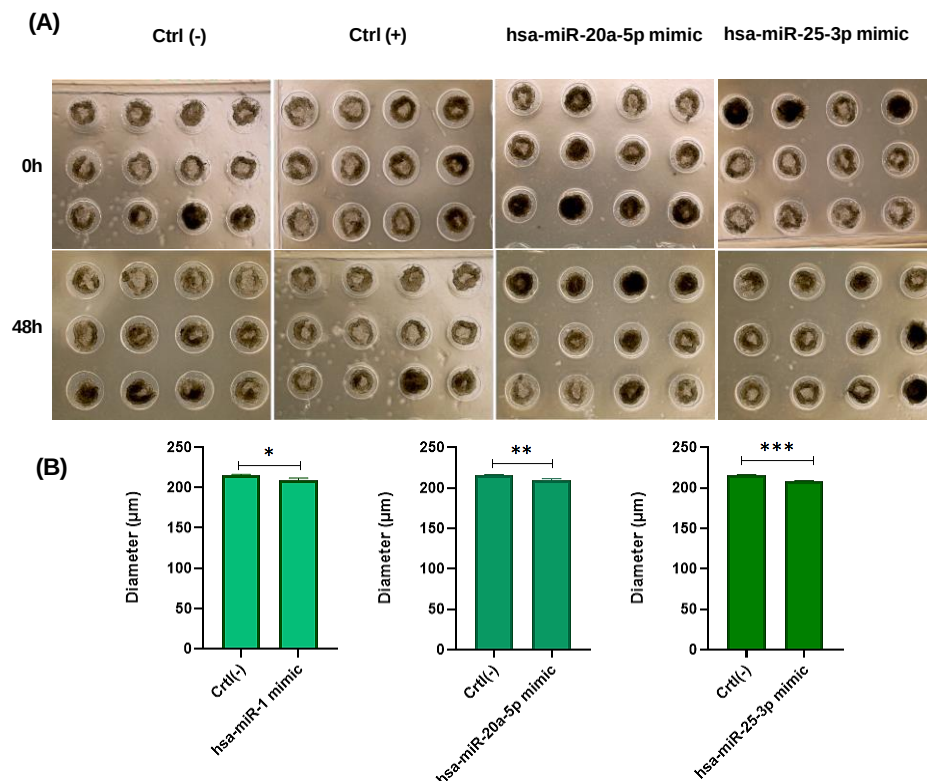


Figure 19 - (A) Microscopic images of each condition tested immediately after transfection (0h) and 48h after transfection, obtained with EVOS™ XL Core Imaging system (4x). **(B)** Spheroid diameter measurements (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

All tested conditions showed a reduction in diameter compared to the negative control (Figure 18B). Significant reductions were observed between the negative control and the following conditions: hsa-miR-1 mimic ($p = 0.0203$), hsa-miR-20a-5p mimic ($p = 0.0064$), and hsa-miR-25-3p mimic ($p = 0.0007$) (Figure 18B).

Figure 20 shows the metabolic activity of spheroids formed by 2.5 μ M LNCaP cells used for transfection, expressed as a fold change relative to the metabolic activity of the negative control, based on luminescence values.

The results suggest that the metabolic activity of spheroids of each tested condition decreases compared to negative control. This trend is observed in hsa-miR-20a-5p mimic and hsa-miR-25-3p ($p < 0.0001$ Figure 20A, B).

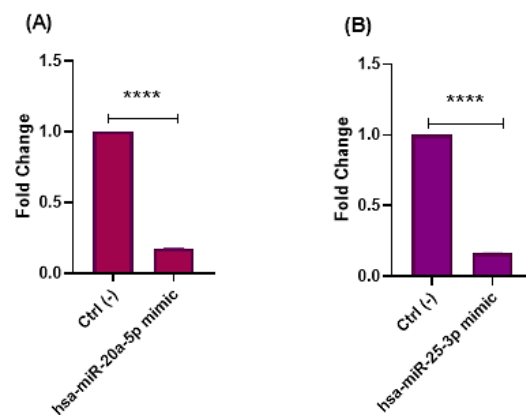


Figure 20 - Metabolic activity for **(A)** has-miR-20a-5p mimic and **(B)** has-miR-25-3p mimic in relation to negative control (fold change) (****: $p < 0.0001$).

Discussion

5. Discussion

The development of the second-generation anti-androgen ENZ, used as the primary therapeutic choice for patients facing recurrence, has significantly improved survival rate among these patients [151]. Despite this progress, a noteworthy percentage of patients with CRPC exhibit primary resistance or eventually develop resistance to this treatment [154]. This highlights an urgent need to explore alternative treatment options and identify new predictive biomarkers for therapy response. Current studies emphasize the multifaceted role of EVs in modulation of the TME and drug resistance acquisition [197]. Moreover, miRNAs derived from EVs have been implicated in the development of resistance to ADT and second-generation anti-androgens such as ENZ, placing them as potential biomarkers for diagnostic, prognostic, and monitoring therapeutic responses [198-200]. There are several scientific arguments for the use of EV-derived miRNAs rather than circulating miRNAs. First, miRNAs found in EVs are more stable because they are protected from degradation and fluctuations in the bloodstream, making them more reliable for diagnosis and prognosis [201]. Additionally, these miRNAs are released in response to the specific cellular environment, providing useful insights into how tumors respond to treatment [201]. This is particularly important for understanding drug resistance. Furthermore, the selective loading of miRNAs into EVs allows for a more accurate analysis of miRNA signatures associated with different therapeutic responses, helping to identify predictive biomarkers [202].

Therefore, the investigation of EV-derived miRNA-mediated mechanisms associated with ENZ resistance is essential for the development of new therapeutic strategies. Identification of these miRNAs can lead to the discovery of potential new biomarkers and to the development of RNA-based therapies targeting miRNA deregulation, thereby addressing the urgent need for effective treatments in CRPC.

In the present study, our previously established ENZ-resistant cell line showed significantly higher metabolic activity compared to LNCaP cells, corroborating findings in the existing literature [203, 204]. Poku and colleagues studied the enzyme PMPMEase (polyisoprenylated methylated protein methyl esterase) in PCa cells and observed that androgen-resistant cells had significantly increased metabolic activity compared to sensitive cells, suggesting a link between increased metabolic activity and drug resistance [203]. Furthermore, Verma et al. showed that resistance in ENZ-treated PCa cells, such as LNCaP, is often associated with metabolic alterations, including increased glycolysis and altered lipid availability [204]. These alterations suggest a different metabolic profile in resistant

cells compared to sensitive cells. Ultimately, these studies highlight metabolic reprogramming as a key feature of ENZ resistance in PCa.

Additionally, the cells used in the present study were quantified for their mRNA expression levels of *AR* and *PSA*, both in 2D and 3D cultures. Interestingly, the trend observed in 2D remained consistent in 3D. All the studied cells showed a decrease in *AR* mRNA expression compared to LNCaP cells, except for the LNCaP 2.5 μ M cells. Regarding *PSA* mRNA expression levels, it was observed that all the concentrations studied showed a reduction in *PSA* mRNA expression compared to LNCaP cells. Overall, the results obtained are consistent with what is described in the literature and demonstrate that ENZ is effectively blocking the *AR* signaling pathway, and consequently the *PSA* pathway, suggesting that these cells have become resistant through *AR*-independent mechanisms. Feng T. and colleagues corroborate these results by reporting a decrease in *AR* and *PSA* mRNA expression levels, among other genes, in ENZ-resistant cells compared to sensitive cells [205]. On the other hand, Hoefer and his team created an ENZ-resistant cell line that utilized mechanisms involving increased *AR* expression, allowing the cells to remain dependent on the *AR* pathways [206]. These results suggest that LNCaP cells have the ability to employ multiple resistance mechanisms in response to ENZ treatment, rather than relying on a single, pre-established mechanism specific to their cell type. Moreover, a significant increase in *AR* expression levels was observed in cell lines exposed to the lowest concentration of ENZ, suggesting that its administration has an immediate effect on the cells. We propose that this initial increase in *AR* expression may serve as a compensatory mechanism by which cells attempt to offset the inhibitory effects of ENZ on the *AR* pathway, possibly in response to the selective pressure imposed within the cellular microenvironment.

As 3D models closely mimic the TME, they provide more accurate evidence of the mechanisms of resistance to therapy, including ENZ [190]. These models facilitate the study of EV dynamics and miRNA expression in a context that reflects the complex interactions and gradients found in solid tumors [191-193]. This approach is fundamental for identifying key molecular biomarkers and resistance mechanisms that are often not detected in 2D cell culture. Therefore, 3D spheroid models formed by cells resistant to different concentrations of ENZ were established to mimic the resistance development process.

All spheroids, whether composed of LNCaP cells or LNCaP ENZ-resistant cells, showed increased compaction and density, as evidenced by their darkening and reduced size. This darkening was suggestive of hypoxic core formation, a feature commonly observed in advanced tumors. Similar results have been reported by other researchers [207] [208] [209]. For example, Cavo M. and colleagues, using different spheroid assembly techniques,

observed that spheroids underwent compaction during the first 10 days of culture, as evidenced by a decrease in diameter along with an increase in darkness and cell density [207]. Gibot et al. also reported similar findings, noting that smaller spheroids (<300 μm) showed a marked reduction in growth over four days, however larger spheroids (650 μm) remained unaffected under the same conditions [208]. These results highlight the critical role of spheroid size in influencing their behavior and response to environmental conditions. Additionally, Godet and colleagues report that spheroids can develop a hypoxic core similar to tumors, making them good study models [209]. Regarding metabolic capacity, an increase was observed from 72 hours to 7 days in all cell lines evaluated. These results are not consistent with what is described in the literature. The literature suggests that ENZ-resistant cells are less glycolytic than parental LNCaP cells [210]. This is because when LNCaP cells are treated with ENZ, they switch from glycolysis to mitochondrial oxidative phosphorylation, which increases their demand for oxygen and the production of ATP [210]. Although this switch helps the cells to proliferate and survive in oxygen-rich conditions, it can become a disadvantage in low-oxygen environments, such as the hypoxic core of spheroids. However, we hypothesized that this increase in metabolic capacity was due to the increase in cell numbers over the days as a result of the more aggressive resistance phenotype exhibited by these cells.

The *in silico* analysis revealed that VEGFA and SMAD7 are common targets of the largest number of miRNAs under study. Notably, the expression dynamics of these targets varied significantly between 2D and 3D models, likely due to the distinct physical and structural conditions of each culture type. These differences may have profound effects on signaling pathways and gene expression patterns [211].

In 2D, the results were as expected, as increased levels of VEGFA were observed in the ENZ-resistant cells compared to LNCaP cells, and VEGFA is a validated target of the miRNAs under study. However, this trend was reversed in 3D models. While the specific expression dynamics of VEGFA in ENZ-resistant CRPC remain poorly defined in the literature, there is emerging evidence suggesting elevated levels of VEGFA in this cancer subtype [212]. Given its critical role in promoting angiogenesis, tumor survival and progression, such an increase is expected [213]. As tumors acquire resistance to ENZ, they may exhibit increased VEGFA expression, contributing to a more aggressive and treatment-resistant phenotype. This overexpression of VEGFA creates a microenvironment that promotes angiogenesis, facilitating tumor growth and allowing cancer cells to evade the effects of AR-targeted therapies. Indeed, Maxwell and colleagues demonstrated that co-inhibition of IL-8 and VEGFA restored tumor response in the presence of ENZ, highlighting the functional significance of their elevated expression in ENZ-resistant models [212].

Furthermore, the combined inhibition of IL-8 and VEGFA resulted in sustained and effective resolution of ENZ-sensitive prostate tumors [212].

Thus, the results obtained from 2D cultures are consistent with what is described in the literature, whereas the findings from 3D cultures present a contrasting narrative. Nonetheless, it is possible to observe that within the ENZ-resistant cells, there is an increase in VEGFA expression levels as the acquisition of resistance to ENZ progresses. Therefore, we hypothesize that these results reflect the immediate impact of ENZ administration on the cells, with a progressive increase in VEGFA expression as resistance is acquired, ultimately supporting tumor survival and progression.

The results obtained for SMAD7 were similar to those observed for VEGFA. In 2D, as expected, SMAD7 levels increased in ENZ-resistant cells compared to LNCaP cells, supporting that SMAD7 is a validated target of the miRNAs under study. In 3D, decreased levels of VEGFA were observed in the ENZ-resistant cells compared to LNCaP cells. Although the literature has not yet described the dynamics of SMAD7 expression in ENZ-resistant CRPC, we hypothesize that this finding may be explained by the crucial role of the TGF- β signaling pathway in cancer progression, metastasis, and therapy resistance [214].

Indeed, RNA-seq data from circulating tumor cells showed significant upregulation of the TGF- β and cyclin D1 pathways in drug-resistant circulating tumor cells [215]. Moreover, key regulators of the TGF- β pathway, such as SMAD3 and cyclin D1, have been identified as important players in ENZ resistance [215]. Additionally, Paller and colleagues showed that, with a single treatment with ENZ, the addition of the TGF- β receptor I kinase inhibitor galunisertib could increase the efficacy of ENZ in inhibiting tumor growth and metastasis in CRPC xenografts [216].

The TGF- β pathway plays a dual and complex role in cancer: while it acts as a tumor suppressor in the early stages of the disease by inhibiting cell proliferation and promoting apoptosis, in advanced stages, many tumors, including PCa, co-opt this pathway to drive malignancy, metastasis, and therapy resistance [214]. Given that SMAD7 inhibits TGF- β signaling, we propose that it may indirectly contribute to ENZ resistance by regulating alternative survival pathways, which are frequently activated in CRPC.

Based on the contrasting results between 2D and 3D, we can assume that, while VEGFA and SMAD7 have been shown to be targets of the miRNAs in study, they are not exclusively regulated by them. Other molecules may probably contribute to their regulation, possibly overlapping with miRNA activity, which emphasizes the complex and multifactorial nature of gene expression control. Nevertheless, these results show the importance of using 3D models to study molecular mechanisms in therapy resistance acquisition, since they bring

a context that more accurately mimics the complex interactions and gradients present in solid tumors, and their results defer from the traditional 2D *in vitro* cells study.

In the same way, the results obtained from quantifying the intracellular and EV-derived miRNAs studied also highlight the importance of using 3D models to study miRNA expression dynamics. Notably, the expression patterns of these miRNAs differed significantly between 2D and 3D models, with certain miRNAs exhibiting altered regulatory behavior in the 3D environment. This highlights the importance of 3D models in revealing the subtle behavior of miRNAs in a more physiologically representative tumor environment.

Although the expression dynamics of hsa-miR-15b-3p, hsa-miR-19a-3p, hsa-miR-20a-5p, hsa-miR-20b-5p, hsa-miR-25-3p and hsa-miR-93-5p in ENZ-resistant CRPC have not yet been described in the literature, previous studies have associated these miRNAs with PCa [217], [218], [219], [220], [221], [222].

In 2D models, hsa-miR-15b-3p showed decreased intracellular expression levels in ENZ-resistant cells compared to LNCaP cells. A similar expression pattern was observed in 3D models, except for ENZ-resistant cells at the lower ENZ concentration, which exhibited increased intracellular expression compared to LNCaP cells. This suggests that ENZ administration results in an acute and immediate effect on the cells, so we hypothesize that the initial spike in miRNA expression may be a compensatory mechanism used by the cells to counteract the inhibitory effects of ENZ, which may be a response to the selective pressure introduced into the cellular microenvironment. However, the expression dynamics of this miRNA derived from EVs was particularly interesting. In 2D, LNCaP 5 μ M cells showed decreased expression compared to LNCaP cells, while in 3D, the opposite was observed, with LNCaP 5 μ M cells showing increased expression compared to LNCaP. Previous studies have linked this miRNA to resistance against another anti-androgen, bicalutamide (BIC), in PCa [217]. Zhang and colleagues explored how miR-15b-3p modulates ferroptosis in response to BIC resistance and its potential as a biomarker for early PCa screening. They found that BIC treatment reduced miR-15b-3p expression levels [217]. Moreover, administering a miR-15b-3p mimic or inhibitor weakened or enhanced BIC sensitivity, respectively, indicating that miR-15b-3p plays a crucial role in BIC resistance [217]. Therefore, a decrease in hsa-miR-15b-3p expression levels is associated with drug resistance, which is consistent with the results observed in the analysis performed in the present study, regarding the EVs miRNA content, strengthening the potential of EVs miRNAs as biomarkers of resistance to therapy.

In 2D models, hsa-miR-19a-3p showed reduced intracellular expression in ENZ-resistant cells compared to LNCaP cells. A similar pattern was observed in 3D models, except that

ENZ-resistant cells at lower ENZ concentrations exhibited increased intracellular expression compared to LNCaP cells. Similar to what was observed for hsa-miR-15b-3p, this suggests that ENZ administration triggers an acute response, where the initial rise in miRNA expression may act as a compensatory mechanism to counter the ENZ inhibitory effects. Regarding EVs-Derived miRNAs, in 2D, LNCaP cells treated with 2.5 μ M ENZ showed higher expression than untreated LNCaP cells, while in 3D, treated cells showed reduced expression of this miRNA. Previous studies, such as Wa et al., found that miR-19a-3p expression significantly decreases in bone metastatic PCa tissues and cells [218]. Furthermore, upregulating miR-19a-3p was shown to suppress, while silencing it promoted, invasion and migration *in vitro* [218]. These findings previously observed by other researchers strengthen the results of the present study, specifically the EV-derived miRNAs dynamics, which are consistently reduced in the 3D setting, showing that hsa-miR-19a-3p is downregulated in ENZ-resistant CRPC, has a tumor-suppressor role in this context and may be a potential biomarker of resistance.

Regarding EVs-derived miRNAs, hsa-miR-20a-5p showed a consistent pattern in both 2D and 3D models, with all ENZ-resistant LNCaP cells presenting lower expression levels compared to LNCaP cells. Intracellularly, the trend remained in 2D, with reduced expression in ENZ-resistant cells. However, in 3D, ENZ-resistant cells at the lowest concentration of ENZ exhibited higher miRNA expression compared to LNCaP cells. This suggests that ENZ treatment triggers an immediate cellular response, and the initial increase in miRNA expression may act as a compensatory mechanism against ENZ's inhibitory effects. Previous research has linked this miRNA to biochemical failure in PCa [219]. Stoen et al. found that high miR-20a-5p expression was negatively associated with biochemical failure, and overexpression in DU145 and PC3 cells enhanced migration and invasion [219]. While this study positions hsa-miR-20a-5p as an oncomiRNA, it does not explain its downregulation in ENZ-resistant CRPC. However, numerous studies have looked at the effect of miR-20a-5p on other carcinomas, reporting that overexpression or deregulation of hsa-miR-20a-5p can affect downstream signaling pathways such as PI3K-Akt, MAPK and TGF- β [223-225]. ENZ resistance can also arise from non-AR-dependent pathways, such as the PI3K-Akt-mTOR pathway, which regulates key processes like cell growth, proliferation, protein synthesis, and programmed cell death [226]. Aberrant activation of this pathway has been linked to antiandrogen drug resistance [226]. Moreover, key regulators of the TGF- β pathway have been identified as important players in ENZ resistance [215]. Therefore, further research into these pathway may reveal new insights and explain our results.

Hsa-miR-20b-5p showed decreased expression levels compared to LNCaP cells, both in 2D and 3D, intracellularly and EVs-Derived miRNAs. This miRNA has been little described in PCa. However, previous studies have reported an association between this miRNA and the EMT transition in PCa [220]. Qi et al. investigated whether and how TGF- β 1 regulates EMT in PCa cells through modulation of the regulatory cycle involving TGFBR2, E2F1 and miR-20b-5p [220]. They found that the expression level of miR-20b-5p was significantly lower in PC3 and DU145 cells than in prostate epithelial cells (RWPE-1) [220]. Furthermore, functional studies revealed that miR-20b-5p plays a critical role in suppressing TGF- β 1-induced migration and invasion by up-regulating E-cadherin and down-regulating vimentin, thereby inhibiting the EMT process [220]. Reduced levels of hsa-miR-20b-5p might potentiate the overexpression of TGF- β 1, which can have an impact on cell proliferation, cell cycle regulation, EMT modulation and acquisition of resistance to treatment, explaining the results obtained.

The results obtained for hsa-miR-25-3p showed a decrease in the expression levels of this miRNA in ENZ-resistant cells compared to LNCaP cells at the intracellular level and in EVs-derived miRNAs levels in 2D. However, the same was not observed in 3D, where it was only possible to see a decrease in the expression levels of this miRNA in ENZ-resistant cells compared to LNCaP cells at the intracellular level. Zoni et al. showed that hsa-miR-25-3p is, among other miRNAs, upregulated in PCa [227]. Furthermore, previous studies have reported that miR-25, which is part of the miR-106b-25 cluster, is upregulated in primary tumors and distant metastasis in PCa [228]. Additionally, Pudova and colleagues demonstrated that hsa-miR-25-3p is associated with lymphatic dissemination, as this miRNA is overexpressed in N1 tumors compared to N0 [221]. These studies present hsa-miR-25-3p as oncogenic, which does not explain the results obtained. These results indicate a potentially distinct role for hsa-miR-25-3p in the context of ENZ-resistant CRPC, suggesting that although it is typically characterized as an oncogenic miRNA in other tumor types, it may function differently in this specific setting, requiring further investigation of its mechanisms and implications in PCa biology.

Lastly, hsa-miR-93-5p showed decreased expression levels in ENZ-resistant cells compared to LNCaP cells, both intracellularly and EV-derived miRNAs, in 2D. Previous research has shown that this miRNA acts as an oncomiRNA in PCa [222, 229]. Yang et al. reported high levels of miR-93-5p in PCa samples compared to normal samples [229]. They also demonstrated that low expression of miR-93-5p can inhibit cell proliferation, migration, invasion, modify the cell cycle and promote early apoptosis of PC-3 cells [229]. In addition, Zhiyanov and colleagues demonstrated that high expression of hsa-miR-93-5p is

associated with cancer aggressiveness and that FBXO31 and MFN2 are targets of this miRNA [222].

Although these studies position hsa-miR-93-5p as an oncomiRNA, they do not explain its negative regulation in ENZ-resistant CRPC. However, FBXO31, a known target of hsa-miR-93-5p, activates ERK and suppresses PI3K-AKT-mediated signaling pathways in PCa [230]. ENZ resistance can also result from non-AR dependent pathways, such as the PI3K-Akt-mTOR pathway, which regulates key processes such as cell growth, proliferation, protein synthesis and programmed cell death [154]. Deregulation of this pathway has been closely linked to resistance against anti-androgen therapies [154]. Given that FBXO31 negatively regulates the PI3K-Akt pathway, its deregulation by decreasing miR-93-5p activity could lead to increased PI3K-Akt signaling, contributing to the resistance mechanisms observed in ENZ-resistant CRPC, thus explaining the results obtained in 2D. Interestingly, in 3D, this miRNA showed a more complex behavior with an irregular dynamic along the concentrations studied. This highlights the importance of using 3D settings that better mimic the complexity of tumor behavior and that can help to distinguish and better select the most promising miRNAs to be tested in a clinical setting.

In an effort to restore the expression levels of miRNAs and potentially reverse the ENZ resistance phenotype, hsa-miR-20a-5p and hsa-miR-25-3p were selected for the transfection assay. This choice was based on the fact that the behavior of these miRNAs in 3D cell culture was the most consistently deregulated among the different concentrations, both in cells and in extracellular vesicles, reinforcing their potential as reliable candidates for further investigation. The transfection of these miRNAs in prostate cancer, in 3D cultures and with the method used, has not been reported in the literature. Indeed, the existing literature on 3D transfection models is limited, and the rare studies that do explore this field consistently report low transfection efficiencies, highlighting the significant challenges in achieving effective miRNA delivery in 3D environments [231]. This highlights the technical complexity of 3D transfection, an area that remains underexplored and requires further research to overcome these challenges. The transfection of these miRNAs was successful, as evidenced by the increased expression levels of hsa-miR-20a-5p and hsa-miR-25-3p, along with the decreased expression of their targets, VEGFA and SMAD7, following the administration of their mimics compared to the negative control. Interestingly, we observed a significant reduction in spheroid diameter and decreased metabolic activity compared to the negative control, after transfection, which suggests that the increased expression of these miRNAs, induced by the mimics, led to reduced cell proliferation, which may be associated to a reversion of the aggressive phenotype associated with ENZ resistance. Therefore, this transfection methodology successfully applied in a 3D setting shows the

potential of future therapies based in restoring miRNAs levels as a way to revert NEZ resistance.

In conclusion, our findings highlight the potential of EV-derived hsa-miR-20a-5p, hsa-miR-20b-5p and hsa-miR-25-3p as reliable predictive biomarkers for the development of ENZ resistance, as evidenced by their consistently downregulated profiles in this context, specifically regarding the EV-derived miRNA levels. As previously mentioned, the miRNAs derived from EVs have been revealed promising to be applied as biomarkers of therapy resistance in clinical settings due to their enhanced stability, as they are protected from degradation in the bloodstream, and their selective loading, which allows for more accurate analysis of miRNA signatures associated with different therapeutic responses. These 3 miRNAs are the most consistently downregulated and, therefore the most promising to be further tested in a clinical setting with patients undergoing enzalutamide treatment until developing resistance.

Notably, these results also highlight hsa-miR-20a-5p and hsa-miR-25-3p as promising therapeutic targets for reversing the resistant phenotype, as restoration of their expression significantly reduced pro-tumorigenic properties. These findings pave the way for future research targeting these miRNAs for therapeutic intervention in ENZ-resistant CRPC.

Conclusion

6. Conclusion and Future Perspectives

ADT is commonly used in advanced and metastatic PCa since this is an androgen-dependent disease for its progression and proliferation. However, most patients develop resistance, progressing to CRPC, which is associated with limited therapeutic options and impaired quality of life. ENZ was designed to overcome this resistance. Despite the therapeutic success of ENZ in improving survival rates, 15-20% of patients exhibit primary resistance, and many others eventually develop resistance, often associated with complex cellular and molecular alterations. Given their crucial role in modulating the TME and facilitating resistance to antineoplastic therapies, EVs have gained significant attention in oncology. They carry multiple active biomolecules, including miRNAs, which can influence other cells under both normal and pathological conditions, such as in PCa. Currently, EV-derived miRNAs are believed to be involved in the acquisition of resistance to ADT, particularly to ENZ. With no effective treatment for ENZ-resistant CRPC, identifying predictive biomarkers of resistance is crucial. Moreover, the analysis of EV-content to identify these biomarkers has been proved promising since their isolation and purification processes are rapidly evolving and allow detection and quantification of biomolecules such as miRNAs in different biofluids. Additionally, due to their selective packaging ability and their role in TME modulation they may be a better mirror and predictor of tumor behavior.

Thus, we aim to identify EV-derived miRNAs associated with the acquisition of ENZ resistance, in order to identify resistance biomarkers that can be applied during patient follow-up, facilitating the early detection of clinical resistance and promoting the translation of basic research into clinical practice. Additionally, this will open up avenues for the development of innovative RNA-based therapies aimed at restoring normal miRNA levels.

On one hand, the results obtained reinforce the critical role of metabolic reprogramming, AR-independent mechanisms and miRNA deregulation in the promotion and establishment of resistance to ENZ. The use of 3D spheroid models, which more accurately replicate the TME, showed significant differences in miRNA expression and cellular behavior compared to 2D cultures, emphasizing the need to study ENZ resistance in more physiologically relevant settings. The consistently deregulated profiles of miRNAs such as hsa-miR-20a-5p, hsa-miR-20b-5p and hsa-miR-25-3p in ENZ-resistant cells in both 2D and 3D models suggest their potential as predictive liquid biopsy biomarkers for the development of ENZ resistance. Additionally, the identification of VEGFA and SMAD7 as key targets of these miRNAs suggests their involvement in the survival and progression of ENZ-resistant tumors. Transfection of hsa-miR-20a-5p and hsa-miR-25-3p mimics was shown to

effectively reduce spheroid size and metabolic activity, highlighting the therapeutic potential of these miRNAs. These results suggest that restoring the deregulated expression levels of these miRNAs may be a viable strategy to overcome ENZ resistance in CRPC. However, further research is needed to fully validate these miRNAs as predictive biomarkers of resistance and as therapeutic targets, as well as to elucidate the wider implications of their regulatory networks.

In the future, 3D spheroid models of the remaining ENZ-resistant concentrations established in the LNCaP-ER *in vitro* cell model (LNCaP 20 μ M, LNCaP 30 μ M) should be established, and the expression dynamics of these deregulated miRNAs analyzed in these cells to complete the EV-derived miRNA profile and improve its predictive ability. It would also be interesting to assess the expression dynamics of VEGFA and SMAD7 at the remaining concentrations in the LNCaP-ER cell model to better understand the role and relevance of these molecules during ENZ resistance acquisition. Moreover, different targets common to the most promising miRNAs should also be quantified to better understand which mechanisms are behind the establishment of ENZ resistance. Subsequently, this EV-derived miRNA profile should be studied in a cohort of CRPC patients undergoing ENZ therapy to monitor the dynamic profile during treatment until resistance acquisition.

Finally, given their potential as reliable predictive biomarkers for the development of ENZ resistance, it would be relevant to modulate hsa-miR-20b-5p expression through the use of mimics in 3D models. Co-transfection of hsa-miR-20a-5p, hsa-miR-20b and hsa-miR-25-3p would also be beneficial, as they have all shown great promise as predictive liquid biopsy biomarkers for ENZ resistance acquisition, and co-transfection offers multiple advantages over single mimic transfection by targeting multiple pathways simultaneously. Advanced phenotypic and molecular studies after transfection as well as the behavior of the spheroids upon enzalutamide administration to better understand a possible reversion in enzalutamide resistance.

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7. **References:**

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Supplementary Table 1- Selected downregulated miRNAs and their validated target mRNAs by strong evidence.

MicroRNAs	Validated Targets
hsa-miR-19a-3p, hsa-miR-20a-5p, hsa-miR-20b-5p, hsa-miR-25-3p, hsa-miR-93-5p	PTEN
hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-20b-5p, hsa-miR-93-5p	VEGFA
hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-25-3p, hsa-miR-93-5p	SMAD7
hsa-miR-15b-5p, hsa-miR-19a-3p, hsa-miR-20a-5p	CCND1
hsa-miR-19a-3p, hsa-miR-20a-5p, hsa-miR-93-5p	TP53INP1 / TGFB2
hsa-miR-19a-3p, hsa-miR-20a-5p, hsa-miR-25-3p	BCL2L11
hsa-miR-19a-3p, hsa-miR-25-3p, hsa-miR-93-5p	KAT2B
hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-20b-5p	CRIM1
hsa-miR-15b-5p, hsa-miR-20a-5p, hsa-miR-93-5p	PURA
hsa-miR-20a-5p, hsa-miR-20b-5p, hsa-miR-93-5p	CDKN1A / MAPK9
hsa-miR-15b-5p, hsa-miR-19a-3p	SOCS3
hsa-miR-19a-3p, hsa-miR-20a-5p	PKNOX1 / MEF2D / SMAD4 / KIT / BMPR2 / MAP3K5 / DNMT1
hsa-miR-19a-3p, hsa-miR-20b-5p	ESR1
hsa-miR-19a-3p, hsa-miR-93-5p	ABCA1 / ZBTB4 / TUSC2
hsa-miR-19a-3p, hsa-miR-25-3p	PRMT5
hsa-miR-15b-5p, hsa-miR-20a-5p	BCL2 / WEE1
hsa-miR-15b-5p, hsa-miR-20b-5p	AKT3
hsa-miR-15b-5p, hsa-miR-25-3p	RECK
hsa-miR-20a-5p, hsa-miR-20b-5p	RB1CC1 / LIMK1 / BAMBI / PPARG / STAT3 / HIF1A
hsa-miR-20a-5p, hsa-miR-93-5p	ITGB8 / ATG16L1 / E2F1 / PHLPP2
hsa-miR-20a-5p, hsa-miR-25-3p	TCEAL1
hsa-miR-25-3p, hsa-miR-93-5p	LATS2
hsa-miR-19a-3p	TLR7 / ERBB4 / PMEPA1 / NPEPL1 / RHOB / ADRB1 / TLR2 / TNF / TF / RAB14 / DPYSL2 / NR4A2 / ATXN1 / ALOX5 / MTUS1 / PSAP / MYCN / MECP2 / SIVA1 / AKT1 / TNFRSF12A / IL10 / TNFAIP3 / FOXP1 / SUZ12 / CD22 / CUL5 / IMPDH1 / SOCS1 / MXD1 / HOXA5 / VPS4B / RAB13 / PIK3CA / MSMD1 / PHLPP1
hsa-miR-15b-5p	TBR1 / IFNG / CHEK1 / KDR / TRIM29 / HNF1A / PEBP4 / INSR / FUT2 / TRIM14 / TGFB1 / SMAD2 / MTSS1 / AGO2 / OIP5 / SMURF1 / BAX / FOXO1 / CCND3 / RAB1A / PPM1D / AXIN2 / CCNE1 / EIF4A1 / MMP9
hsa-miR-20a-5p	KIF26B / REST / VEGFA / EPAS1 / RBL2 / RGS5 / DUSP2 / PPP2R2A / EGR2 / MYC / ETV1 / TSG101 / BNIP2 / PKD1 / GJA1 / MAP2K3 / MAP3K12 / E2F3 / ABL2 / CCND2 / RUNX3 / MCL1 / ANKH / PRKG1 / EGLN3 / RUNX1 / RB1 / NRAS / RBL1 / APP / UBE2C / TGFB1 / TIMP2 / IRF2 / NFKB1B / ZFYVE9 / FBXO31 / PTPRO / SIRPA / DAPK3 / ARHGAP12
hsa-miR-20b-5p	ARID4B / MYLIP / HIPK3 / EFN2 / BRCA1 / EPHB4 / FZD6
hsa-miR-93-5p	RPS6KA4 / ZNRF3 / FOXO3 / ANG / STK11 / PTENP1 / MMP3 / DAB2 / SLC2A4 / PDCD4 / RHOC / CXCL8 / RAB11FIP1 / CERS2 / FOXA1
hsa-miR-25-3p	CDH1 / TNFSF10 / CCL26 / TP53 / MAP2K4 / ATP2A2 / CPEB1 / REV3L / HAND2 / RGS3 / DSC2 / CYP2B6 / SEMA4C / MDM2 / FBXW7 / WDR4 / DHFR / EZH2 / KLF4 / CDKN1C / ERBB2

Scientific Outputs

In the scope of the Master Dissertation:

Ferreira M, Morais M, Medeiros R, Teixeira AL, (2024) microRNAs as promising therapeutic agents against Prostate Cancer Resistant to Castration – where are we now?, *Pharmaceutics* (submitted manuscript)

Tavares I, Morais M, Dias F, **Ferreira M**, Martins G, Fernandes R, Bidarra S. J., Medeiros R, Teixeira AL, (2024) Extracellular vesicles derived-microRNA profile dynamic in a 3D spheroid enzalutamide-resistance Prostate Cancer Model, *International Journal of Biological Macromolecules* (Submitted manuscript)

Ferreira M, Morais M, Tavares I, Dias F, Bidarra S, Medeiros R, Teixeira AL, (2024) Modelos *in vitro* de Cancro da Próstata resistente à castração: aplicação de miRNAs derivados de vesículas extracelulares para a deteção precoce de resistência à terapia (presented at Encontro Ciência 2024 – Encontro com a Ciência e Tecnologia em Portugal, - Porto, 3rd July 2024)

Ferreira M, Morais M, Tavares I, Dias F, Bidarra S, Medeiros R, Teixeira AL, (2024) Modelos *in vitro* de Cancro da Próstata resistente à castração: aplicação de miRNAs derivados de vesículas extracelulares para a deteção precoce de resistência à terapia (presented at Presented at the 6^o Encontro de Jovens Investigadores, LPCC, 24th September, Porto, Portugal)

Other Scientific Outputs:

Morais M*, Dias F, Figueiredo P, **Ferreira M**, Mikkonen KS, Teixeira AL, Medeiros R. (2024) Do we have evidence for the multi-targeted therapy effectiveness against Castration Resistant Prostate Cancer (CRPC)? - a miRNA-profile definition approach and its potential as biomarker of response against Silver Nanoparticles (AgNPs) functionalized with Glucose (G-AgNPs) (submitted manuscript)

Morais M*, Figueiredo P, Dias F, **Ferreira M**, Mikkonen KS, Teixeira AL, Medeiros R. (2024) A endocitose caveolar é responsável pelo efeito seletivo das Nanopartículas de Prata Sensíveis e Resistentes à Terapia de Bloqueio Androgénico (awarded Best Oral Communication at IPO Summit, 11th May 2024, IPO Porto)

