

DOUTORAMENTO

CIÊNCIAS BIOMÉDICAS

Silver Nanoparticles meet Prostate
Cancer cells: nanomedicine applied to
treatment optimization and its effects on
microRNAs' dynamic

Mariana Gomes Morais

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SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR



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SILVER NANOPARTICLES MEET PROSTATE CANCER CELLS:
NANOMEDICINE APPLIED TO TREATMENT OPTIMIZATION AND ITS EFFECTS ON
MICRORNAS' DYNAMIC

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“How, Alan Turing thought, does the soft-walled,
jellied, symmetrical cell
become the asymmetrical horse? It was just before dusk,
the sun's last shafts doubling the fence posts,
all the dark mares on their dark shadows. It was just
after Schrödinger's *What is Life?*
not long before Watson, Franklin, Crick, not long before
supper. How does a chemical soup,
he asked, give rise to a biological pattern? And how
does a pattern shift, an outer ear
gradually slough its fur, or a shorebird's stubby beak
sharpen toward the trout?”

Linda Bierds

Declaração de Honra

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referência. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

A handwritten signature in black ink, reading 'Mariana Gomes Morais'. The script is cursive and fluid, with the first name 'Mariana' being more prominent and the last name 'Morais' following in a similar style.

(Mariana Gomes Morais)

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De acordo com o disposto no ponto 2 do artigo 31 do Decreto-Lei n.º 65/2018, de 16 de agosto, o autor declara que na elaboração desta tese foram incluídos dados das publicações abaixo indicadas. O autor participou ativamente na conceção e execução dos trabalhos que estiveram na origem dos mesmos, assim como na sua interpretação, discussão e redação.

According to the relevant national legislation, the author declares that this thesis includes data from the publications indicated below. The author participated actively in the conception and execution of the work that originated that data, as well as in their interpretation, discussion, and writing.

The following articles were used to support this thesis:

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- Almeida C, Teixeira A.L, Dias F, **Morais M**, Medeiros R (2023), Extracellular Vesicles as Potential Therapeutic Messengers in Cancer Management, *Biology* (12) ([10.3390/biology12050665](https://doi.org/10.3390/biology12050665))
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“Let us be certain of who we want to be. Let us choose for ourselves our path in life, and let us try to strew that path with flowers.”

Emilie Du Chatelet

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Abstract

Despite the major advances in the comprehension of cancer biology and in the development of new anticancer therapies, two major concerns remain: treatment resistance acquisition and the lack of biomarkers that can predict it. In Prostate Cancer (PCa), these are the two main challenges of clinical practice. Castration Resistant Prostate Cancer (CRPC) is characterized by a poor prognosis and an impaired life quality. Moreover, the available therapies for CRPC patients are limited and show low impact on overall survival. While in a therapy naïve setting, PCa cells show a major dependence on the androgens pathway to proliferate and evade apoptosis, in metastatic CRPC, defects in DNA repair genes and alteration in PI3K/mTOR, MAPK and Wnt signaling pathways are commonly reported. The application of nanotechnology in the biomedical field with the ability to design drug carriers that specifically target the cancer cell and encapsulate drugs that can multi-target different pathways, allows the development of a multi-targeted approach that can help overcome treatment resistance. Moreover, silver nanoparticles (AgNPs) arise as a promising approach since their physical-chemical properties and the ability to functionalize their surface chemistry give them antitumor properties demonstrated in different tumor models. Thus, the aim of this work was to study the application of AgNPs functionalized with glucose (G-AgNPs) in PCa, through an *in vitro* study in CRPC cells (PC-3 and Du-145) and androgen deprivation therapy (ADT) sensitive cells (LNCaP), analyzing their cytotoxic effect, their uptake, molecular mechanisms and toxicity. Additionally, we established an associated microRNA (miRNA) profile in order to propose potential new predictive biomarkers regarding this new nanomedicine therapeutic strategy. The AgNPs synthesized showed an average size of 61 nm and moderate monodispersity, with a slight increase after functionalization, and a spheric shape. Moreover, when redispersed in culture medium, these characteristics remained stable. AgNPs and G-AgNPs were cytotoxic only to CRPC cells and not to ADT sensitive ones, with a higher effect after functionalization. We further proved that this selective effect was due to the uptake of AgNPs, since LNCaP cells do not express Caveolin 1 nor Caveolin 2, therefore not showing any caveolae-dependent endocytosis, which is their key entrance pathway. Indeed, when modulating the caveolae-dependent endocytic pathway, we were able to influence the cytotoxic capacity of AgNPs, increasing the cytotoxic effect in LNCaP cells by inducing caveolae-dependent endocytosis and inhibiting the cytotoxic effect in Du-145 and PC-3 by blocking this pathway. This further proved that the impact selective targeting has in increasing the cytotoxic capacity of AgNPs. Moreover, we proved that functionalization of AgNPs with glucose enhanced the cytotoxic effect, mostly due to an increased uptake. After uptake through endocytosis, AgNPs were encapsulated into lysosomes where the lysosome acidic environment and its enzymes

degrade the AgNPs capping and Ag^+ induces an increase of reactive oxygen species (ROS) that damages different cellular organelles, such as mitochondria, which exhibited membrane depolarization. This leads to a cell cycle blockage in the S-phase and a proliferation blockage and cell death induction through apoptosis. Moreover, we observed that AgNPs and G-AgNPs displayed a lower toxicity against HepG2, a liver cell line, comparing to PC-3 and Du-145, which comes as a first indicator of low hepatotoxicity of these nanoparticles. We further analyzed the molecular pathways deregulated in response to AgNPs and G-AgNPs through a wide-screening approach of 799 miRNAs and an *in silico* analysis of their validated targets. The *in silico* analysis highlighted the same pathways in both AgNPs and G-AgNPs and in both cell lines and revealed the involvement of multiple pathways directly or indirectly related to the oxidative stress induced by an increase of ROS production, in the response of cells to AgNPs. Moreover, pathways such as AGE-RAGE, PI3K-Akt and mTOR, FOXO, JAK STAT and HIF1 were shown to be involved and are reported to be disrupted in the most advanced forms of PCa. Finally, we proposed a miRNA profile composed by 2 downregulated miRNAs (hsa-miR-1283 and hsa-let-7i-5p) and 16 upregulated miRNAs (hsa-miR-1-3p, hsa-miR-1200, hsa-miR-1228-3p, hsa-miR-1272, hsa-miR-187-3p, hsa-miR-203a-5p, hsa-miR-494-5p, hsa-miR-495-5p, hsa-miR-517c-3p, hsa-miR-519a-3p, hsa-miR-518e-3p, hsa-miR548g-3p, hsa-miR-572, hsa-miR-590-3p, hsa-miR-637 and hsa-miR-648) that were systematically highly deregulated among cell lines and between AgNPs and G-AgNPs, and can predict response to this potential future therapeutic strategy. Through this study we propose that drug development should focus on a specific targeting of tumor cells allied to a multiple target of deregulated pathways in cancer, to achieve better therapeutic results and surpass resistance. We showed that the administration of G-AgNPs as a future therapeutic approach in CRPC is promising, since they can be selectively uptaken through caveolae-dependent endocytosis and this uptake can be enhanced through glucose functionalization. Moreover, they are able to target multiple pathways that are known to be deregulated in CRPC. In the future, *in vivo* studies are needed to confirm these results and assess their biocompatibility. Finally, we showed that miRNAs can be potential biomarkers of response of PCa cells to this therapeutic approach and future 3D and *in vivo* studies should focus on narrowing the profile and proving its capability to predict response to G-AgNPs.

Keywords: Castration Resistant Prostate Cancer, Glucose functionalized Silver Nanoparticles, caveolae-dependent endocytosis, multi-targeted therapy, microRNAs

Resumo

Apesar dos grandes avanços na compreensão da biologia tumoral e do desenvolvimento de novas terapias anticancerígenas, dois principais problemas persistem: a aquisição de resistência às terapias e a falta de biomarcadores que conseguem monitorizar e prever esta resistência. No Cancro da Próstata (CP), estes são dois dos grandes desafios encontrados na prática clínica. De facto, o cancro da próstata resistente à castração (CPRC) é caracterizado por um mau prognóstico e qualidade de vida limitada. Para além disso, as terapias existentes para doentes com CPRC são limitadas e mostram um baixo impacto na sua sobrevivência global. Se por um lado, antes de qualquer abordagem terapêutica as células de CP apresentam grande dependência da via do androgénio para proliferar e escapar à apoptose, no CPRC metastático, alterações nos genes de reparação de DNA e nas vias de sinalização de PI3K/mTOR, MAPK e Wnt são comumente reportadas. A aplicação da nanotecnologia no campo biomédico com a capacidade de *design* de veículo de fármacos que tem como alvo específico a célula tumoral e encapsulam agentes terapêuticos direcionados para diferentes vias, permite o desenvolvimento de uma abordagem *multi-targeted* capaz de ultrapassar a resistência às terapias atuais. Assim, as nanopartículas de prata (AgNPs) surgem como uma abordagem promissora, uma vez que as suas propriedades físico-químicas e capacidade de funcionalização da sua superfície são responsáveis pelas propriedades antitumorais já reportadas em diferentes modelos tumorais. O objetivo deste trabalho foi estudar a aplicação de AgNPs funcionalizadas com glucose (G-AgNPs) em CP, através do estudo *in vitro* em células CPRC (PC-3 e Du-145) e sensíveis à terapia de bloqueio de androgénios (TBA) (LNCaP), analisando o seu efeito citotóxico, internalização, mecanismo de atuação e toxicidade. Adicionalmente, estabelecemos um perfil de microRNAs (miRNAs) associados à administração das G-AgNPs de forma a propor potenciais novos biomarcadores preditores de resposta a esta nova potencial abordagem terapêutica. As AgNPs sintetizadas apresentam um tamanho médio de 61 nm e moderada monodispersão, com um aumento ligeiro após funcionalização, bem como uma forma esférica. Para além disso, quando redispersas em meio de cultura celular, estas características permaneceram estáveis. As AgNPs e G-AgNPs mostraram-se citotóxicas apenas nas células CPRC e não nas células sensíveis à TBA, com um efeito maior após funcionalização. Este efeito seletivo contra as CPRC deve-se à não internalização das AgNPs pelas células LNCaP, já que as mesmas não expressam as caveolinas 1 e 2 (Cav1 e 2), não apresentando endocitose cavéola-dependente. De facto, ao modular a atividade desta via, fomos capazes de influenciar o efeito citotóxico das AgNPs e G-AgNPs, aumentando o seu efeito e internalização nas LNCaP ao induzir esta via, e inibindo o seu efeito nas PC-3 e Du-145,

bloqueando-a. Estes dados mostraram o impacto do *targeting* seletivo na capacidade citotóxica das AgNPs. Após a internalização por endocitose, as G-AgNPs são encapsuladas em lisossomas onde o ambiente ácido lisossomal e as suas enzimas degradam o *capping* das AgNPs e a Ag^+ induz um aumento da produção de espécies reativas de oxigénio (ROS) que danificam diferentes organelos celulares, tais como a mitocôndria que apresenta uma despolarização da membrana. Isto leva a uma paragem do ciclo celular na fase S e a um bloqueio da proliferação e indução de morte celular por apoptose. Para além disso, observamos que as AgNPs e G-AgNPs apresentam uma toxicidade mais baixa contra as células HepG2, uma linha celular de fígado, comparado com PC-3 e Du-145, o que se apresenta como um primeiro indicador de baixa hepatotoxicidade destas nanopartículas. Para além disso, analisamos as vias moleculares desreguladas em resposta às AgNPs e G-AgNPs, através de uma abordagem de *wide-screening* de 799 miRNAs e uma análise *in silico* dos genes alvo validados. A análise *in silico* revelou as mesmas vias potencialmente desreguladas após administração das AgNPs e G-AgNPs em ambas as linhas celulares, mostrando que a funcionalização das AgNPs com glucose aumenta o efeito citotóxico, maioritariamente por um aumento de internalização. Adicionalmente, revelou o envolvimento de diversas vias direta ou indiretamente relacionadas com stress oxidativo induzido pelo aumento de produção de ROS na resposta das células às AgNPs. Vias moleculares como AGE-RAGE, PI3k-Akt e mTOR, FOXO, JAK STAT e HIF1 estão envolvidas nesta resposta e estão reportadas como desreguladas nas formas mais avançadas de CP. Finalmente, propomos um perfil de miRNAs composto por 2 miRNAs sub-expressos (hsa-miR-1283 e hsa-let-7i-5p) e 16 sobre-expressos (hsa-miR-1-3p, hsa-miR-1200, hsa-miR-1228-3p, hsa-miR-1272, hsa-miR-187-3p, hsa-miR-203a-5p, hsa-miR-494-5p, hsa-miR-495-5p, hsa-miR-517c-3p, hsa-miR-519a-3p, hsa-miR-518e-3p, hsa-miR548g-3p, hsa-miR-572, hsa-miR-590-3p, hsa-miR-637 e hsa-miR-648) que se apresentam altamente desregulados de forma sistemática nas duas linhas celulares após administração de AgNPs e G-AgNPs, com potencial de predição de resposta para esta futura estratégia terapêutica. Através deste estudo, propomos que o desenvolvimento de fármacos deve focar-se no direcionamento específico para as células tumorais, aliado ao *multi-targeting* das vias desreguladas no cancro, de forma a conseguir melhores resultados terapêuticos e ultrapassar a resistência à terapia. Mostramos que a administração de G-AgNPs como futura abordagem terapêutica no CPRC é promissor, já que podem ser seletivamente internalizadas através da endocitose cavéola-dependente e que esta internalização é aumentada através da funcionalização com glucose. Para além disso, as G-AgNPs são capazes de atingir diversas vias moleculares desreguladas no CPRC. No futuro, estudos *in vivo* são necessários para confirmar estes resultados e avaliar a sua biocompatibilidade. Finalmente, mostramos que

os miRNAs são potenciais biomarcadores de resposta das células de CP a esta abordagem terapêutica e futuros estudos em 3D e *in vivo* devem focar-se em estreitar este perfil e comprovar a sua capacidade de prever a resposta às G-AgNPs.

Palavras-chave: Cancro da Próstata Resistente à Castração, Nanopartículas de Prata funcionalizadas com Glucose, Endocitose cavéola-dependente, terapia-multi-dirigida, microRNAs

List of Abbreviations

A

ADT - Androgen Deprivation Therapy
AGE – Advanced glycation endproducts
AgNPs – Silver Nanoparticles
AGO2 – Argonaute 2
AHNAK – Desmoyokin
AKT – Protein Kinase B
AMPK – AMP-activated Protein Kinase
AR – Androgen Receptor
ASCL1 – Achaete-Scute Family
ATCC – American Type Culture Collection
ATP – Adenosine Triphosphate

B

Bcl-2 – B-cell lymphoma 2
Bcl-xl – B-cell lymphoma-extra large
BHLH – Transcription Factor 1
BMP4 – Bone Morphogenetic Protein-4
BRAF – Raf Murine Sarcoma Viral Oncogene Homolog B
BRCA – Breast Cancer gene
BrdU – Bromodeoxyuridine
BSA – Bovine Serum Albumin
BTG2 – BTG Anti-Proliferation Factor 2

C

cAMP – Adenosine 3',5'-cyclic monophosphate
Cav – Caveolin
CCND1 – Cyclin D1
CCND2 – Cyclin D2
CDK6 – Cell division protein kinase 6
CDKN1A – Cyclin Dependent Kinase Inhibitor 1A
CDKN1B – Cyclin Dependent Kinase Inhibitor 1B
cDNA – complementary DNA
CELF1 – CUGBP Elav-Like Family Member 1

C-myc - Cellular- Myelocytomatosis
COP9 – Constitutive photomorphogenesis 9
COPS6 – COP9 Signalosome Subunit 6
COPS8 – COP9 Signalosome Subunit 8
CRPC – Castration-Resistant Prostate Cancer
Ct – Threshold cycle
CYP17A1 – Cytochrome P450 17A1

D

DC – Detergent compatible
DHT – Dihydrotestosterone
DMEM – Dulbecco's Modified Eagle Medium
DNA – Deoxyribonucleic acid
DLS – Dynamic Light Scattering
DPPC – Dipalmitoyl-phosphatidyl choline
Dyn2 – Dynamin 2

E

EDTA - Ethylenediamine tetra acetic acid
EDX – Energy-dispersive X-ray spectroscopy
EGFR – Epidermal growth factor receptor
EMA – European Medicines Agency
EMT - Epithelial Mesenchymal Transition
EPR - Enhanced Permeability and Retention
ERG – ETS-related gene
ERK – Extracellular Signal-Regulated Kinase
ESMO - European Society of Medical Oncology
ESR – Erythrocyte sedimentation rate
ETS – Erythroblast Transformation Specific
ETV1 – ETS Variant Transcription Factor 1
ETV4 – ETS Variant Transcription Factor 4
EU – European Union

F

FASN – Fatty acid synthase
FBS – Fetal bovine serum
FDA – Food and Drug Administration

FDG-PET - 18F-fluorodeoxyglucose-positron emission tomography
FDR – False Discovery Rate
FGFR – Fibroblast Growth Factor Receptors
FLI1 – Friend leukemia integration 1
FOXA1 – Forkhead box protein A1
FOXO – Forkhead box transcription factors
FTIR – Fourier Transform Infrared Spectroscopy

G

GADD45 - Growth arrest and DNA-damage-inducible 45
G-AgNPs – Glucose Functionalized AgNPs
GG-NER – Global genome nucleotide excision repair
GIGYF1 – GRB10 Interacting GYF Protein 1
GLUT1 – Glucose transporter 1
GPS1 – G Protein Pathway Suppressor 1
GTPase - Guanosine triphosphatases

H

H₂DCFDA – 2',7'-dichlorodihydrofluorescein diacetate
HER2 - Human epidermal growth factor receptor 2
HIF1 – Hypoxia Inducible Factor 1
HIP1 – Huntingtin-interacting protein 1
HK2 – Hexokinase 2
HNRNPU – Heterogenous nuclear ribonucleoprotein U
HNSCC – Head and neck squamous cell carcinoma
HRAS - Harvey Rat sarcoma virus

I

IC₅₀ – Half maximal Inhibitory Concentration
IDH1 – Isocitrate dehydrogenase 1
IGF1 – Insulin-like growth factor 1
IGF1R – Type 1 insulin-like growth factor receptor
IL13 – Interleukin 13
IL2 – Interleukin 2
IL6 – interleukin 6
ISUP - International Society of Urological Pathology

J

JAK – Janus kinase

K

KEGG – Kyoto Encyclopedia of Genes and Genomes

KLHL15 – Kelch Like Family Member 15

KRAS - Kirsten rat sarcoma virus

L

LBD – Ligand binding domain

LDHA – Lactate dehydrogenase

LHRH - Luteinizing hormone-releasing hormone

LRRC58 – Leucine Rich Repeat Containing 58

M

MAP1B – Microtubule-associated protein 1B

MAPK – Mitogen-activated protein kinase

MCL – Markov clustering

MCL1 – Myeloid cell leukemia-1

MDR – Multidrug resistant

mCRPC – Metastatic Castration Resistant Prostate Cancer

MCT – Monocarboxylate transporters

MDM2 – Mouse double minute 2 homolog

MQ – MilliQ

miRNA – MicroRNA

MKNK2 – MAPK Interacting Serine/Threonine Kinase 2

MMP2 – Metalloproteinase-2

MMP9 – Metalloproteinase-9

mpMRI – Multi-parametric magnetic resonance imaging

mRNA – Messenger RNA

mTORC – Mammalian target of rapamycin complex

MT1F – Metallothionein 1F

N

NACC1 – Nucleus Accumbens Associated 1

NADH - Nicotinamide adenine dinucleotide-hydrogen

NADPH - Nicotinamide adenine dinucleotide phosphate

NEUROG1 – Neurogenin 1
NHE – Sodium-hydrogen antiporters
NF-kB – Factor nuclear kappa B
NOTCH – Neurogenic locus notch homolog protein
NPs – Nanoparticles
NTRK1 – Neurotrophic tyrosine kinase receptor 1
NTRK3 – Neurotrophic tyrosine kinase, receptor, type 3
NUFIP2 – Nuclear FMR1 Interacting Protein 2

O

OS – Overall Survival

P

PAM – Partitioning Around Medoids
PARP – Poly ADP Ribose Polymerase
PCa – Prostate Cancer
PCA3 – Prostate Cancer Antigen 3
PCWG2 – Prostate Cancer Working Group 2
PDGFAB/BB - Platelet-derived growth factor AA/BB
PET-CT - Positron emission tomography-computed tomography
PI – Propidium Iodide
PIN – Prostatic intraepithelial neoplasia
PIK3CA – Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α
PIK3CB - Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit β
PI3K – Phosphoinositide 3-kinase
PPI – Protein-protein interaction
Pol II – Polymerase II
PSA – Prostate Serum Antigen
PSCA – Prostate stem cell antigen
PSMA – Prostate-specific membrane antigen
PTEN – Protein phosphatase and tensin homolog
PTK6 – Protein tyrosine kinase 6

R

RAD51C - Restriction site associated DNA 51 Paralog C
RAGE – Receptors for advanced glycation endproducts

RAS – Rat sarcoma
RB1 – Retinoblastoma protein
RECIST – Response Evaluation Criteria in Solid Tumors
RIPA – Radioimmunoprecipitation Assay
RISC – RNA-induced silencing complex
RNA – Ribonucleic acid rpm, rotations per minute
ROS – Reactive Oxygen Species
RPMI – Roswell Pearl Memorial Institute
RUNX – Runt-related transcription factor

S

SEM – Standard Error of Mean
siRNA – Small interfering RNA
SOD2 – Superoxide dismutase 2
SOX4 – Sex-determining region Y-related high-mobility group box 4
SPOP – Speckle-type Poz protein
SPR – Surface plasmon resonance
SREBP-1 – Sterol regulatory element-binding protein 1
SRCAP – Snf2 Related CREBBP Activator Protein
SRRM2 – Serine/Arginine Repetitive Matrix 2
STAT – Signal transducer and activator of transcription
STRINGapp – Search Tool for the Retrieval of Interacting Genes

T

TAOK1 – Thousand and one amino-acid kinase 1
TBS-T – Tris-buffered saline with Tween
TCGA - The Cancer Genome Atlas
TC-NER – Transcription-coupled nucleotide excision repair
TEM – Transmission Electron Microscopy
TGF- β - Transforming growth factor beta
TGX – Tris-Glycine extended
TLR4 – Toll-like receptor 4
TMPRSS2 - Transmembrane serine protease 2
TMRE – Tetramethylrhodamine, Ethyl Ester
TNF- α – Tumor necrosis factor
TNPO1 – Transportin 1

TNRC6A – Trinucleotide Repeat Containing Adaptor 6A
TP53 – Tumor protein p53
TRIB3 – Tribbles homolog 3
TRITC – Tetramethylrhodamine
TXNIP – Thioredoxin Interacting Protein

U

uPA – Urokinase plasminogen activation
uPAR – Urokinase plasminogen activation receptor
USA – United States of America
UV-Vis – Ultraviolet-visible

V

VEGF – Vascular endothelial growth factor

W

WEE1 – WEE1 G2 Checkpoint Kinase
WHO – World Health Organization

X

XIAP - X-linked inhibitor of apoptosis protein
XRD – X-ray diffraction analysis

Z

ZIP1 – Zinc-regulated transporter/iron-regulated transporter-like protein 1
ZNF264 – Zinc Finger Protein 264
ZNF460 – Zinc Finger Protein 460

#

3'UTR – 3' Untranslated Region
223Ra – Radium-223

INTRODUCTION

1. Cancer: from worldwide epidemiology to current challenges

During the XXI century, cancer will become the major obstacle to a rise in life expectancy and the leading cause of early death all over the world [1]. According to GLOBOCAN data, in 2020, there was an estimated 19.3 million new cancer cases and about 10.0 million related deaths [2]. In the previous year, World Health Organization (WHO) estimated cancer as the first or second leading cause of death before 70 years old in 112 of 183 countries, and third or fourth in another 23 countries [3]. By 2040, 28.4 million cancer cases are expected and, by 2070, 34 million are estimated, doubling the current number of cases [1, 3]. This rise in incidence and mortality reflects the aging and growth of the population as well as changes in the prevalence and distribution of the main risk factors [4].

One quarter of all cancer cases occur in Europe, even though this continent hosts only one tenth of the world's population [5]. By 2020, there was an estimate of 4.0 million new cancer cases and 1.9 million cancer deaths, with a considerable geographic variability, and in 2018, the economic burden of this disease in the European Union (EU) was of almost 97 billion [6]. In fact, global spending on anticancer drugs surpassed the \$150 billion in 2018 and is expected to exceed \$240 billion by 2023.

Patients' financial burden is considered one of the main struggles in oncologic care, being associated with increased risk of non-compliance to treatment and negative impact in quality of life of the patient and family [7]. Moreover, new cancer drugs are normally marketed at very high prices, which is not strongly correlated with clinical benefit [7]. Davis and collaborators have shown that in Europe, only 35% of newly approved drugs showed significant improvements in survival and only 10% in quality of life of cancer patients [8]. Similarly, Fojo and colleagues showed that 71 chemotherapeutic agents licensed between 2002 and 2012 reported an improvement of median overall survival and progression-free survival of only 2.1 and 2.3 months, respectively [9].

Treatment failure remains one of the main challenges of oncology practice. According to Wong and collaborators, who analyzed 406 038 entries of clinical trial data for over 21 143 compounds from 1st January 2000 to 31st October 2015, the oncology field showed a success rate of only 3.4% in clinical trials [10]. Moreover, the landscape of current treatment options is strongly marked by resistance phenotypes acquisition and lack or limited response. Nowadays, it is possible to classify the different cancer treatments in six different categories: surgery, chemotherapy, radiotherapy, hormone therapy, targeted agents and immunotherapy [11]. Mechanisms behind chemotherapy resistance depend on different factors such as the deregulation of pathways related with cell death inhibition,

deoxyribonucleic acid (DNA) damage repair, drug metabolism and uptake or efflux of drugs [12]. Radiotherapy, on the other hand, loses its efficiency in hypoxic tumors, since they are not sensitive to ionizing radiation because they do not generate the reactive oxygen species (ROS) responsible for triggering the death of cancer cells [13, 14]. Resistance mechanisms in hormone therapy, an alternative treatment approach to hormone dependent tumors, such as prostate or breast cancer, are typically caused by alterations in specific signaling pathways responsible for their dependence on hormones and activation of alternative signaling pathways [15]. Regarding target therapies, which are more precise and directed to deregulated key molecules in the tumor, their high rates of resistance acquisition are mainly due to genetic and epigenetic alterations in the targets, activation of alternative pathways and phenotype alterations [16]. Finally, the modulation of the immune system (immune therapy) was assumed as the next landmark of cancer treatment, but, its benefic effects are restricted to a limit number of cancer patients and resistance development often lies on the loss of T cell function, downregulation of tumor antigen presentation that leads to lack of T cell recognition and development of escape mutation variants in the tumor [17].

Oncologists normally rely on the concepts of tumor response and disease progression to the management of solid tumors. While the first one assesses the therapeutic efficacy, the second one is a key sign of treatment failure, commonly used to determine when to change therapy [18]. The Response Evaluation Criteria in Solid Tumors (RECIST) group established in 2000, later reviewed in 2009, defined the criteria for progression as a 20% increase in unidimensional measurement or appearance of new lesions which is equivalent to a 73% increase in the volume of a spherical tumor [19]. Nevertheless, in clinical practice, changing therapy normally weighs a different number of factors, such as tumor burden, cancer-related symptoms, and drug toxicity [18]. The use of tumor markers to help establish disease progression is also still debated. In Prostate Cancer, for example, Prostate Serum Antigen (PSA) has been a cornerstone of prostate cancer drug development, but the Prostate Cancer Working Group 2 (PCWG2) has now recommended that although an increase in PSA levels can be analyzed as an endpoint in clinical trials, it should not be used as a criterion to discontinue treatment, due to the high rate of false positives [20]. Therefore, the lack of precise and sensitive biomarkers that can monitor and predict therapy response and early relapse is another key challenge in oncology practice.

Thus, these clinical challenges defined the focus of oncologic research in the last years to a better comprehension of the biology and mechanisms behind the disease, not only of the tumor cell, but also of the whole tumor microenvironment, in the hope to define better molecular targets and molecular biomarkers that can address these challenges.

2. The Hallmarks of Cancer: from a single cell to the tumor microenvironment

Carcinogenesis has traditionally been described as a multistage process that etiologically involved mutations in several genes whose function is keeping a balance between cell proliferation and apoptosis [21]. Thus, cancer cells can be seen as transformed cells, with acquired changes that allows them to form tumors and behave in different ways depending on the main genetic changes behind this transformation [22]. Indeed, the foundation of cancer research was set in the discovery of mutations that produce oncogenes, with dominant gain of function, and tumor suppressor genes, with recessive loss of function [23].

However, in 2000, Hanahan and Weinberg, brought up a concept that would revolutionize the way one looks at cancer: the Hallmarks of Cancer (Figure 1-A) [23]. According to these two investigators, tumorigenesis was a multistep process and each of these steps would reflect genetic alterations that would progressively transform normal cells into malignant derivatives. Even though there is a huge number of possible genetic alterations, they suggested that there would be a small number of molecular, biochemical, and cellular traits shared by almost all types of human cancers. The first approach of the Hallmarks, in 2000, referred 6 acquired capabilities of cancer cells: self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Moreover, they presented genomic instability as an enabling characteristic: a means that would enable evolving populations of premalignant cells to reach the other six biological endpoints [23].

In the next decade, research evolved to show that the biology of tumors could not be completely understood by focusing on the traits of cancer cells, but it should also pay attention to the contribution of the tumor microenvironment [24]. Therefore, in 2011, Hanahan and Weinberg updated the Hallmarks of Cancer, with two new emerging hallmark capabilities, which can ease the development and progression of most human cancers: major reprogramming of cellular energy metabolism and active evasion by cancer cells from attack and elimination by immune cells (Figure 1-B). Additionally, the two researchers added one enabling characteristic: the inflammatory state of premalignant and malignant lesions driven by immune cells [24]. In 2022, Hanahan brought up the question regarding the need to incorporate additional features to this conceptual model that would have come

up in the last ten years of research (Figure 1-C). Therefore, he proposed the accrescent of one acquired capability: unlocking phenotypic plasticity; and two enabling characteristics: non-mutational epigenetic reprogramming and polymorphic microbiomes. Moreover, he highlighted the functional contribution of senescent cells of different origins to the development and progression of cancer [25].

Overall, over the years, the way researchers look at cancer kept on changing. While first, cancer was looked upon as an abnormal cell that would proliferate in an uncontrolled manner and eventually earn the capacity to metastasize, nowadays, cancer is comprehended as an ecosystem composed of several different populations of cells that cooperate to sustain tumor survival. Tumor microenvironment is now defined as the transformed parenchyma and the associated stroma composed by heterogeneous and interactive populations of cancer cells and cancer stem cells, along with a multiplicity of recruited stromal cell types, and its integral role in tumorigenesis and malignant progression is widely known and studied [25].

The aim of the cancer hallmarks proposed by Hanahan and Weinberg has contributed to this, by propelling the debate, discussion and experimental research of the cancer research community having set the trends of research in the last years. Indeed, in the last decade, research regarding the emerging hallmark of the major reprogramming of cellular energy metabolism has been the object of increasing interest.

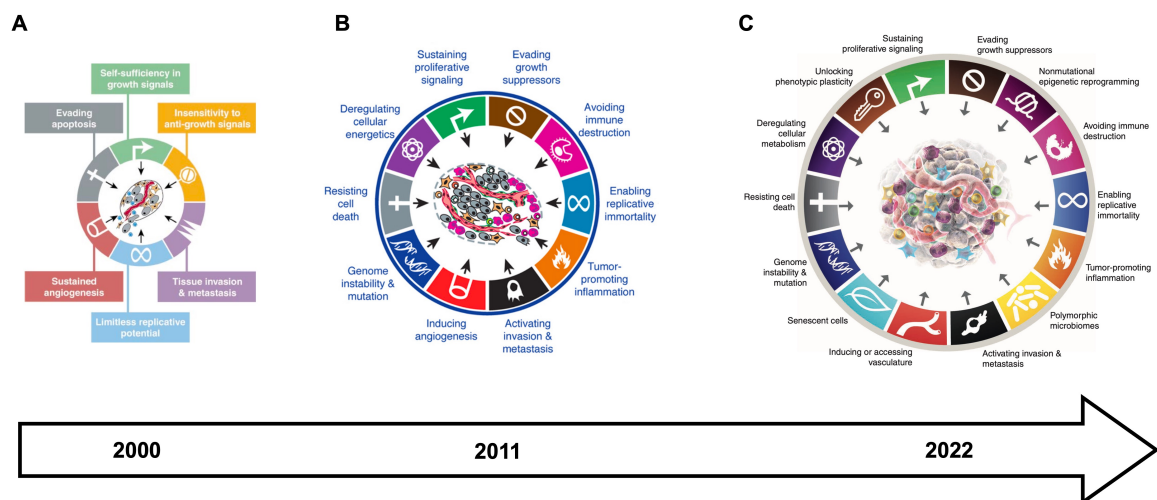


Figure 1 - The evolution of the Hallmarks of Cancer proposed by Hanahan and Weinberg. (A) The six initial Hallmarks proposed in 2000; (B) Update of the Hallmarks of cancer in 2011 with two new emerging hallmark and one enabling characteristic; (C) Last version of the Hallmarks of cancer, proposed by Hanahan in 2022 with one new acquired capability and two enabling characteristics. Adapted from Hanahan, D. and Weinberg, R [23-25].

2.1. Deregulation of metabolism in Cancer

Cancer cells display several alterations in their metabolic pathways that will support the acquisition and maintenance of their malignant phenotype. In a metabolic perspective, cancer cells face two possible circumstances: on one hand, if there is an optimal supply of nutrients, their main goal will be to use these nutrients to ease the assimilation of carbon into macromolecules (proteins, nucleic acids and lipids) to support cell growth and proliferation; on the other hand, if the environment is harsh and resources are limited, cells will need to optimize nutrients' use and modulate the microenvironment to get more nutrients [26].

The most common and studied alterations in cancer metabolism are glucose metabolism, lipid biosynthesis and amino acids and nucleic acids production [27]. The Warburg effect has been described since 1920s, stating the preference of cancer cells to use glycolysis followed by lactate fermentation instead of oxidative phosphorylation, even when oxygen is present [28]. Production of essential compounds such as serine, glycerol 3-phosphate, ribose-5-phosphate and nicotinamide adenine dinucleotide phosphate (NADPH) is highly dependent on glucose metabolism and glycolysis allows a faster way to fulfill these requirements while at the same time providing lactate which will acidify the tumoral microenvironment [29]. Regarding lipids biosynthesis, cancer cells have high requirement of fatty acids and cholesterol for the synthesis of cell membrane and production of important lipidic molecules [30]. Thus, there is an increase of both their synthesis and uptake which demands large amounts of precursors such as acetyl CoA and NADPH which come from glucose metabolism [31]. Additionally, cancer cells require large amounts of amino acids, nucleotides, and polyamines. Glutamine is one of the major sources for the biosynthesis of non-essential amino acids as well as purine and pyrimidine bases and of nitrogen [32]. Besides glutamine, serine is involved in the biosynthesis of purine and pyrimidine, being produced from 3-phosphoglycerate, whose production is dependent on glycolysis [33]. Finally, arginine and ornithine are precursors of polyamines which are involved in proliferation, resistance to apoptosis and metastasis [34].

All these requirements of cancer cells are achieved through a deregulation of different signaling pathways, being the phosphoinositide 3-kinase/ protein kinase B (PI3K/AKT), Rat sarcoma-extracellular signal-regulated kinase (RAS-ERK) and mammalian

target of rapamycin complex (mTORC) key in metabolic deregulation [31]. AKT/PI3K enhances the expression of several enzymes involved in glycolysis, it enhances fatty acids uptake, it strengthens the sterol regulatory element-binding protein 1 (SREBP-1) and it increases the uptake of glutamine by stimulating glutaminase and glutamate dehydrogenase [29]. Kirsten rat sarcoma virus (KRAS) also increases expression of key glycolytic enzymes and affects lipids' metabolism increasing expression of Fatty acid synthase (FASN) and stimulating lipogenesis through ERK2 activation [31]. Cellular-Myelocytomatosis (C-myc), on its turn, causes increased expression of major glycolytic enzymes, influences the metabolism of amino acids and nucleotides, enhancing the expression of glutamine transporters and of several enzymes involved in the biosynthesis of purine and pyrimidine bases [31]. mTORC influences the major pathways deregulated in metabolism, since it stimulates glycolysis through induction of hypoxia inducible factor 1 (HIF-1) expression, lipid synthesis through SREBP expression induction and protein synthesis strengthening the conversion of glutamine to glutamate, production of α -ketoglutarate and polyamines' biosynthesis [27].

These pathways have been widely studied in the oncology for the last several years to develop new therapeutic approaches and prognostic biomarkers. However, despite some promising results, the therapies available are not effective and none of the potential biomarkers made it to the clinical setting yet.

2.1.1. The Warburg Effect

Glucose is indeed central in cancer metabolism, not only as an energy source but also as a provider of biosynthetic intermediates, interwinding with the other metabolic pathways. Therefore, glucose metabolism has been, from an early stage of research, extensively studied.

The Warburg effect, or aerobic glycolysis, has been firstly described almost one hundred years ago, by Otto Warburg [35]. In normoxic conditions, differentiated cells metabolize glucose into pyruvate through glycolysis. Pyruvate then enters mitochondria into the tricarboxylic acid cycle being transformed in carbon dioxide and NADH, which then fuels oxidative phosphorylation producing ATP [36]. Only in hypoxic conditions, glycolysis is followed by lactic fermentation, with large amounts of lactate produced. However, in cancer cells, there is an accelerated conversion of glucose to lactate, even in the presence of abundant oxygen [37]. Even though, Otto Warburg firstly attributed this to a mitochondrial dysfunction, these was shown not to be true, since in most tumors, there is an upregulation

of glycolysis without mitochondrial dysfunction. In fact, oxidative phosphorylation continues normally, producing as much ATP as it would in normal tissues with the same amount of oxygen [38].

While in some cases, the Warburg Effect can be driven by mitochondrial dysfunctions such as mutations in the Krebs cycle enzymes, most of the times, different signaling pathways combine to sustain this effect [39]. Activation of HIF-1 along with oncogenes such as cMyc, mTORC1, Akt and KRas, inactivation of tumor suppressor genes such as p53, inactivation of AMP-activated protein kinase (AMPK) signaling pathway, downregulation of several microRNAs and sirtuins, or allosteric regulation by glycolytic intermediates as well as interactions with tumor microenvironment and cancer-associated fibroblasts or even the maintenance of the intra-extracellular pH gradient, are major drivers of aerobic glycolysis [38].

On one hand, the Warburg Effect gives an answer to the high energetic demands of cancer cells. Despite only generating 2 moles of ATP per glucose molecule (in opposition to the 32-33 generated from the complete oxidation), cytosolic ATP is generated approximately 100x faster than in mitochondria, providing a higher amount of ATP as long as there is a sustained glucose supply [40]. This is guaranteed, in part, by the upregulation of glucose transporters such as glucose transporter 1 (GLUT-1) [38].

On the other hand, the catabolism of glucose to pyruvate provides different intermediates for the macromolecular biosynthesis of lipids, nucleotides, proteins and hexoamine [41]. Glucose-6-phosphate is diverted to the pentose phosphate pathway to generate pentose phosphate for ribonucleotide synthesis and NADPH. Glyceraldehyde-3-phosphate is used for the biosynthesis of cell membrane components, phospholipids and triglycerols. 3-phosphoglycerate is diverted to the one-carbon metabolism pathway to generate serine and glycine [38].

Finally, Warburg effect can also guarantee redox homeostasis, since the NADPH produced keeps the antioxidant power of glutathione increasing radio-resistance [42]. Moreover, the pentose phosphate pathway, derived from glycolysis, diminishes ROS production which and reduces reactive oxygen species production which promotes increased survival of cancer cells [38].

Warburg effect leads to a high production of lactate and is the main contributor to the high lactate levels present in the tumor microenvironment. In fact, lactate and consequent extracellular acidosis is considered one major oncometabolite involved in the activation of different pathways such as the PI3K-Akt-mTOR and HIF1, needed for malignant progression and therapy resistance [43]. Its exportation from the cytosol is

performed by monocarboxylate transporters (MCT) together with sodium-hydrogen antiporters (NHE) and carbonic anhydrases, which are reported to be upregulated [38].

Taking advantage of one key feature of Warburg Effect, the high glucose consumption of tumor tissues, an imaging technique called 18F-fluorodeoxyglucose-positron emission tomography (FDG-PET) arose which uses ^{18}F , a positron-emitting radioisotope, that can be analyzed with PET and the quantity of glucose in a certain area can be measured and located when combined with CT scan technology. Nowadays, PET is used in clinical practice with several advantages to cancer diagnosis and immunotherapy effectiveness assessment. However, it presents limitations since high glucose is not exclusive of cancer, therefore, infections and inflammations, muscular activity, brown fat's metabolism and changes in response to bone marrow-stimulating cytokines can lead to false positives [26].

Indeed, a switch to a Warburg-type glucose metabolism is also observed in rapidly proliferating mammalian cells, such as pluripotent stem cells, immune cells and endothelial cells, acutely regenerating tissues, and mature human red blood cells [44, 45]. In oncology, the Warburg effect is present in 70-80% of human tumors and seems to be, not a mere adaptation to harsh conditions, but a key feature of the metabolic reprogramming that drives the malignant phenotype [38]. Urological tumors, such as prostate cancer are tumors in which the Warburg Effect correlates with progression and therapy resistance [28].

3. Prostate Cancer

Prostate Cancer (PCa) represents the second most frequent cancer and the fifth leading cause of cancer death among men worldwide, with an estimate of 1.4 million new cases and 375 000 deaths by 2020 [3]. This incidence is globally heterogeneously distributed, being higher in transitioned than in transitioning countries, with the highest rates found in Northern and Western Europe, the Caribbean, Australia/ New Zealand, Northern America, and Southern Africa [46]. In more than half of the countries in the world (112/185) PCa is the most frequently diagnosed cancer in men and it is the leading cause of cancer death among men in 48 [4]. In Portugal, PCa represents 20% of all new cancer cases, being the most frequent neoplasia in men, and it is the third cause of cancer related death in men [5]. PCa risk factors include age, race, family history and germline mutations, which are considered nonmodifiable and metabolic syndrome, obesity and smoking which are possible modifiable [46].

Around 60-75% of PCa cancers arise from the peripheral zone of the prostate and studies have shown that both luminal and basal cells from the prostatic epithelial can be the origin cell for PCa [47]. The cell proliferation with dysplasia along the duct of luminal cells is denominated prostatic intraepithelial neoplasia (PIN), being the first stage of PCa progression. PIN progresses to localized prostate adenocarcinoma, that will later become locally invasive carcinoma when the basal cell layer is degraded, and cells invade [48]. PCa mainly metastasizes to the lymph nodes adjacent to the primary tumor, firstly, and followed by the liver, lungs, and bones (Figure 2) [49].

Primary PCa's great heterogeneity among patients can also be observed regarding molecular abnormalities and that determines different aspects of its clinical course such as resistance to therapies. Mutations in tumor protein p53 (TP53), loss of Retinoblastoma protein (RB1), mutations and amplifications of Androgen Receptor (AR), inactivation of Protein phosphatase and tensin homolog (PTEN), amplifications of Myelocytomatosis (MYC) and Transmembrane serine protease 2- Erythroblast Transformation Specific related gene (TMPRSS2-ERG) gene fusions have been extensively related in PCa (Figure 2) [50]. In 2015, The Cancer Genome Atlas (TCGA) project performed a large study in 333 primary prostate carcinomas on a genomic, transcriptomic and epigenomic level and 74 % of the tumors could be molecularly classified in one of seven subtypes defined by specific gene fusions (ERG, ETS Variant Transcription Factor 1 (ETV1), ETV4 and Friend leukemia integration 1 (FLI1)) or mutations (Speckle-type Poz protein (SPOP), Forkhead box protein A1 (FOXA1), Isocitrate dehydrogenase 1 (IDH1)), with ERG fusion-positive tumors being subdivided into two methylation subtypes (lower levels of methylation and hypermethylation) [51]. Moreover, 19% of the tumors showed defects in DNA repair genes and 19% had alteration in key effectors of both PI3K and MAPK pathways [51]. They also showed key alteration in the PI3K/mTOR pathway with frequent inactivation of PTEN and rare activation of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit β (PIK3CB), AKT1, and MTOR, and small Guanosine triphosphatases (GTPases), such as Harvey Rat sarcoma virus (HRAS) and V-Raf Murine Sarcoma Viral Oncogene Homolog B (BRAF) [51]. Genomic profile of metastatic PCa has been shown different from localized PCa (Figure 2) [50]. In a study involving 150 patients with metastatic Castration Resistant Prostate Cancer (mCRPC), 63% of patients showed mutations in AR, 57% of patients had ETS gene fusions, 53% of patients had mutations in TP53 and 41% in PTEN gene. All in all, 89% had clinically actionable genomic aberration, 71% in the AR signaling pathway, 49% in the PI3K signaling pathway, 18% in Wnt signaling pathway and 23% in DNA repair pathways [52].

3.1. Diagnosis, staging and treatment of Prostate Cancer

In the 1980's, the introduction of the PSA test as a way to monitor and detect PCa revolutionized this disease's diagnostic and management as well as incidence and mortality trends [53]. PSA is a serine protease produced by the prostate epithelium and secreted into seminal fluid, able to be quantified through immunoassay laboratory techniques both at free unbound form and bound to the protease inhibitor α 1-antichymotrypsin [54]. Evidence regarding early detection and management of PCa through PSA testing and the reducing risk of men's dying from this tumor is clear. Indeed, the wide use of PSA testing has been shown to have contributed to the 40-50% decline in PCa mortality in the United States of America (USA) between 1993-2015 [54]. However, it is important to note that studies assessing diagnostic power of PSA have shown high sensitivity but lower specificity [55]. In fact, the extensive use of PSA testing in asymptomatic men and subsequent biopsy led to an overdiagnosis of tumors that would never cause symptoms or harm in a man's lifetime. Moreover, the consequent unnecessary treatment has several risks regarding impotence, urinary leakage and bowel dysfunction, with a dramatic impact in the quality-of-life [54]. Therefore, in the last years, different guidelines have been proposed regarding the usage of PSA in PCa screening [53-55]. The European Society of Medical Oncology (ESMO), in its 2020 guidelines regarding PCa diagnosis, treatment and follow-up has recommended against population-based PSA screening, advising early PSA testing only to men older than 50 or 45 years if they are African Americans or have family history of PCa, or older than 40 years old if BRCA1/2 carriers. Moreover, it also advised against screening for PCa in men with a life expectancy of less than 10 years if asymptomatic [56].

Besides PSA, Food and Drug Administration (FDA) has already approved other biomarkers that provide information regarding risk of future disease and treatment outcomes [57]. Isoforms and cleavage forms of PSA as well as its kinetic characterization can be used for screening, prognosis and recurrence prediction, improving PSA performance [57]. Prostate Cancer Antigen 3 (PCA3) is a noncoding mRNA overexpressed in 95% of PCa, prostate-specific and its expression is not impacted by benign non-prostate tissue or prostate volume. It is correlated with tumor volume and Gleason score but its prognostic value and ability to predict PCa presence still lacks clinical validation [58]. The inclusion of the abnormal fusion of TMPRSS2-ERG, which is present in 50% of PCa patients, in conjunction with PCA3 and PSA in the algorithm MiPS was found to be more specific than the individual variables [59]. Indeed, the use of algorithms that combine clinical data with PSA and human kallikrein 2, produced by the same secretory prostatic epithelial cells of PSA, can improve prediction of high-grade disease detection in biopsy [57]. The

urokinase plasminogen activation (uPA) a precursor for serine protease and its receptor (uPAR) for degradation of extracellular matrix has been associated with distant metastases and poor prognosis as well as Prostate stem cell antigen (PSCA) which is an indicator of high-risk disease and metastasis [60]. Finally, there are three commercially available RNA-based genetic panels: Prolaris[®], Oncotype DX[®], and Decipher[®] validated in PCa patients, but performance is affected due to the heterogeneity of PCa molecular landscape [57].

Therefore, since the risk of a clinically significant PCa relates to factors such as age, ethnicity, family history as well as the clinical parameters of PSA levels, free/total PSA ratio and the findings on digital rectal examination, both PCa risk calculator and/or Multiparametric magnetic resonance imaging (mpMRI) are recommended to confirm the need for biopsy in men with high PSA levels [56]. Biopsy, along with MRI will lead to a PCa staging. Newly diagnosed PCa commonly presents as multifocal disease with histopathological distinct features. Gleason grading system is the most common method for PCa grading, measuring from 1 to 5 the similarities among healthy prostate gland and tumor tissue. During biopsy, several samples are taken from different regions of the gland and the two most common grades are summed to form a Gleason score (which can vary from 2 to 10) [47]. In the consensus conference of 2014, the International Society of Urological Pathology (ISUP) issued guidelines for the grading of PCa, grouping the Gleason Scores in 5 groups to improve risk stratification [61]. Moreover, men with intermediate/high risk localized disease ($\geq T2$ and $\geq G7$) are recommended to have imaging for nodal or metastatic disease through whole body MRI, choline-positron emission tomography-computed tomography (PET-CT) and prostate-specific membrane antigen (PSMA)-PET-CT [56].

PCa treatment will, naturally, depend on the stage of the disease (Figure 2) [47]. In low risk localized PCa, watchful waiting with delayed hormone therapy when there is symptomatic progression can be an option for men not suitable or not wanting treatment with a curative intent [56]. On the other hand, active surveillance with close monitoring (using PSA, biopsies and MRI) with an intent of delaying curative intent when disease progresses can also be an option [56]. Radical prostatectomy or radiotherapy (external beam or brachytherapy) are the main options regarding men with intermediate-risk localized disease [56]. In high-risk localized disease and locally advanced disease, androgen deprivation therapy (ADT) or hormone therapy, is the key therapeutic strategy, along with radiotherapy and, in cases of high-risk disease, pelvic lymphadenectomy [62]. This treatment affects the androgen pathway since both androgens and androgen receptors are important regulators in the proliferation and development of PCa [63]. AR is a nuclear ligand transcription factor that is part of the steroid hormone nuclear receptor family and whose

main ligands are the male sexual hormones, such as testosterone and dihydrotestosterone (DHT) [64]. When AR binds to androgens, its conformation changes and it translocate to the nucleus where it dimerizes and binds to specific androgen response elements, regulating target genes transcription [65]. This will trigger cell proliferation and inhibit apoptosis, contributing to the development of PC. Thus, through ADT, the androgens levels are downregulated or their binding to receptor is inhibited and consequently the cell proliferation decreases and apoptosis rate increases [47]. This downregulation can be achieved both by luteinizing hormone-releasing hormone (LHRH) agonists and antagonists that will reduce serum levels of androgens or through anti-androgens that inhibit the binding of DHT to the AR [66]. ADT has shown great results, with a remission of disease in about 90% of patients. However, in 2-3 years, disease progresses to the most advanced form of the disease, the Castrate-Resistant Prostate Cancer (CRPC) [67]. In the last 20 years, different therapies have been approved that changed the treatment landscape of CRPC, which currently involves chemotherapeutic agents, second generation antiandrogens, agents targeting bone metastasis and Poly ADP Ribose Polymerase (PARP) inhibitors [68]. While docetaxel and cabazitaxel, chemotherapeutic agents, inhibit microtubules, acting systemically in CRPC patients, second generation antiandrogens arose when it was proved that most CRPC tumors are not hormone independent and re-activation of AR signaling is a key driver of resistance and progression [69]. Abiraterone Acetate irreversible inhibits Cytochrome P450 17A1 (CYP17A1), antagonizing the production of testosterone and DHT [63, 70]. Enzalutamide is an AR inhibitor with an affinity that is higher than that of earlier generation anti-androgens and active in the presence of AR amplification and overexpression, since it prevents AR nuclear translocations, DNA binding and coactivator recruitment [65, 71]. Apalutamide acts as a selective competitive silent antagonist of the AR, via the ligand-binding domain, being structurally and pharmacologically like enzalutamide, but with advantages such as higher antiandrogenic activity as well as reduced central nervous system distribution [72]. On the other hand, most patients with CRPC have bone metastases, and bone-only disease is seen in approximately 40–50% of these patients [73]. In these men, ²²³Radium is recommended. Radium is a “calcium mimetic” that accumulates mainly in areas of bone with increasing turnover, such as osteoblastic metastasis. Its emitted alpha particle radiation will cause DNA double-strand breaks in the targeted region without damage in the surrounding tissue [74]. Additionally, bisphosphonate or denosumab, which are known to retard bone resorption, are recommended in patients with bone metastasis [56].

More recently, oloparib, a PARPinhibitor, was approved for treatment of CRPC patients with mutations in BRCA1 and BRCA2 [56]. In March 2023, ESMO updated its

guidelines regarding PCa management, including the usage of Lutetium-177 (^{177}Lu)-PSMA-617 in men with cancer expressing PSMA [75]. This radiolabeled small molecule binding with high affinity to PSMA enabling a selective beta particle radiotherapy with fewer secondary effects [76].

Nevertheless, all these new therapeutic agents are not of curative intent, offering an increase of overall survival of only 4-5 months, making both understanding the resistance mechanisms behind CRPC and the development of new therapeutic agents, urgent needs [56, 75].

3.2. Mechanisms behind Castrate Resistant Prostate Cancer

Tumor heterogeneity is present both in naïve PCa and following treatment, contributing to CRPC progression and drug heterogeneity with the mechanisms behind resistance to ADT being split into two key classes: AR-dependent and AR-independent (Figure 2).

Among AR dependent mechanisms are AR mutations, AR splicing variants, AR amplification and overexpression, posttranslational regulation of AR, coactivators or corepressor modification and intratumoral steroid hormone synthesis [77]. About 15-20% of CRPC patients present AR mutations, including single point mutations, pre-termination, deletions, and insertions [52]. These mutations normally lead to a lower AR antagonist binding affinity or the transformation of the AR antagonists in agonists. F876L mutation, for example, leads to an enzalutamide turnover from antagonist to agonist, causing resistance [78]. On the other hand, AR alternative splicing can lead to constitutively active AR proteins, with lack of the ligand binding domain (LBD) to which the second-generation antiandrogens bind, leading to resistance [79]. AR-v7 is one of the most common AR variants, not only facilitating nuclear localization and DNA binding of AR even in the absence of androgens and promoting cell growth and survival [78]. AR overexpression can be caused by AR amplification, enhanced transcription activation or increased stability, with 80% of patients showing high AR gene copy numbers from which, 20-30% showed AR amplification [80]. Posttranslational mechanisms of AR include deregulation of acetylation, methylation, phosphorylation and ubiquitination patterns, which are mostly associated with an increase of AR activity [79]. Coregulators (coactivators and corepressors) are in the control of transcriptional activity of AR. Normally, they ease its transcriptional function even in androgen absence. Their modification results in aberrant stimulation of AR and a higher transcriptional response [78]. Finally, upon inhibition of serum testosterone concentrations

by ADT, PCa can produce intracrine androgens, with the amount of DHT in prostatic samples after ADT remaining the same values before treatment [80].

The different genomic profile of mCRPC, which presents mutations and genomic aberrations in TP53, PI3K, Wnt signaling pathways and DNA repair pathways, can present clues to the AR independent mechanisms of CRPC progression [52]. Indeed, most CRPC tumors present activation of the PI3K-AKT-mTOR pathway, which can compensate for the inhibited AR and need for androgens, since it can promote tumor cell proliferation and survival through, for example, the activation of B-cell lymphoma 2 (Bcl-2) [80]. On the other hand, Mulholland and collaborators have observed that 90% of metastatic CRPC samples had an activation of RAS/MAPK signaling which is a downstream mediator to the cellular responses to different growth signals, resulting in the promotion of proliferation and expression of Epithelial Mesenchymal Transition (EMT) markers which are associated with migration and metastasis capacity [79, 81]. Wnt pathway has been reported as activated in 18% of CRPC cases and is associated with neuroendocrine differentiation, induction of EMT, promotion of bone metastasis and interaction with different molecules and pathways such as the PTEN, PI3K and mTOR [52, 82]. Janus kinase - Signal transducer and activator of transcription (JAK-STAT) pathway can be activated by the binding of both BRCA1 and AR to STAT3 or by interleukin-6 (IL-6), whose levels have been shown to be higher in PCa, and it will upregulate genes related to apoptosis inhibition and angiogenesis such as vascular endothelial growth factor (VEGF), Bcl-2, B-cell lymphoma-extra-large (Bcl-xl) and survivin [79]. Apart from these, the hedgehog and Transforming growth factor beta (TGF- β) pathways as well as glucocorticoid receptors (GR) and Cell-surface Adhesion Molecules activation or alterations in the PTEN, RB1, TP53 genes and DNA repair genes have been shown to promote CRPC progression [78-80].

If, on one hand, the multitude of resistance mechanisms behind ADT resistance shows the complexity of CRPC, on the other hand, these altered pathways provide several new targets that can be explored to design new therapeutic strategies. Additionally, the activation of pathways due to the acquisition of ADT resistance will inevitably lead to a tumor composed of cells with characteristics of enhanced proliferation, migration capabilities and disrupted metabolism, that will turn the tumor more aggressive and invasive.

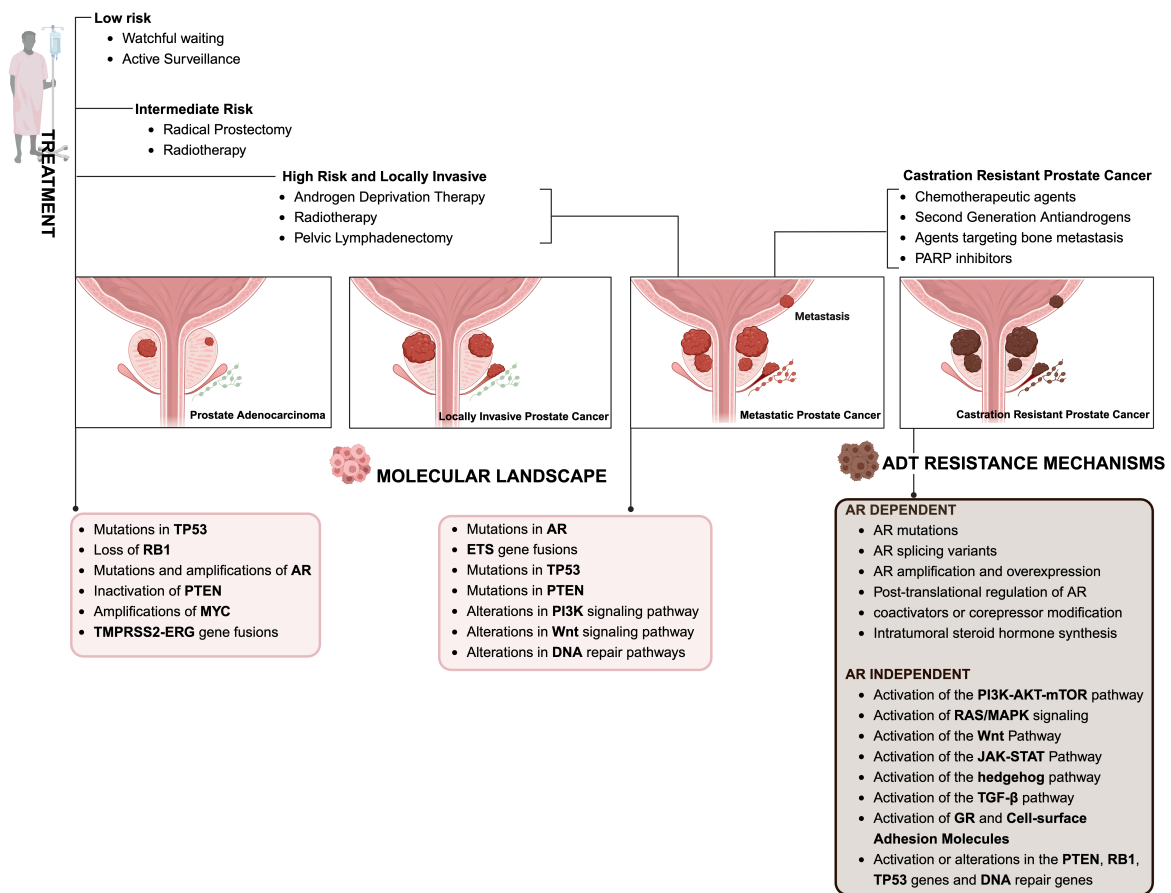


Figure 2 – Prostate cancer evolution from Prostate Adenocarcinoma to Metastatic and Castration Resistant. During the different stages of the disease, and depending on risk stratification, treatment modalities will be different. Moreover, its molecular landscape in the Adenocarcinoma stage is different from the Metastatic stage. In CRPC, resistance mechanisms can be AR dependent or AR independent, with different pathways being activated.

3.3. Glucose Metabolism disruption in Prostate Cancer Progression

The metabolism of glucose disruption in PCa is peculiar due to one of the key functions of the prostate gland – the production of citrate, an intermediate of the Krebs cycle, that is part of the composition of the seminal liquid [83] (Figure 3). Therefore, normal epithelial cells show a very inefficient energetic metabolism. In other organs, epithelial cells metabolize glucose to Acetyl-CoA through glycolysis. Acetyl-CoA will then react with oxaloacetate creating citrate which will undergo successive transformations in the Krebs

cycle and produce aminoacid precursors and a great amount of NADH which will then be used to generate ATP through oxidative phosphorylation [84].

In order to preserve the acetate needed for the composition of the seminal liquid, prostate epithelial cells show an impairment of the mitochondrial enzyme aconitase which oxidizes citrate [83]. This impairment is achieved by an accumulation of zinc due to the overexpression of the zinc-regulated transporter/iron-regulated transporter-like protein 1 (ZIP1) [85]. Thus, in these cells, citrate is the final product of glucose metabolism and due to Krebs cycle inhibition, and consequent oxidative phosphorylation impairment, these cells obtain their energy mainly through glycolysis [84, 86].

Therefore, through the development of PCa the energetic demands increase and, according to Franklin and Costello, completion of the Krebs cycle and subsequent ability to produce much more ATP is an early event of PCa carcinogenesis [87]. This is achieved through an AR-mediated metabolic reprogramming and loss of zinc- transporters during oncogenic transformation [88].

It is only in the late stage of PCa, in a metastatic and ADT resistant phenotypes, that the Warburg effect will take place, due to the need for readjustment of bioenergetics and metabolism according to an increase energetic need, during cancer progression [89]. This shift will, on one hand contribute to a more aggressive phenotype and, on the other hand, be supported by the mutational burden of CRPC [90]. The conversion of pyruvate into lactic acid, for example, is supported by and increased expression of lactate dehydrogenase (LDHA) which can confer treatment resistance to PCa [91]. Indeed, increased lactic acid production will consequently lead to an increased export of lactic acid to the tumor microenvironment, which plays an important role promoting angiogenesis, metastasis, resistance to hypoxia and suppression of anticancer immunity [92, 93]. Additionally, expression of other enzymes involved in glycolysis such as hexokinase 2 (HK2), phosphofructokinase and aldolase are also increased [90]. Interestingly, HK2 is particularly increased in tumors with loss of PTEN and consequent activation of the signaling pathway PI3K-AKT, since this enzyme is one of its downstream targets [94]. As in other tumors, the Warburg Effect energetic efficiency is achieved through an increased glycolytic flux that is supported by an increased expression of GLUT1, GLUT3 and GLUT4 which are correlated with poor prognosis in advanced treatment resistant PCa [90]. Indeed, while FDG-PET has not been considered a useful diagnostic tool for early PCa, it is used to assess metastatic nodules in a more aggressive PCa setting [95]. Besides the application in imaging techniques, the increased glucose influx in CRPC cells, may come as an opportunity in the therapeutic design field.

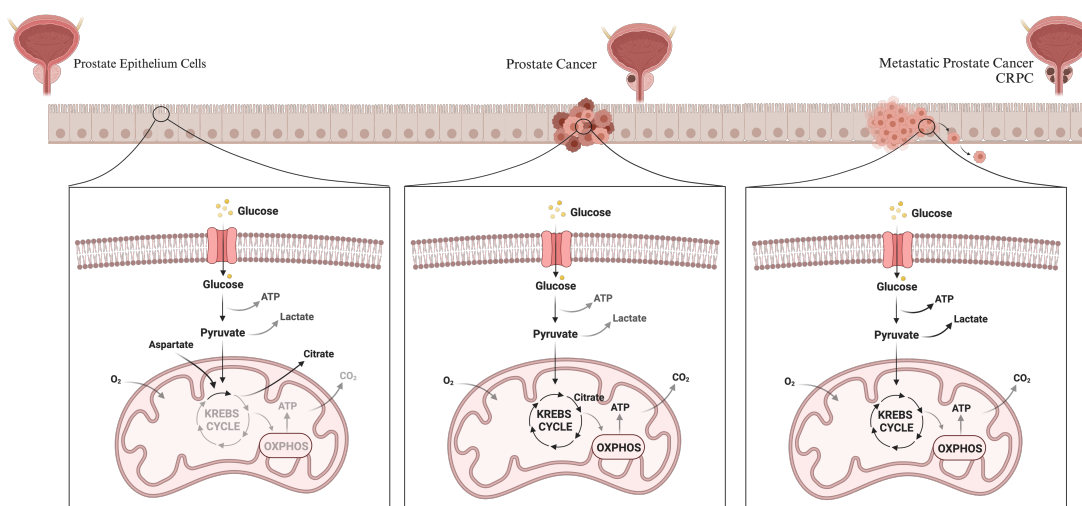


Figure 3 – Glucose metabolism pathways in prostate epithelial cells are differently activated during the stages of disease. In healthy prostate epithelial cells, Krebs cycle is impaired to accumulate citrate used in the production of seminal fluid. In the initial stages of PCa, Krebs Cycle is reactivated with oxidative phosphorylation. It is only in the metastatic and CRPC that Warburg effect is observed (adapted from Morais M. [28])

In the CRPC setting, where new therapeutic approaches are an urgent need, the increased glucose influx may be a mechanism of directing a new therapeutic agent to these cells. On the other hand, given the complexity of mutations and activated signaling pathways, apart from the AR, existing in the CRPC context, a new therapeutic agent would, ideally, be able to disrupt all of them in a more systemic manner. The advent of nanomedicine opened the doors to these possibilities.

4. Nanomedicine approach to new cancer therapies – Silver Nanoparticles role

The possibility of the application of nanostructures to medicine has been seriously considered since 1959, when the Nobel prize winner Richard Feynman gave a talk regarding the concept of nanotechnology, entitled: “There is plenty of room at the bottom” [96]. Nanomedicine has, from then, arisen as the nanotechnology that is applied to health and medicine, being extensively researched regarding oncology due to the current challenges that exist in treating cancer with drugs of low specificity, rapid clearance and biodegradation and limited targeting [97]. The usage of nanoparticles (NPs) that can work at a molecular level is the main advantage of cancer nanomedicine [98]. NPs are defined as small, organized structures ranging in size from 1 to 100 nm and can be categorized depending on their composition as polymeric, metallic, magnetic, carbon nanotubes,

liposomes, dendrimers or quantum dots [98]. Metal NPs constitute an extensive variety of NPs with different metal ions conjugated with organic or inorganic moieties that can be further functionalized [99]. Silver Nanoparticles (AgNPs) have been rising attention in different biomedical fields, including oncology due to their unique physical-chemical properties, namely their nanoscale size, high surface-to-volume ratio, different chemical composition and ability to attach different targeting ligands to their surface [11].

AgNPs synthesis can be achieved by two different approaches: top-down or bottom-up [100]. Top-down refers to the synthesis from bulk materials using physical forces such as mechanical, electrical and thermal energy. Though it leads to AgNPs with uniform particle size distribution and high purity, the absence of stabilizer and capping agents increases the risk of agglomeration. Moreover, it needs complex equipment and external energy, being a long process [101]. On the other hand, the bottom-up approach consists in the construction of clusters to obtain AgNPs from molecular components using nucleation and growth processes and it can be split into chemical and biological synthesis [100]. In the chemical synthesis Ag^+ ions gain electrons from reducing agents and convert in their metallic form that aggregates to form AgNPs. Chemical synthesis' main advantages are the convenience and simplicity of the equipment, its high yield as well as being timesaving and having a controllable route, but the chemicals used may cause environmental pollution [102]. Biological synthesis relies on the usage of fungi, bacteria and yeasts as well as plant sources as capping agents, reducing agent or stabilizing agent to reduce Ag^+ [102]. This method is more environmentally friendly and cheap but is characterized by a low yield that depends on the organism type, enzyme activity and cellular growth, the complete mechanism behind the synthesis is not completely known and the isolation of the AgNPs from the microorganisms is complex to perform the NPs without damaging or disrupting their physicochemical integrity [103].

The control and optimization of the synthesis' parameters of AgNPs is achieved through their characterization which assesses its morphology, composition, surface chemistry and area. Ultraviolet-visible (UV-Vis) spectrophotometry allows the control of AgNPs synthesis' success, since AgNPs produce a strong absorbance peak, called surface plasmon resonance (SPR), at around 400 nm [11]. Its structure, size, shape and dispersity can be assessed through Transmission electron microscopy (TEM), X-ray diffraction analysis (XRD) and Dynamic light scattering (DLS) and its composition can be analyzed through Fourier transform infrared spectroscopy (FTIR) and Energy-dispersive X-ray spectroscopy (EDX) [102].

Indeed, the optimization and modulation of these physical and chemical characteristics will allow the design of AgNPs that can be selectively uptaken by cancer cells, have antitumor effects and show low toxicity in healthy tissue.

4.1. AgNPs' uptake mechanisms

There are two major steps regarding AgNPs uptake, where NPs perform better than other drugs: their entrance in the body and their cellular uptake [104].

Routes of administration of NPs can include inhalation, intravenous injection and topical delivery [105]. Moreover, when in contact with physiological fluids and physical barriers, NPs can undergo processes of aggregation and/or dissolution, as well as modifications of their properties [106]. Indeed, interactions of NPs with proteins present in serum can, for example, lead to the formation of a protein corona over their surface and increase their size [107]. In the intravenous route, the pathophysiological differences of tumor and normal tissues lead to a primary accumulation of AgNPs in the later ones, an occurrence entitled enhanced permeability and retention (EPR) effect. EPR effect is caused, on one hand, by the irregular and vast neovascularization of tumors with significant abnormalities in structure and function of tumor blood vessels. On the other hand, the inefficient drainage of lymphatic systems in solid tumor tissues leads to the retention of the NPs and a higher chance to passively target their delivery [108].

After achieving tumor tissue, AgNPs will need to translocate through cell membranes and target sub-cellular organelles, being endocytosis, which is commonly deregulated in cancer, the main pathway to achieve it [109]. The electrostatic, hydrophobic, and specific ligand-receptor interactions between AgNPs and cell surface leads to the formation of invaginations from the cell membrane around AgNPs (budding). Its consequent pinch off will form endocytic vesicles (early endosomes) that will work as sorting machine carrying the AgNPs to different organelles, to recycling endosomes or evolving to lysosomes [104]. NPs can be sorted into the cells through different endocytic mechanisms: phagocytosis or pinocytosis (which can be subclassified into macropinocytosis, clathrin-mediated and caveolae-mediated endocytosis) [110].

Phagocytosis is usually associated with the uptake of large particles and, it is mostly done by specialized cells such as macrophages, monocytes, dendritic cells, osteoclasts, eosinophils and neutrophils, but fibroblasts, epithelial and endothelial cells also possess some phagocytic activity [105]. Macropinocytosis also allows the uptake of bigger particles by cells that have no phagocytic activity. Through large vacuoles, the macropinosomes, particles are internalized, and, after, there is a decreased in macropinosomes pH's and they

will fuse with late endosomes or lysosomes [110]. Clathrin-mediated endocytosis is the most well studied endocytosis mechanisms, and it is associated with the recruitment of clathrin through the activation of certain receptors, such as epidermal growth factor receptor (EGFR), that will trigger the formation of clathrin coated vesicles. After pinching off, they will transport the cargo to the different organelles [104]. On the other hand, caveolae-mediated endocytosis is not as well studied involving the presence of proteins called caveolins that will form caveolae structures – flask or omega shaped invaginations. In endothelial cells, caveolae perform trans-endothelial transport and in tumor cells they will fuse with early endosomes or caveosomes and be sorted into the golgi apparatus or endoplasmic reticulum [109].

Several factors influence the endocytic pathway by which an AgNP can be internalized. Regarding size, larger NPs (> 500 nm) will be internalized by phagocytosis or macropinocytosis [105]. Clathrin vesicles are normally bigger (about 120 nm) than caveolae vesicles (about 60 nm), therefore, smaller NPs tend to be internalized through caveolae-mediated endocytosis [111, 112]. Spherical shape NPs have been correlated with a higher rate of internalization, and cationic NPs are more efficiently internalized in comparison to neutral and anionic NPs [113]. Interestingly, anionic charged NPs seem to be more easily uptaken by caveolae-mediated endocytosis and cationic NPs prefer clathrin-mediated endocytosis [110]. Nevertheless, the uptake mechanism is highly dependent on the cell type, as well [11].

4.2. Mechanisms behind AgNPs phenotypic and molecular effect

After entering cells through endocytic mechanisms, AgNPs will be primarily located in endosomes and lysosomes, where the “lysosome-enhanced Trojan horse effect” will trigger their cytotoxicity. In a somehow ironic manner, cells’ primary security process, the lysosomal acidic environment and proteases that are responsible for engulfing and degrading foreign material, will be the ones dissolving AgNPs structure and capping into Ag⁺ ions [114]. Consecutive steps of cytotoxicity will then involve cell surface adhesion, alterations of the properties of membrane and damage induction, interactions with DNA, deterioration of enzymes and reactive oxygen species (ROS) formation [11].

Indeed, the trigger of ROS production and oxidative stress is one of the most common referred cytotoxic mechanisms of AgNPs and can be done both in a direct (through electron donation to molecular oxygen, generating O²⁻) or indirectly (through mitochondrial damage) [115]. In mitochondria, on one hand, Ag⁺ binding to thiol groups impairs the

antioxidants enzyme activity, such as glutathione, and on the other hand, they will alter the enzymatic production of ROS, inhibit the electron transport chain and decrease the expression of enzymes that neutralize ROS such as superoxide dismutase and catalase [116]. Still in mitochondria, AgNPs will trigger a depolarization of mitochondrial membrane, needed for canonical protein import mechanisms, ATP production and responses to cellular calcium concentration flux [117-119]. Moreover, AgNPs have been shown to inhibit the electron transport chain enzymes in complex I, II, III and IV [120] (Figure 4).

Regarding cytoplasmic membrane, Ag^+ ions will interact electrostatically with membrane phospholipids and proteins, causing the depolarization and destabilization of the membrane, making it more fluid and expanded [121]. Indeed, different authors have reported membrane blebbing, and an increase of the leakage of cytosolic enzyme lactate dehydrogenase [122-126] (Figure 4).

AgNPs show the ability to induce different damages in the DNA, including DNA fragmentation, mutations, formation of DNA ladders, DNA bases' oxidation, single or double strand breaks, crosslinks or adducts formation, condensation and fragmentation of chromatin [116, 117, 122, 127, 128]. Besides, several studies have reported the deregulation of different genes such as Restriction site associated DNA 51 Paralog C (RAD51C), growth arrest and DNA-damage-inducible 45 (GADD45), VEGF, metalloproteinase-2 (MMP2) and metalloproteinase-9 (MMP9), HIF1 α , AKT, tumor necrosis factor- α (TNF- α), Interleukin-6 (IL-6), human epidermal growth factor receptor 2 (HER2), Platelet-derived growth factor AA/BB (PDGFAB/BB), factor nuclear kappa B (NF- κ B), p53, p21, p27, Bax, cyclins, caspase-3, caspase 8 and caspase-9 [100, 115, 118, 129-138]. All this culminates in a blockage of cell cycle and cell death through the trigger of apoptotic mechanisms [11] (Figure 4).

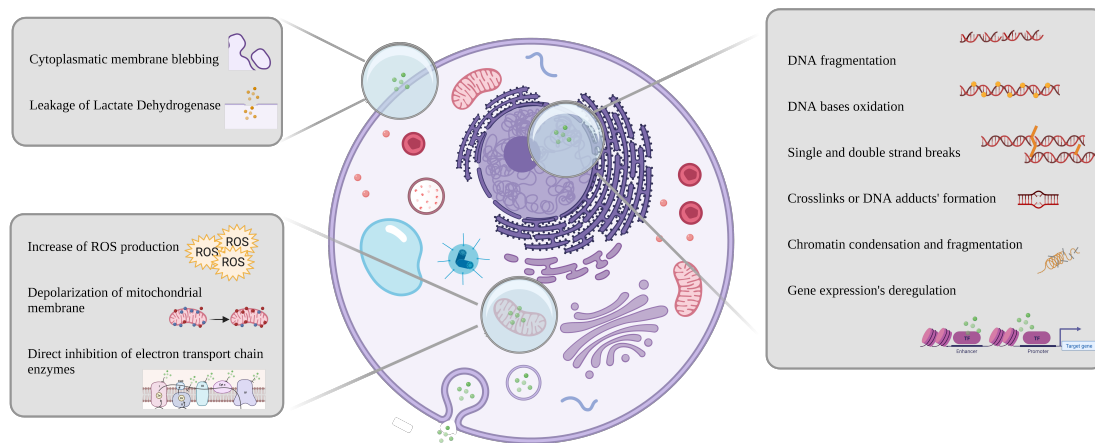


Figure 4 - AgNPs mainly induce damages in three locations of the cell: nucleus, cytoplasmic membrane, and mitochondria. These damages will lead to cell cycle arrest and consequently to apoptosis. Adapted from Morais M [11].

4.3. AgNPs current applications in the cancer field

It is important to notice that AgNPs in the oncology field have not been exclusively studied as a single cytotoxic agent, with their potential being explored to other possible applications.

On one hand, its theranostic potential has been analyzed by different authors [100, 139]. Indeed, due to their high conductivity, catalytic activity and plasmonic properties, they have been explored as biosensors [115]. Indeed, AgNPs SPR peak can be utilized to lower detection limits and monitor disease progression; their boosted Rayleigh scattering is brighter, shows lower background and higher resistance to photobleaching than the fluorophores currently in use and they also show enhanced fluorescence capacity due to their size, photostability and strong quantum confinement [139]. One example of a possible application of AgNPs as biosensors are the nuclear-targeted AgNPs synthesized by Austin and collaborators, that, incubated them with human oral squamous carcinoma cells and, using light-scattering dark-field microscopy, was able to capture their phagocytic activity and cellular clusters [140]. Directed to the same tumor model, Zhou and collaborators have developed a localized SPR nanosensor, using an AgNPs array that can sense serum p53 protein and distinguish healthy from head and neck squamous cell carcinoma (HNSCC) individuals [141].

On the other hand, the usage of AgNPs in combinatory treatments has also been highly explored, with the aim to obtain synergistic effects by targeting different vulnerabilities of the cancer cells [101]. Zhang and Gurunathan have administered salinomycin (an antimicrobial agent with antitumor properties) and AgNPs to human ovarian cancer cells

and were able to achieve synergistic cytotoxic effects with an enhanced mitochondrial dysfunction, autophagy and apoptosis [142]. These combinations are particularly interesting to eradicate multidrug-resistant (MDR) cancer cells since AgNPs can target several metabolic and cellular characteristics of MDR cells, such as their high tolerance to oxidative stress, increased anti-apoptotic capacity, altered signaling pathways and raised activation of DNA repair machinery. Moreover, they seem to be able to impair drug efflux through an inhibition of Pgp transporters [101]. Kovács and collaborators have shown that different chemotherapeutic drugs (methotrexate, cisplatin, carmustine, bleomycin, vinblastine, and verapamil) in combination with AgNPs showed synergistic cytotoxic effects against colon adenocarcinoma cells, due to a decreased Pgp expression that would lead to a higher intracellular concentration of the drugs inside the cells [143].

The usage of AgNPs to potentiate radiotherapy effects has also been reported [115]. This enhancement of radiotherapy effects can be due to the high energy absorption of AgNPs that triggers the Auger cascade, Compton and photoelectric effects and the release of secondary electrons; due to an induction of ROS production through the free electrons transference to oxygen molecules and due to their plasmonic capacity that makes AgNPs scatter and absorb photons that can be used in radiotherapy [101]. Indeed, AgNPs in combination with radiotherapy administered to rats with gliomas was able to inhibit cellular proliferation and induce apoptosis, leading to an increased life span and no apparent toxicity [144]. This synergism is particularly interesting in hypoxic tumors, where AgNPs can alternatively enhance radiosensitivity through mitochondrial damage, autophagy and apoptosis induction [145].

Even though the antitumor capacity of AgNPs, as well as their theranostic capacity and potential in combinatory schemes have been extensively studied and proved in different tumor models, its biocompatibility is one major concern when looking at their possible application in the oncology field [11]. AgNPs toxicity and pharmacokinetics has been studied by several different authors in *in vitro* and using animal models, but results are conflicting [115]. On one hand, several report an accumulation in different organs such as liver, kidney, spleen, lungs, heart and testis with histopathological alterations, biochemical disturbances, genotoxicity and immunotoxicity [146-149]. On the other hand, several studies report a lower toxicity in normal cells compared with tumoral cells and different studies have stated no detectable histopathological and biochemical alterations [150-154]. However, these conflicting results show the need to clarify this topic for each AgNP designed before its possible clinical application.

Nonetheless, in the last years, evidence has been accumulating regarding AgNPs potential as an anticancer agent that can target multiple pathways, known to be deregulated

in CRPC [100, 115, 118, 129-138]. Moreover, AgNPs possess a natural ability to better target tumor tissue due to the EPR effect that can be further enhanced through surface chemistry modifications that can take advantage of tumor features, such as the enhanced glucose consumption of tumor cells. Therefore, AgNPs can be a potential therapeutic strategy regarding CRPC.

Nevertheless, when considering a new therapeutic approach, the ability to monitor and predict patients' response and resistance has been considered an increasingly important factor. The screening of potential biomarkers at an early stage of drug development, can help to adjust the whole process, reduce costs and improve success rate [155].

5. microRNAs and their potential as biomarkers in a liquid biopsy approach

The molecular profiling of tumors has been shown to improve diagnosis, follow-up and therapy monitorization of patients [156]. However, the standard procedure to obtain the sample is tissue biopsy [156]. Biopsies are invasive techniques that show limitations in acquiring tumor samples with enough quantity and quality to profile the tumor. Therefore, monitorization of a tumor response and relapse is a challenge, since repeating this invasive procedure throughout time is not feasible. Additionally, biopsies of the tumor do not take into consideration tumor heterogeneity and in a metastatic case, where tumors spread spatially and temporarily, multiple biopsies would be needed to properly stage the patient [157].

In an attempt to address this issue, the concept of liquid biopsy has arisen, changing the focus from analyzing tumor tissue to biological fluids, such as blood, mucosa, pleural effusions, urine and cerebrospinal fluid [158]. This concept, especially in the case of blood, takes its principle in the fact that these biological fluids will contain tumor cells or tumor cell products derived from most of the tumor cells, providing higher sensitivity and a minimal invasive approach that will enable repeated sampling and help monitor treatment response and relapse [159].

Among the biological components found in blood are platelets, circulating tumor cells, cell-free DNA, proteins, mRNA, microRNAs (miRNAs) and extracellular vesicles [160]. While initially research focused mostly on circulating tumor cells, these are relatively rare, and their collection and analysis is complex and not very effective. Therefore, in the last years, other biomolecules have raised attention, specifically the miRNAs [160].

MiRNAs are a class of short RNAs with a length of 19-25 nucleotides that are able to regulate gene expression post-transcriptionally, through sequence-selective targeting of mRNAs that can lead to their inhibition or degradation, depending on the level of complementarity [161]. MiRNAs derive from intergenic and intronic regions of both coding and non-coding genes and are transcribed in the nucleus by RNA polymerase II (Pol II) as a long primary RNA. They are then cleaved by a Microprocessor and exported to the cytoplasm where they will be further processed by DICER1, being transformed in double strand miRNAs, that will be unwounded by a helicase, reaching its final form of mature miRNAs [162]. MiRNAs can circulate in biofluids through two different mechanisms: inside protein complexes, such as the RNA-induced silencing complex, where they associate to argonaute 2 (AGO2) or inside extracellular vesicles [163]. Both protect miRNAs from RNase degradation and make them extremely stable to unfavorable conditions, such as fluctuations in temperature and pH, which highlights their biomarker potential [164].

They post-transcriptionally regulate the expression of thousands of protein-coding genes, which presents them as key regulators of different biological processes such as differentiation and development, metabolism, proliferation and apoptosis [161]. In a cancer context, miRNAs can act as oncomiRNAs when they target tumor suppressor genes and inhibit their expression, or act as tumor suppressor, by targeting oncogenes [165]. Interestingly, their expression patterns alter in pathological conditions such as cancer, being reproducible and conservative among same species individuals, which, along with their stability and the fact that they can be easily quantified makes them great biomarkers candidates [166]. Indeed, there are several major revisions summarizing the potential of different miRNAs in the diagnosis, prognosis stratification and therapy monitorization of different neoplasias [160, 167, 168]. However, there are very few miRNAs found deregulated in one single tumor model, which can be explained by the fact that each miRNA regulates several mRNAs and a single mRNA can be detected by several miRNAs [169]. Therefore, there is a need to look to a profile of miRNAs that can increase the specificity while not losing sensitive power, when defining a biomarker strategy.

Some studies have reported a deregulation of miRNAs expression upon AgNPs exposure. Eom and collaborators, for example, have shown through a microarray analysis that 63 miRNAs were altered by AgNPs exposure in Jurkat T cells. These miRNAs correlated with genes such as metallothionein 1F (MT1F) and tribbles homolog 3 (TRIB3) which are associated with DNA damage and apoptosis [170]. In another study, AgNPs led to a downregulation of hsa-miRNAs-126, -155 and -146 but did not affect DNA methylation in endothelial cells [171]. Brzóška and collaborators found similar results regarding DNA methylation patterns in HepG2 cells, reporting the deregulation of 6 miRNAs related with

tumor proliferation inhibition and tumorigenesis [172]. Interestingly, ROS, one of the key players in the mechanisms behind AgNPs effect has been shown to modulate miRNA expression through regulating miRNA biogenesis, transcription, and epigenetic modifications. On one hand, ROS can alter DNA methylation and modify histones which is associated with deregulated miRNAs. Moreover, through regulation of ROS-responsive transcription factor such as c-myc, p53, NFkB and HIF-1 α , it can activate miRNA transcription. Finally, it can affect miRNAs processing through deregulation of the expression of processing machinery such as DICER [173].

Thus, given the interplay between AgNPs' damage and miRNAs deregulation, and due to the miRNAs shown potential as biomarker in a liquid biopsy approach, miRNAs present themselves as a good option for a future therapy response monitorization in an AgNPs therapeutic approach design.

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OBJECTIVES

The aim of this work is to study the application of AgNPs functionalized with glucose (G-AgNPs) in PCa as a future therapeutic approach that can help surpass therapy failure (figure 5). The need for new therapeutic approaches that can address tumors that are as aggressive, metastatic, and resistant to current therapies is one of the main challenges in oncology field, specifically in PCa management. AgNPs have been studied as an attractive future therapeutic strategy due to their ability to damage multiple cellular organelles and target several signaling pathways deregulated in the most advanced forms of this tumor model.

Therefore, through an *in vitro* study, we intend to explore the administration of AgNPs and G-AgNPs to PCa cells resistant to ADT and analyze their cytotoxic effect, uptake mechanism, molecular mechanism, and toxicity. Additionally, we intend to establish an associated miRNA profile to define potential predictive biomarkers regarding this new therapeutic strategy.

This study will allow us, not only, to explore the potential of G-AgNPs as a future therapeutic strategy in therapy resistant forms of cancer, but also to define a miRNA profile of response to this therapy, that can help define which patients will better respond to it and monitor that response.

Specific objectives

1. Synthesize and characterize AgNPs functionalized with glucose (G-AgNPs) and further study their cytotoxic and cellular effect in PCa cells resistant to ADT.
2. Evaluate the uptake mechanism of AgNPs and G-AgNPs by ADT sensitive and resistant PCa cells and its importance for their cytotoxic effect.
3. Evaluate the molecular mechanisms behind the cytotoxic effect of AgNPs and G-AgNPs through a miRNA-profile definition approach with a potential as biomarkers of response to therapy monitorization.

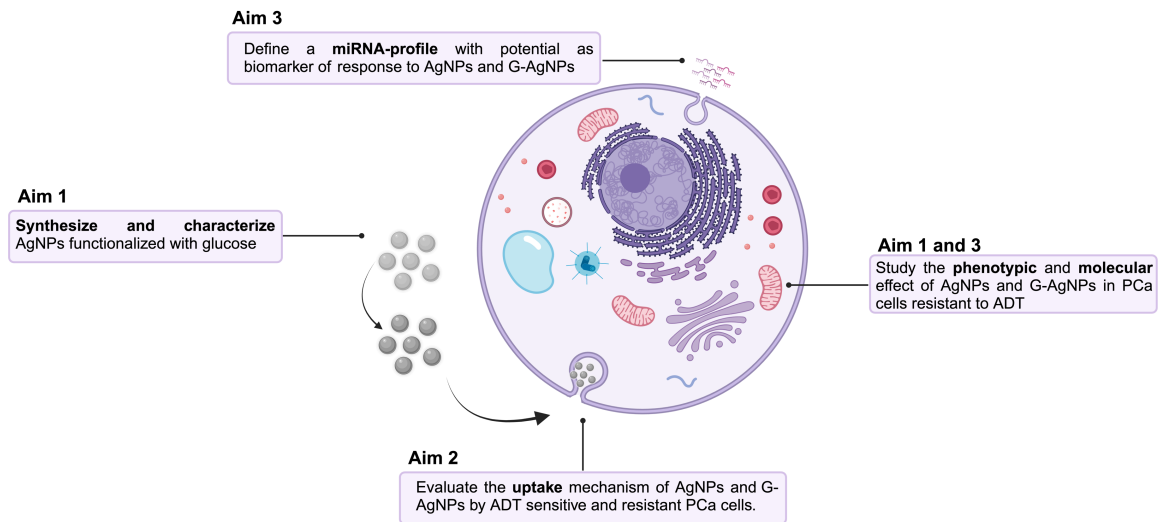


Figure 5 - Study of the application of AgNPs functionalized with glucose in PCa as a future therapeutic approach that can help surpass therapy failure.

RESULTS

In order to answer to the proposed objectives, this section will be divided into three topics:

1. Glucose-Functionalized Silver Nanoparticles as a Potential New Therapy Agent Targeting Hormonal Resistance Prostate Cancer Cells

This chapter will focus on the synthesis and characterization of AgNPs functionalized with glucose (G-AgNPs) with a further study on their cytotoxic and cellular effect in PCa cells resistant to ADT;

2. Glucose-functionalized Silver Nanoparticles (G-AgNPs) uptake by caveolae-dependent endocytosis is responsible for their selective effect towards Castration Resistant Prostate Cancer

This chapter will focus on the role of the uptake mechanism of AgNPs and G-AgNPs in their cytotoxic effect in ADT resistant cells and lack of effect in ADT sensitive cells;

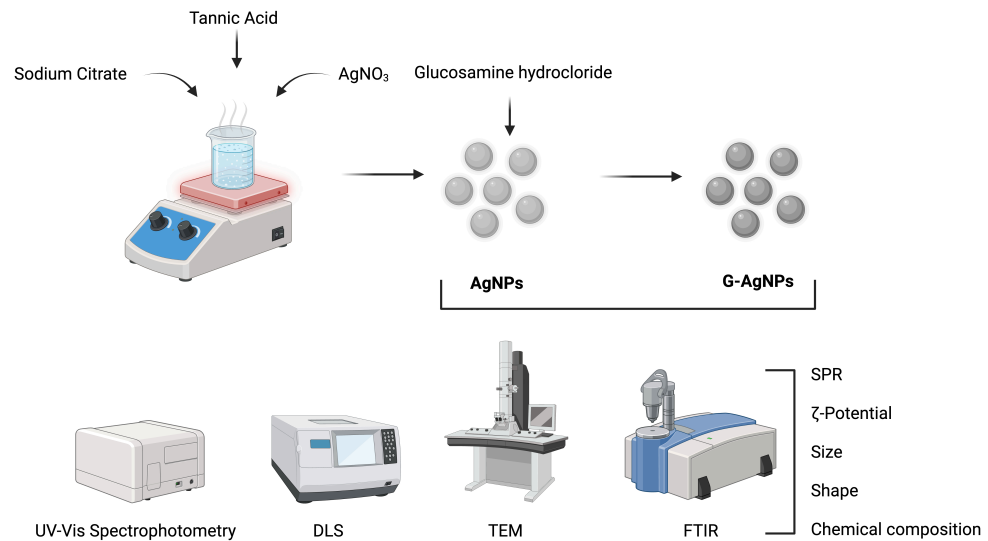
3. Do we have evidence for the multi-targeted therapy effectiveness against Castration Resistant Prostate Cancer (CRPC)? - a miRNA-profile definition approach and its potential as biomarker of response against Silver Nanoparticles (AgNPs) functionalized with Glucose (G-AgNPs)

This chapter will focus on the definition of a miRNA-profile of response to AgNPs and G-AgNPs and its ability to uncover the molecular mechanisms behind the cytotoxic effect of AgNPs and G-AgNPs in PCa resistant to ADT.

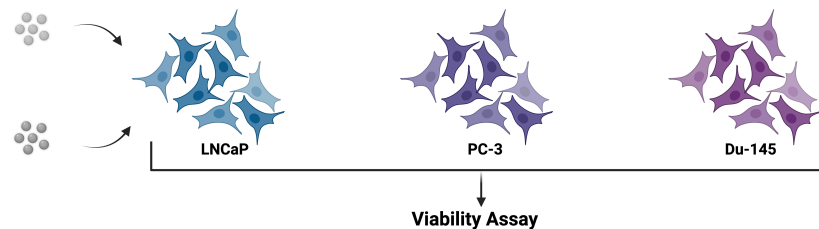
1. Glucose-Functionalized Silver Nanoparticles as a Potential New Therapy Agent Targeting Hormonal Resistance Prostate Cancer Cells

1.1. Study design

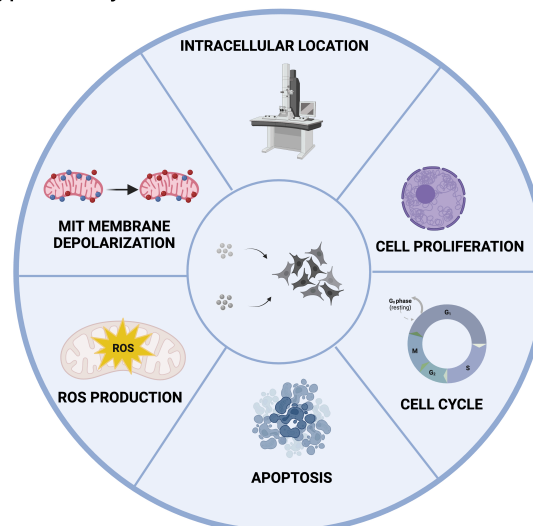
Phase I: Synthesis and characterization of G-AgNPs



Phase II: IC_{50} determination in PCa cell lines



Phase III: Phenotypic Assays



Glucose-Functionalized Silver Nanoparticles as a Potential New Therapy Agent Targeting Hormone-Resistant Prostate Cancer cells

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Purpose: Silver nanoparticles (AgNPs) have shown great potential as anticancer agents, namely in therapies' resistant forms of cancer. The progression of prostate cancer (PCa) to resistant forms of the disease (castration-resistant PCa, CRPC) is associated with poor prognosis and life quality, with current limited therapeutic options. CRPC is characterized by a high glucose consumption, which poses as an opportunity to direct AgNPs to these cancer cells. Thus, this study explores the effect of glucose functionalization of AgNPs in PCa and CRPC cell lines (LNCaP, Du-145 and PC-3).

Methods: AgNPs were synthesized, further functionalized, and their physical and chemical composition was characterized both in water and in culture medium, through UV-visible spectrum, dynamic light scattering (DLS), transmission electron microscopy (TEM) and Fourier-transform infrared spectroscopy (FTIR). Their effect was assessed in the cell lines regarding AgNPs' entering pathway, cellular proliferation capacity, ROS production, mitochondrial membrane depolarization, cell cycle analysis and apoptosis evaluation.

Results: AgNPs displayed an average size of 61nm and moderate monodispersity with a slight increase after functionalization, and a round shape. These characteristics remained stable when redispersed in culture medium. Both AgNPs and G-AgNPs were cytotoxic only to CRPC cells and not to hormone-sensitive ones and their effect was higher after functionalization showing the potential of glucose to favor AgNPs' uptake by cancer cells. Entering through endocytosis and being encapsulated in lysosomes, the NPs increased the ROS, inducing mitochondrial damage, and arresting cell cycle in S Phase, therefore blocking proliferation, and inducing apoptosis.

Conclusion: The nanoparticles synthesized in the present study revealed good characteristics and stability for administration to cancer cells. Their uptake through endocytosis leads to promising cytotoxic effects towards CRPC cells, revealing the potential of G-AgNPs as a future therapeutic approach to improve the management of patients with PCa resistant to hormone therapy or metastatic disease.

Keywords: castration-resistant prostate cancer, hormonal therapy, therapy resistance, Warburg Effect



Plain Language Summary

Prostate cancer (PCa) is the second most frequent and the fifth cause of cancer-related death in men worldwide. When patients stop responding to androgen deprivation therapy, they progress to castration-resistant prostate cancer (CRPC), which is associated with poor prognosis and limited therapeutic options. Thus, the present study evaluated the cytotoxic potential of silver nanoparticles (AgNPs) functionalized with glucosamine in PCa cell lines, since in the last years, several researchers have shown that AgNPs can be useful as anticancer drugs. In our study, we successfully synthesized and functionalized AgNPs and we proved them to be stable in cell culture medium, which is important because they can remain in circulation intact until they get to tumor location. We observed that AgNPs were cytotoxic to CRPC cell lines and not to the sensitive ones and that glucosamine functionalization increased their cytotoxic capacity. With this study, we showed that the nanoparticles enter the cells through endocytosis and increase the production of reactive oxygen species, inducing damage in mitochondria. The damage induced by the nanoparticles lead to a blockage of cell cycle and, consequently, of cell proliferation inducing apoptosis. These results reveal the potential of glucosamine functionalized AgNPs as a future therapeutic approach to improve the management of patients with CRPC or metastatic disease.

Introduction

Prostate cancer (PCa) is the second most frequent and the fifth cause of cancer-related death in men worldwide.^{1,2} Nearly 79% of the patients are diagnosed at an early and localized stage but, 35–60% of these patients experience disease recurrence.³ In cases of advanced disease, androgen deprivation therapy (ADT) is the first-line treatment, suppressing AR signalling and inhibiting PCa cells' proliferation.⁴ Despite the high early response rates, the heterogeneity of PCa cells ultimately leads to their adaptation to androgen deprivation and reactivation of AR signalling even under a low level of serum androgen with patients progressing to castration-resistant prostate cancer (CRPC) within 2 to 3 years.⁵ Progression to CRPC is burdened with poor prognosis and impaired quality of life, being the estimated mean survival of these patients 15–36 months.^{6,7} In the last years, the upcoming of second-generation antiandrogens (such as enzalutamide and abiraterone), chemotherapy (such as docetaxel and cabazitaxel) and radionuclide therapy (radium-223) have improved overall survival (OS).⁸ Nevertheless, the increase in the median PCa-specific survival in mCRPC with the introduction of the novel therapies only increases 2.8–4.8 months, making overcoming resistance one of the major challenges in these patients' management.^{9–11}

There are different molecular mechanisms behind the acquisition of resistance to ADT.⁶ Among them is the disruption of glucose metabolism, since AR regulates genes related to glucose consumption and increases the activity of several glycolytic enzymes. One example of such is GLUT1's expression, which is associated with poor prognosis and whose gene promotor directly binds to AR.^{12–15} Prostate cells show, indeed, a distinctive metabolism, with unique alterations during tumor development and progression.¹⁶ Due to the prostate epithelial cells' function of producing and secreting citrate that will be part of the seminal fluid, in normal conditions, Krebs cycle is interrupted and cells produce energy mostly through a glycolytic pathway.¹⁷ During tumor development, Krebs cycle is reactivated in consequence of the cancer cells higher energetic demands.¹⁸ In the most advanced stages of the disease, there is a preference for cancer cells to metabolize glucose through glycolysis and lactate fermentation instead of Krebs cycle and oxidative phosphorylation, also called the Warburg Effect.¹⁹ This is a faster method to obtain energy and acidifies the microenvironment of the tumor; however, it is energetically inefficient. Therefore, it is normally followed by an increase in glucose consumption by cancer cells.²⁰ This increased glucose uptake poses as an opportunity of targeting CRPC cells in the development of new therapy approaches.

The steady development of nanotechnology and nanoscience in the last years has brought up a particular interest in the development of metallic nanoparticles directed to biotechnology and biomedicine in different medical fields, including oncology, with various nanoparticles (NPs) having been approved for clinical use, either by the Food and Drug Administration in the United States or the European Medicines Agency (EMA) in the European Union.^{21–23} Due to their nanoscale size, high surface-to-volume ratio, different chemical composition and the ability to attach different targeting ligands to their surfaces, silver nanoparticles (AgNPs), which are small, organized structures ranging from 1 to 100 nm in size with about 20–15,000 atoms, have been emerging as a potential strategy to improve treatment in different tumor models, including the overcome of therapy-resistant cancer phenotypes.^{24,25} In fact, AgNPs nanoscale size allows them to cross tissue barriers since the tissue junction between capillaries ranges from 150 to 200 μ M. Moreover, while

larger particles (>200 nm) accumulate in the liver and spleen, being quickly removed from the bloodstream by macrophages of the reticuloendothelial system, smaller AgNPs are limited to tissue extravasation and renal clearance.²³ When inside the tumor cells, they trigger different processes that result in the activation of signaling pathways that lead to apoptosis.^{25,26} These characteristics show AgNPs' potential as anticancer agents, for example, in therapies' resistant forms of cancer. Their long-term toxicity is yet not fully understood, with contradictory data having been presented throughout the years. Nevertheless, in a recent review, Ivlieva and collaborators concluded that the toxicity is mostly due to the liberation of Ag^+ and not from the nanoparticle itself, showing the importance of a good stability of the AgNPs for its safety.²⁷ Additionally, Maziero and her team have shown that AgNPs stabilized with Gum Arabic Protein revealed adverse effects in zebrafish species but not in Sprague Dawley rats for 28 days in doses up to 10.0 mg/kg which shows the potential of their usage and the importance of their study.²⁸ Thus, the purpose of this study was to synthesize and characterize AgNPs functionalized with glucose (G-AgNPs) and further study their cytotoxic and cellular effect in PCa cells resistant to ADT.

Materials and Methods

Materials

Silver nitrate $\geq 99\%$ (AgNO_3), sodium citrate dihydrate, tannic acid, and D-(+)-glucosamine hydrochloride were purchased in Sigma-Aldrich. Roswell Park Memorial Institute-1640 medium (RPMI-1640), fetal bovine serum (FBS), penicillin–streptomycin and trypsin-EDTA were purchased from Gibco[®]. Trypan Blue, which allows for the evaluation of cytoplasmic membrane damage, was purchased from VWR TM. Resazurin, which allows for the evaluation of cell viability and IC_{50} determination was purchased from ACROS Organic[™] – Fisher Scientific[®]. Bromodeoxyuridine (BrdU), which allows for the evaluation of cell proliferation capacity, was purchased from ROCHE[®]. Propidium iodide (PI), which allows for the evaluation of cell cycle, was purchased from Sigma-Aldrich[®]. Tetramethylrhodamine (TMRE) kit, which allows for the evaluation of mitochondrial membrane potential, was purchased from Abcam[®]. The 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), which allows to quantitatively assess reactive oxygen species, was purchased from Millipore[®].

Synthesis of AgNPs

An aqueous solution containing 5 mM sodium citrate and 5 mM tannic acid at final volume of 100 mL was mixed and heated at 100 °C, for 15 min under vigorous stirring, as described by Bastus et al.²⁹ After starting to boil, 1 mL of 25 mM AgNO_3 was added into this solution. The resulting AgNPs were purified by centrifugation at 20,000 g for 15 min and further redispersed in MilliQ (MQ) water.

Functionalization of AgNPs with Glucosamine

After synthesis, the functionalization of AgNPs with glucosamine was done by ligand exchange.²⁹ For that, 1 mg of AgNPs was redispersed with 2 mL of 2 mM sodium citrate containing 5 mM glucosamine hydrochloride, and the solution was stirred for 24 h at room temperature. Finally, the resulting G-AgNPs were washed, redispersed in MQ-water, and stored at 4 °C.

Characterization of AgNPs

The average hydrodynamic diameter, polydispersity index, and ζ -potential of the AgNPs were measured by dynamic light scattering (DLS), using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK). For this, the samples were diluted in MilliQ-water at concentration of 100 $\mu\text{g/mL}$. The morphology of AgNPs was confirmed by transmission electron microscopy (TEM, Jeol JEM-1400, Jeol Ltd., Tokyo, Japan), using an acceleration voltage of 80 kV. For the sample preparation, a droplet of AgNPs' suspension was placed on a carbon-coated copper grid, blotted using a filter paper, and then air-dried before analysis. To acquire the UV–visible spectra, AgNP dispersions at concentration of 15 $\mu\text{g/mL}$ were placed in a cell, and the analysis was performed in the 300–700 nm range at room temperature, using an UV-visible spectrophotometer (UV-1800 Shimadzu) with the UV probe 2.70 software. Additionally, the AgNPs were characterized using a Fourier transform infrared spectroscopy (FTIR) instrument (SpectrumOne, PerkinElmer, Turku, Finland), equipped with a universal attenuated total reflectance (ATR) accessory. The ATR-FTIR spectra were recorded at

room temperature between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1} and number of 64 scans, and the baseline was corrected using the built-in software. The stability of AgNPs was evaluated after incubation of 500 $\mu\text{g/mL}$ of AgNPs with RPMI supplemented with 10% fetal bovine serum (FBS), at 37 °C for 2 h. For this, 200 μL were withdrawn at 5, 15, 30, 60, 90 and 120 min, and diluted in water to evaluate changes on the size, PDI and ζ -potential of AgNPs over time. All the experiments were performed in triplicates.

Cell Culture and Treatments Conditions

PC-3 and LNCaP cells (ATCC[®] CRL-1435TM RRID:CVCL_0035 and ATCC[®] CRL-1740TM RRID:CVCL_1379, respectively) were obtained from ATCC and Du-145 cells (ATCC[®] HTB-81: RRID:CVCL_0105) were kindly granted by Professor Cármen Jerónimo from IPO-Porto Research Center (Portugal). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and were maintained in a humidified incubator at 37°C, 5% CO_2 and 95% humidity. Cells were routinely grown in tissue culture flasks and harvested with a solution of trypsin-EDTA 0.05% (Gibco[®], Gaithersburg, MD, USA), while in a logarithmic growth phase. When cells had reached 80–90% confluence, cells were trypsinized with trypsin-EDTA 0.05% (Gibco[®], Gaithersburg, MD, USA). The cell pellets were resuspended in growth media, and cells were counted using trypan blue solution (VWRTM) and an automatic cell counter (EVETM – NanoEntek). All cell lines were routinely tested, each 2 weeks, for mycoplasma presence and were found to be free from contamination.

AgNPs and G-AgNPs IC_{25} and IC_{50} Determination by Resazurin Assay

Cells were cultured in 96-multi-well plates at a density of 2×10^5 cells per mL and treated with increasing concentrations of AgNPs and G-AgNPs, ranging from 600 to 3000 $\mu\text{g/mL}$ for 24h. Then, cells' viability was determined by a cell viability assay using Resazurin sodium salt (ACROS OrganicTM – Fisher Scientific[®], MA, USA). Two biological replicates were made for all the conditions, with seven technical replicates each. The IC_{50} of both AgNPs and G-AgNPs was determined using The Quest GraphTM IC_{50} Calculator, with a four-parameter logistic regression model, using the seven replicates and the average of the IC_{50} was calculated. The same was done for the IC_{25} determination, using the following equation: $\text{IC}_{25} = \left(\frac{25}{100-25} \right)^{\frac{1}{\text{Hill Slope}}} \times \text{IC}_{50}$. The following assays were made using the IC_{25} and IC_{50} values determined for each cell line.

Determination of Cellular Localization of AgNPs and G-AgNPs by Transmission Electron Microscopy (TEM)

Cells were cultured in 6-multi-well plates at a density of 2×10^5 cells per mL and treated with the determined IC_{25} for 24 hours. Then, cells were harvested with a solution of trypsin-EDTA and fixated. Cells were then further processed, and TEM images were collected by the Histology and Electron Microscopy platform from I3S Porto using a Jeol JEM 1400 Transmission Electron Microscope.

Cell Proliferation Assay – BrdU

BrdU assay was performed to evaluate cell proliferation. During the assay, BrdU, which is an analog of the nucleoside thymidine, is incorporated into replicating DNA, thus being an indicator of cellular proliferation. BrdU assay was performed using the Cell Proliferation ELISA, BrdU kit, according to the manufacturer instructions (Roche Diagnostics[®], Germany). Briefly, cells were grown overnight in 96-well microplates and incubated with either AgNPs or G-AgNPs and 5'-bromodeoxyuridine (BrdU) solution at a final concentration of 0.01 mM for 24 h. The optical density of proliferating cells (positive for BrdU) after ELISA assay using anti-BrdU-specific antibodies was evaluated at the microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg Germany). Results were expressed as percentage of control (100%). Two biological replicates and five technical replicates were performed for each condition.

Cell Cycle Analysis

Cell cycle analysis was performed by measuring the DNA content of the treated and untreated cells using Propidium Iodide (PI) by flow cytometry. Briefly, cells were grown in 6-well plates (2 wells per condition) and incubated with AgNPs and G-AgNPs for 24 hours. Then, culture medium was collected, and the cells detached with trypsin/EDTA and centrifuged at 160 g for 3 minutes. The pellet was washed with PBS 1× (Gibco®, Gaithersburg, MD, USA) and fixed by slowly adding 3 mL of cold 70% (v/v) ethanol for 15 minutes at 4°C. Fixed cells were centrifuged and the pellet obtained was washed with cold PBS. Subsequently, the cells were stained with a solution containing Triton X-100 (1.1%, v/v), PI and RNase A (50 and 100 µg/mL, respectively) in PBS. The cell cycle analysis was made using the Cytomics FC500 flow cytometer (Beckman Coulter) and the CXP software. The percentage of cells in the sub-G1 phase of the cell cycle was calculated from the total 10,000 cells (100%) in the assay, and the percentage of cells in G0/G1, S and G2/M phases was calculated from the total cells excluding the sub-G1 cells. Three technical replicates were performed for each condition.

Cell Apoptosis Assay – FITC Annexin V and PI

Apoptosis was evaluated through the Annexin V-FITC Apoptosis Detection kit (Abcam) according to manufacturer's protocol. Briefly, cells were grown in 6-well plates (2 wells per condition) and incubated with AgNPs and G-AgNPs for 24 hours. Then, cells were detached using Accutase enzyme cell detachment medium (Thermo Fisher Scientific) and stained with both recombinant annexin V conjugated to fluorescein (FITC annexin V) and red-fluorescent propidium iodide (PI) nucleic acid binding dye. Data analysis was performed through CXP Software in a FC500 Beckman Coulter flow cytometer.

Detection of Intracellular Reactive Oxygen Species – DCFH₂-DA Assay

Intracellular ROS formation was assessed by measuring the fluorescence of DCF, the oxidation product of the non-fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA). Briefly, cells were grown in 96-well plates at a density of 2.0×10^5 cells per mL were treated with the IC₂₅ and IC₅₀ of AgNPs and G-AgNPs for 24h. Three hours prior to completion of treatment, 150 µM *tert*-butyl hydrogen peroxide (Sigma-Aldrich®, USA) was used as positive control. Then, cells were incubated with 10 µM DCFH₂-DA (Merck Millipore®, USA) for 30 minutes at 37 °C, in the dark. Live cells were then imaged with filter set appropriate for fluorescein (FITC) using a fluorescence microscope OLYMPUS IX51 (Tokyo, Japan). The oxidation of DCFH₂-DA was detected by the increase in fluorescence which is proportional to the amount of intracellular ROS generated. The intracellular mean fluorescence intensity was quantified using the ImageJ software (National Institutes of Health, USA).

Mitochondrial Membrane Potential Assay (TMRE)

To assess the alterations on mitochondrial membrane potential, an important parameter of mitochondrial function used as an indicator of cell death, active mitochondria were labeled with tetramethylrhodamine, ethyl ester (TMRE) (Abcam, Cambridge, UK) according to the manufacturer's instructions. Cells were plated in 96-well plates with clear flat bottom and black sides at a density of 2.0×10^5 cells per mL and treated with the IC₂₅ and IC₅₀ of AgNPs and G-AgNPs for 24h. A positive control, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of mitochondrial oxidative phosphorylation, was used at the concentration of 100 µM for 10 minutes. After that, the cells were incubated with 500 nM of TMRE for 20 minutes at 37 °C, 5% CO₂, followed by one washing step with 100 µL of PBS containing 0.2% bovine serum albumin (BSA). A volume of 100 µL of PBS/0.2% BSA was added to each well, and the fluorescence was measured in a microplate fluorescence reader (Flx 800, Biotek) with excitation/emission: 549/575 nm. After background subtraction, the results were expressed as percentage of control (100%). Additionally, live cells were imaged with filter set appropriate for tetramethylrhodamine (TRITC) using a fluorescence microscope OLYMPUS IX51 (Tokyo, Japan). Two biological replicates and five technical replicates were performed for each condition.

Statistical Analysis

Using the sample *t*-test, the Mann–Whitney and the Kruskal–Wallis tests (depending on whether the results followed or not a normal distribution), the results were analyzed using SPSS26 software (release 26, SPSS Inc., Chicago, IL/USA) and their graphical representation was constructed using GraphPad Prism 6 software. *p*-values <0.05 were considered statistically significant.

Results

Synthesis and Characterization of AgNPs

To synthesize the AgNPs, sodium citrate and tannic acid were used as reducing agents, as previously done by Bastús et al.²⁹ After adding the aqueous solution of silver nitrate (AgNO₃) into the previous mixture, the solution changed its color from light yellow to dark brown within minutes, indicating the presence of AgNPs. The AgNPs were centrifuged and washed with MQ water before redispersing them with MilliQ (MQ) water. The ligand exchange of the resulting AgNPs with the amino-terminated glucose molecule (glucosamine) was performed in order to functionalize the AgNPs with glucose (G-AgNPs).²⁹ For that, the as-prepared AgNPs were mixed with glucosamine in a sodium citrate solution under vigorous stirring, and further centrifuged and washed to remove the excess of glucosamine. This ligand exchange is a spontaneous reaction that occurs due to the strong bonds formed between the amino groups on the glucosamine and the COOH/OH groups in the citrate/tannic molecules on the AgNP surface.²⁹ In addition, the plain AgNPs were also incubated in sodium citrate as control. The resulting AgNPs were further characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM), UV–Vis spectroscopy, and Fourier transform infrared spectroscopy (FTIR) (Figure 1).

The molar ratio of tannic acid and sodium citrate (1:1) used in the AgNP synthesis was chosen to obtain AgNPs with an average size of 61 ± 10 nm and moderate monodispersity (Figure 1A), given by their PDI values lower than 0.25. The surface charge of plain AgNPs was highly negative (-59 ± 6 mV), mainly due to the free COOH/OH groups of tannic acid and sodium citrate on the AgNP surface (Figure 1B). After functionalization with glucosamine, the size of G-AgNPs (64 ± 11 nm) and PDI did not change significantly. However, the ζ -potential of G-AgNPs slightly increased (-50 ± 4 mV), which indicates some replacement of sodium citrate/tannic acid by glucosamine. The optical characterization of the AgNPs (Figure 1C) showed a maximum UV-Vis absorption at around 412 nm due to the surface plasmon resonance.³⁰ Moreover, the G-AgNPs presented a red-shift in surface plasmon resonance band at 563 nm, suggesting a small increase in the size and polydispersity of the functionalized AgNPs.^{29,30} Besides the effect on reducing the polydispersity of AgNPs, the addition of high molar amount of tannic acid also influences the morphology of the resulting AgNPs. The TEM images showed that AgNPs presented roughly round shape (Figure 1D), because of the high amount of tannic acid added to the reaction mixture for the AgNP synthesis.²⁹ The FTIR analysis was also conducted in order to determine the functional groups present on the surface of the AgNPs and evaluate the differences between the plain AgNPs and the glucosamine-functionalized AgNPs (Figure 1E). The G-AgNPs spectrum showed stretching vibrations of C–H bonds from CH₂ groups at 2980 and 2900 cm⁻¹, C–N stretch on aromatic amines at 1390 cm⁻¹, stretching of the glycosidic linkage (C–O–C) and C–OH in the sugar ring at 1245 cm⁻¹, and deformation vibrations of C–O bonds in secondary alcohols at 1065 cm⁻¹.^{31,32} Therefore, these results suggested that the functionalization of AgNPs with glucosamine was successful.

Evaluation of the AgNP Stability

The synthesized NPs are known to remain stable dispersed in water for over a month. However, the magnitude and specificity of protein adsorption onto the surface of NPs in physiological environment can be influenced by the composition, size, shape and surface charge of the particles, which will ultimately affect their cellular uptake and biodistribution.^{33,34} Therefore, it is important to study its stability in these conditions. Since adsorption of proteins of nanoparticles usually happens in the first minutes of incubation, we performed a stability test of AgNPs and G-AgNPs for up to 2 hours. In this way, the stability of the AgNPs was evaluated in relevant biological media by measuring the changes on the size, PDI and ζ -potential of the NPs. For that, the AgNPs were incubated in RPMI, supplemented with

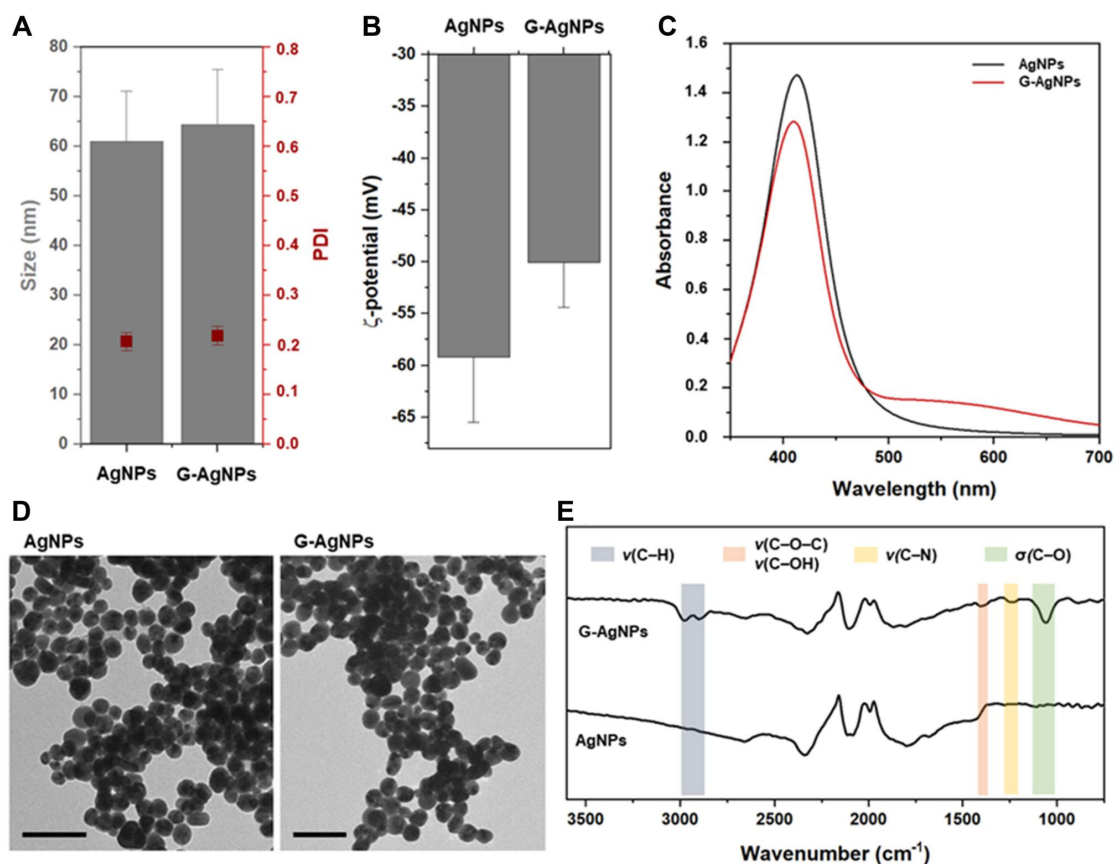


Figure 1 Characterization of AgNPs and G-AgNPs by means of (A) Size and PDI; (B) ζ -potential, and (C) UV-Vis spectra. (D) TEM images of AgNPs and G-AgNPs (Scale bars = 100 nm). (E) ATR-FTIR spectra of AgNPs and G-AgNPs. Error bars represent mean $\pm$ SD ($n \geq 3$).

10% of FBS at 37 °C, for 2 h (Figure 2). In the first 30 min of incubation, a slight increase in the NP size from ca. 60 nm to 110 and 100 nm for AgNPs and G-AgNPs, respectively, indicating some protein adsorption onto the NPs' surface due to the FBS proteins (Figure 2A). Furthermore, the size of the AgNPs kept constant over time, along with steady PDI values (Figure 2B), suggesting that the AgNPs remained monodispersed after incubation with the FBS containing cell culture medium. In addition, the surface charge of the NPs shifted to about -17 mV after the first 5 min of incubation, due to the protein adsorption on their surface. Overall, the stability of AgNPs can be ascribed to the presence of FBS in cell culture medium that can improve the colloidal stability of NPs.^{34,35}

IC₂₅ and IC₅₀ Determination

To perform the in vitro study, three cell lines were used: one hormone-sensitive cell line, LNCaP and two AR- and hormone-resistant cell lines Du-145 and PC-3. The cytotoxic effect of both AgNPs and G-AgNPs in the three cell lines used in the study was determined using the resazurin assay, and IC₂₅ and IC₅₀ were calculated. Regarding the LNCaP cell line, it was not possible to determine the IC₂₅ and IC₅₀ since there was no consistent effect of both AgNPs and G-AgNPs in these cells (Figure 3A). Regarding Du-145 and PC-3 cell lines, one can see that the viability of both cell lines decreases with the increase in both AgNPs' and G-AgNPs' concentration (Figure 3B and C), thus allowing the determination of the IC₂₅ and IC₅₀ (Table 1).

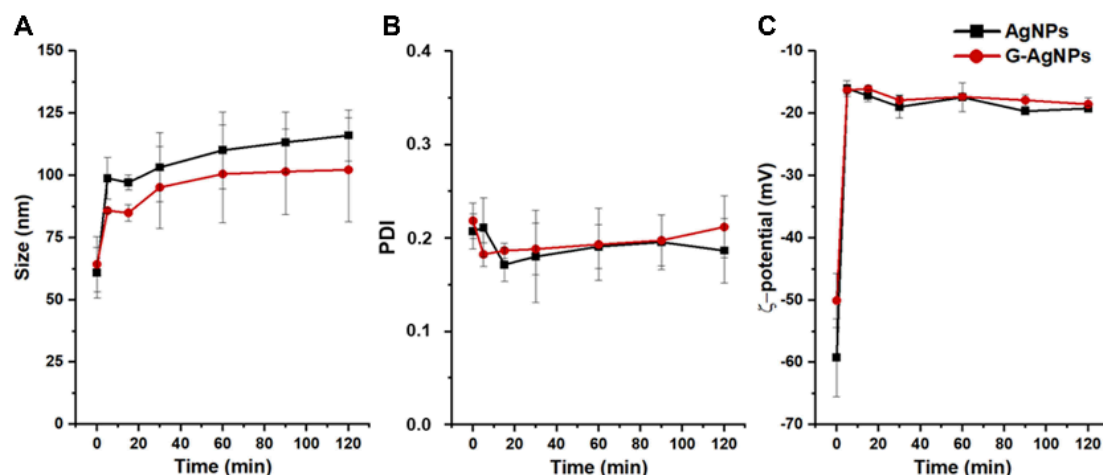


Figure 2 Stability of AgNPs in RPMI supplemented with 10% of FBS after 2 h incubation, at 37 °C: effect on the size (A), PDI (B) and ζ -potential (C) of AgNPs. Errors bars represent mean $\pm$ SD (n = 3).

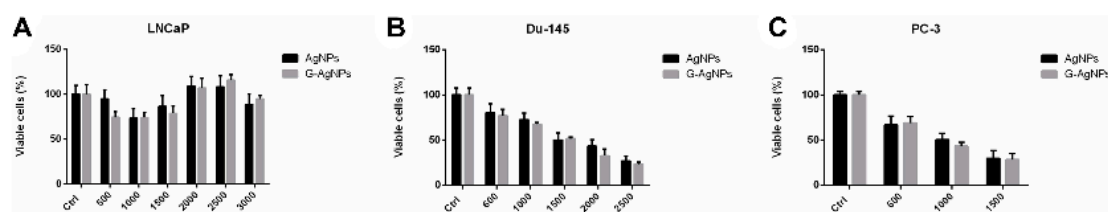


Figure 3 Evaluation of cell viability, by Resazurin Assay, upon treatment with AgNPs and G-AgNPs for 24 hours in LNCaP cell line (A) Du-145 cell line (B) and PC-3 cell line (C). Results are expressed as percentage of control (untreated cells), as mean $\pm$ SEM.

It is important to note that for both cell lines, the IC_{25} and IC_{50} are lower for G-AgNPs than AgNPs, suggesting an increased cytotoxic effect of the AgNPs after functionalization (Table 1). Moreover, both IC_{25} and IC_{50} in AgNPs and G-AgNPs are lower in PC-3 cell line when compared to Du-145. The determined values of IC_{25} and IC_{50} were then used in the following phenotypic assays.

AgNPs and G-AgNPs Intracellular Localization

To further confirm AgNPs and G-AgNPs internalization by both Du-145 and PC-3 cells, we performed a TEM analysis after administration of AgNPs (Figure 4A–C and H–J) and G-AgNPs (Figure 4D–G and K–M) at IC_{25} , to Du-145 and PC-3, respectively (Figure 4). One can observe that nanoparticles enter the cells through endocytosis (Figure 4J-1) and are trapped in lysosomes (Figure 4J-2). Inside the cell, they seem to be dispersed, since they can be observed in the

Table 1 IC_{25} and IC_{50} of AgNPs and G-AgNPs in Du-145 and PC-3 Cell Lines

Cell Line	Silver Nanoparticles	IC_{25} (μ g/mL)	IC_{50} (μ g/mL)
Du-145	AgNPs	919	1579
	G-AgNPs	753	1528
PC-3	AgNPs	511	911
	G-AgNPs	499	860

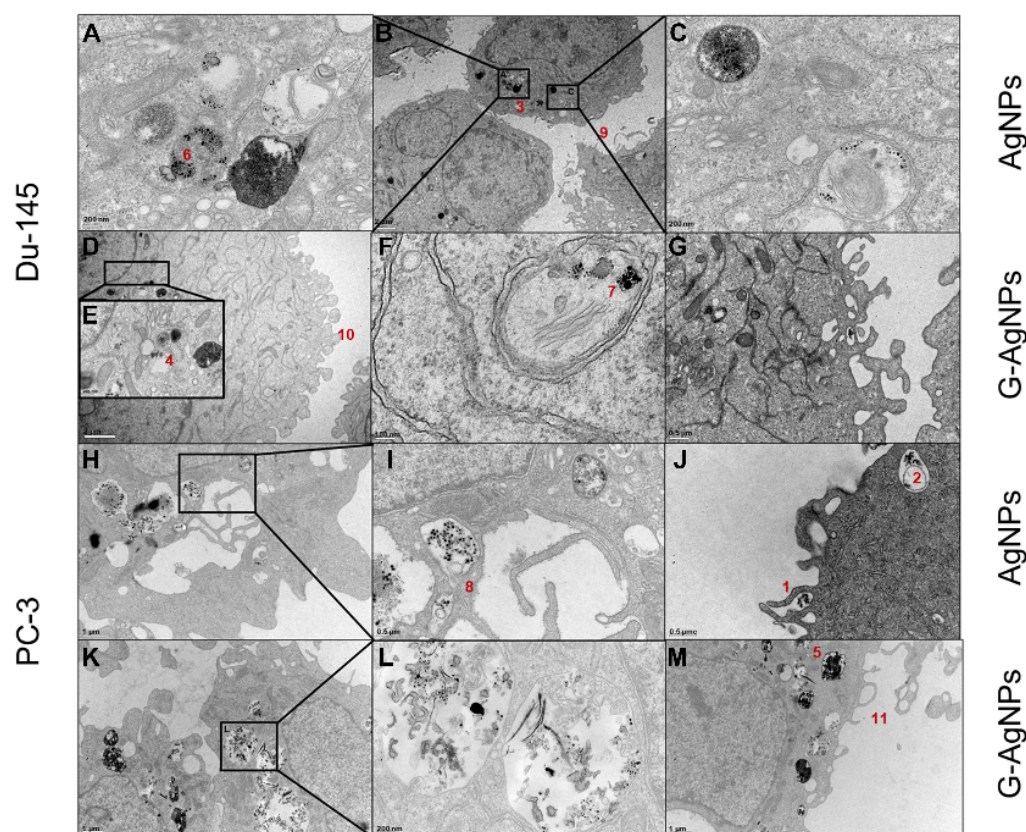


Figure 4 TEM images of Du-145 cells treated with IC_{25} of AgNPs (A–C) or G-AgNPs (D–G) and of PC-3 cells treated with IC_{25} of AgNPs (H–J) and G-AgNPs (K–M). From each sample, three pictures were taken with different amplifications. Thus, scale bars in B and D are 2 μ m, in H, K and M are 1 μ m, in G, I and J are 0.5 μ m, in A, C, E and L are 200 nm and in F is 100 nm. 1 – Nanoparticles entering the cells through endocytosis; 2 – Nanoparticles trapped in lysosomes; 3 – Nanoparticles dispersed throughout the cytoplasm; 4 – Nanoparticles localized in the nucleus; 5 – Nanoparticles localized in the mitochondria; 6–8 – Nanoparticles maintain the monodispersed nature inside the cell; 9–11 – Membrane blebbing of the cells.

cytoplasm (Figure 4B-3), close to the nucleus (Figure 4E-4) and close to mitochondria (Figure 4M-5). It is important to observe that both nanoparticles remain monodispersed inside the cell, even if highly concentrated in the lysosomes (Figure 4A-6, F-7 and I-8). Additionally, in both cells, there is evidence of membrane blebbing, a known apoptotic feature (Figure 4B-9, D-10 and M-11).

AgNPs and G-AgNPs' Effect in Cellular Proliferation

To analyze the cellular proliferation after administration of the NPs, the BrdU assay was performed. In the Du-145 cell line, the IC_{25} and IC_{50} of AgNPs were able to inhibit the proliferation of the cells to 33.05% and 27.21%, respectively (Figure 5A and B). Regarding G-AgNPs, the IC_{25} and IC_{50} were able to inhibit proliferation of the cells to 27.21% and 14.12%, respectively (Figure 5A and B). In the PC-3 cell line, the IC_{25} and IC_{50} of AgNPs were able to inhibit the proliferation of the cells to 43.65% and 25.68%, respectively (Figure 5C and D). Regarding G-AgNPs, the IC_{25} and IC_{50} were able to inhibit proliferation of the cells to 34.84% and 19.21%, respectively (Figure 5C and D). Interestingly, for both IC_{25} and IC_{50} , G-AgNPs had a significantly higher power of cellular proliferation inhibition when compared to

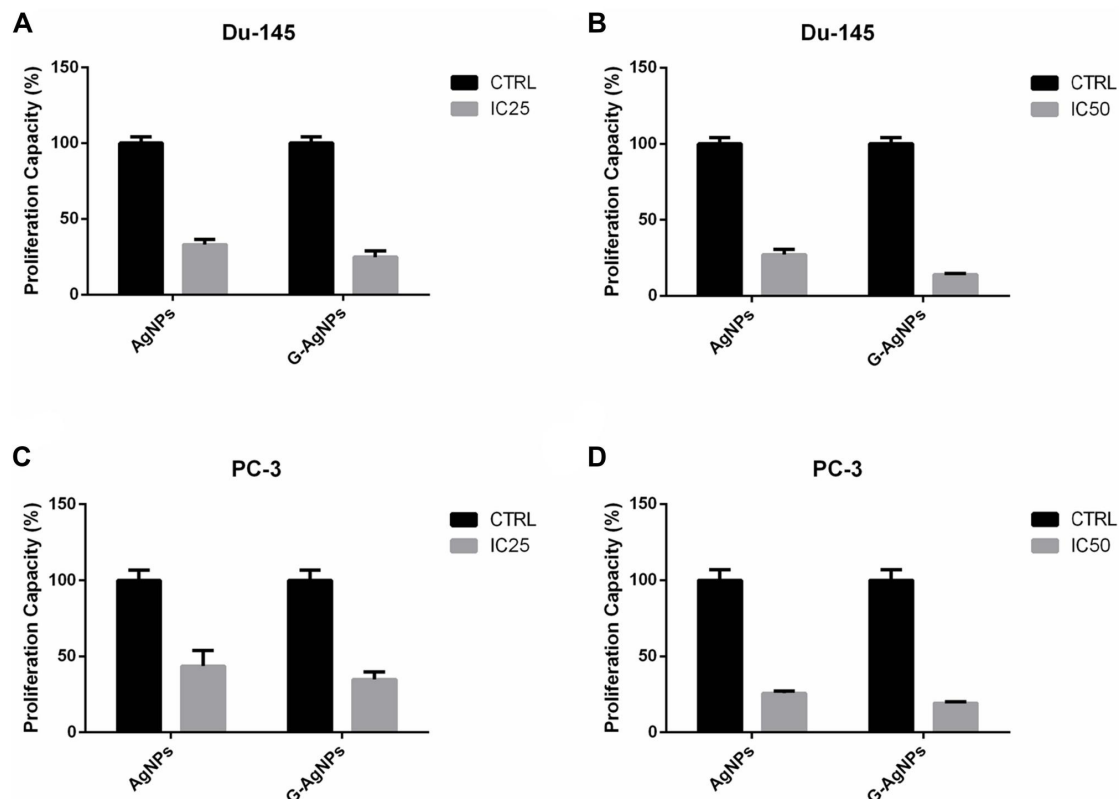


Figure 5 Evaluation of cell proliferation, by BrDu Assay, upon treatment with AgNPs and G-AgNPs in the Du-145 cell line using the determined IC₂₅ (A) and the determined IC₅₀ (B); and in the PC-3 cell line using the determined IC₂₅ (C) and the determined IC₅₀ (D). Results are expressed as percentage of control (untreated cells), as mean $\pm$ SEM.

AgNPs (24.85% vs 33.05%, $p = 0.009$ for IC₂₅ and 14.12% vs 27.21%, $p < 0.001$ in Du-145 cell line and 34.84% vs 43.65%, $p = 0.125$ and 19.21% vs 25.68%, $p < 0.001$ in PC-3 cell line).

AgNPs and G-AgNPs' Effect in Cell Cycle

To analyze possible cell cycle disruption following AgNPs or G-AgNPs administration, propidium iodide (PI) staining analysis by flow cytometry was used and further evaluation of the apoptosis status of the cells was performed (Figure 6).

In the Du-145 cell line, the IC₂₅ of AgNPs and G-AgNPs increased the number of cells in the S phase (28.62% vs 18.94% and 24.93% vs 18.94%, respectively) and subsequently decreased the number of cells in G2/M (10.1% vs 14.61% and 9.27% vs 14.61%, respectively). Additionally, there was a significant increase in the number of cells in subG0 phase for both IC₂₅ of AgNPs and G-AgNPs (4.23% vs 0.89% and 3.19% vs 0.89%). This effect was also observed for the IC₅₀ of AgNPs and G-AgNPs, with more accentuated differences. There was an increase in the number of cells in the S phase (40.51% vs 18.94% and 32.02% vs 18.94%, respectively) and subsequent decrease the number of cells in G2/M phase (5.28% vs 14.61% and 6.21% vs 14.61%, respectively). The number of cells in subG0 phase for both AgNPs and in G-AgNPs was significantly higher (43.23% vs 0.89% and 53.09% vs 0.89%). Regarding the PC-3 cell lines, the same effect was observed: the IC₂₅ of AgNPs and G-AgNPs increased the number of cells in the S phase (33.26% vs 12.65% and 38.21% vs 12.65%, respectively) and subsequently decreased the number of cells in G2/M (14.53% vs 26.53% and 11.08% vs 26.53%, respectively). This trend was also observed for the IC₅₀ of AgNPs and G-AgNPs, with more accentuated differences. There was an increase in the number of cells in the S phase (42.06% vs

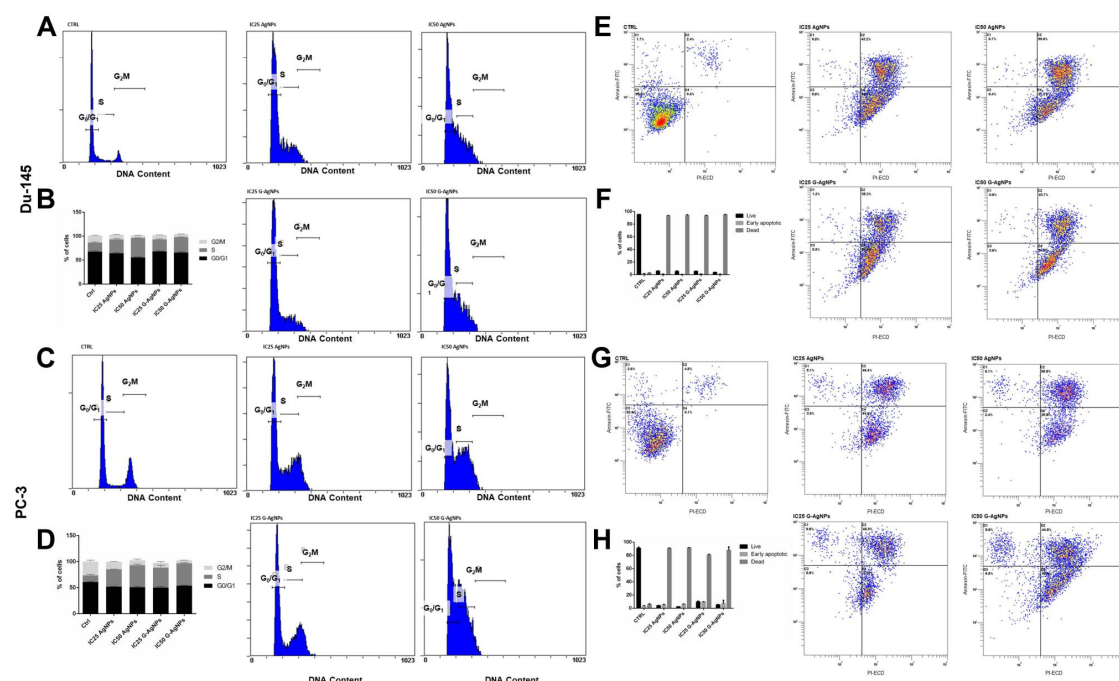


Figure 6 (A) Representative DNA histograms of cell cycle analysis assessed using a PI stain and flow cytometry of Du-145 cells treated with AgNPs and G-AgNPs in the determined IC₂₅ and IC₅₀; (B) quantitative analysis of cell cycle disruptions of Du-145 cells treated with AgNPs and G-AgNPs in the determined IC₂₅ and IC₅₀; (C) Representative DNA histograms of cell cycle analysis assessed using a PI stain and flow cytometry of PC-3 cells treated with AgNPs and G-AgNPs in the determined IC₂₅ and IC₅₀; (D) Quantitative analysis of cell cycle disruptions of PC-3 cells treated with AgNPs and G-AgNPs in the determined IC₂₅ and IC₅₀; (E) Representative flow cytometry images of apoptosis evaluation in Du-145 cells treated with AgNPs and G-AgNPs in the determined IC₂₅ and IC₅₀; (F) Quantitative analysis of live, early apoptotic and dead cells of Du-145 cells treated with AgNPs and G-AgNPs in the determined IC₂₅ and IC₅₀; (G) Representative flow cytometry images of apoptosis evaluation in PC-3 cells treated with AgNPs and G-AgNPs in the determined IC₂₅ and IC₅₀; (H) Quantitative analysis of live, early apoptotic and dead cells of PC-3 cells treated with AgNPs and G-AgNPs in the determined IC₂₅ and IC₅₀. Data are expressed as mean $\pm$ SEM.

10.30% and 42.89% vs 10.30%, respectively) and subsequently decrease in the number of cells in G2/M phase (9.17% vs 22.39% and 5.78% vs 22.39%, respectively). Furthermore, when the apoptotic state of the cells was assessed one can see that most cells marked positive for Annexin and PI confirming their apoptotic state after administration of both AgNPs and G-AgNPs.

AgNPs and G-AgNPs' Increase Cells' ROS Production

To analyze ability of the NPs to induce reactive oxygen species (ROS) production by cells, the DCFH2-DA assay was performed. In the Du-145 cell line, the IC₂₅ and IC₅₀ of AgNPs were able to induce about 2.1- and 2.8-fold increase in the cell mean fluorescence compared to control, respectively (Figure 7A and B). Regarding G-AgNPs, they have a similar effect when compared to AgNPs, with a fold increase in the cell mean fluorescence related to control of about 2.0 and 2.7, for the IC₂₅ and the IC₅₀, respectively (Figure 7C and D). In the PC-3 cell line, the IC₂₅ and IC₅₀ of AgNPs were able to increase the cell mean fluorescence to 2.2- and 2.8-fold increase compared to control cells, respectively (Figure 7E and F). Regarding G-AgNPs, the IC₂₅ have a similar effect of AgNPs IC₂₅ (2.2-fold increase) and the IC₅₀ were able to increase the cell mean fluorescence of 2.3-fold increase when compared to control (Figure 7G and H).

AgNPs and G-AgNPs' Effect in Mitochondria Depolarization

To analyze the mitochondria membrane damage after administration of the NPs, the TMRE assay was performed. Since this dye is positively charged, it accumulates in the negatively charged mitochondria. When mitochondria is depolarized, its membrane potential decreases and it does not incorporate the dye (Figure 8C and F). In the Du-145 cell line, the IC₂₅ and

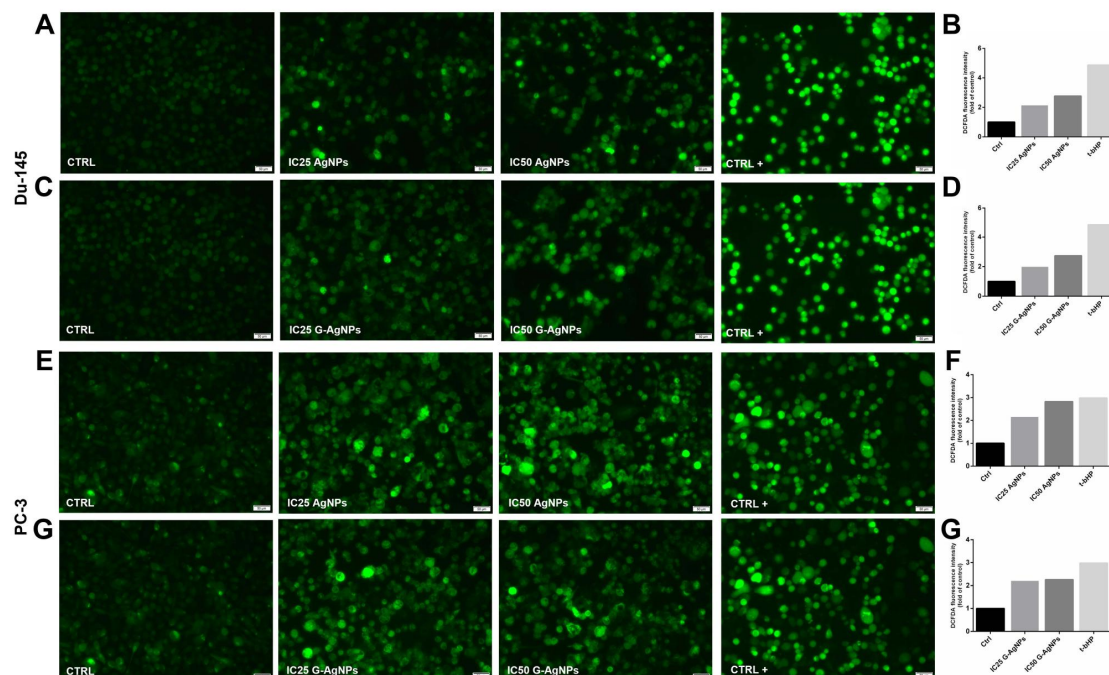


Figure 7 Evaluation of ROS production, by DCFH2-DA Assay, in the Du-145 cell line upon treatment with the determined IC₂₅ and IC₅₀ AgNPs (**A** and **B**) and upon treatment with the determined IC₂₅ and IC₅₀ G-AgNPs (**C** and **D**); and in the PC-3 cell line upon treatment with the determined IC₂₅ and IC₅₀ AgNPs (**E** and **F**) and upon treatment with the determined IC₂₅ and IC₅₀ G-AgNPs (**G** and **H**). (**A**, **C**, **E** and **G**) Representative images (10×) of DCFH2-DA labeled mitochondria (Photographs taken using an Olympus IX51 microscope). (**B**, **D**, **F** and **H**) Graphical representation of the DCFH2-DA fluorescence signal intensity in the different tested conditions. T-bhp is used as positive control. Results are expressed as percentage of control (untreated cells), as mean ± SEM.

IC₅₀ of AgNPs were able to depolarize the cells' mitochondria to 32.74% and 10.36%, respectively (Figure 8A and B). Regarding G-AgNPs, the IC₂₅ and the IC₅₀ were able to depolarize the cells' mitochondria to 30.13% and 6.96%, respectively (Figure 8A and B). In the PC-3 cell line, the IC₂₅ and IC₅₀ of AgNPs were able to depolarize the cells' mitochondria to 49.54% and 49.39%, respectively (Figure 8D and E). Regarding G-AgNPs, the IC₂₅ and IC₅₀ were able to depolarize the mitochondria of the cells to 47.45% and 38.13, respectively (Figure 8D and E). Interestingly, for IC₅₀, after G-AgNPs administration, there were significantly less cells with polarized mitochondria than after AgNPs administration in the PC-3 cell line (38.13% vs 49.39%, $p = 0.012$).

Discussion

The poor prognosis, short overall survival, and the low quality of life of CRPC patients added to the limited therapeutic options available are major concerns in PCa management and make the development of new therapeutic strategies imperative.⁶ The mechanism behind the acquisition of resistance to hormone therapy in patients that progress to CRPC is complex and not completely understood, but the importance of glucose metabolism deregulation has already been shown, and can be used as an advantage for the development of new therapeutic approaches.¹⁶ In the last years, the development of metallic nanoparticles has raised increased interest in the scientific community and AgNPs, specifically, have already shown cytotoxic potential in different tumor models.^{25,36–38} The increased glucose consumption in more advanced forms of PCa can be an opportunity to design AgNPs that specifically target cancer cells, through a glucose functionalization.

In the present study, AgNPs were successfully synthesized and further functionalized with glucosamine, showing physical and chemical characteristics that optimize their uptake by cancer cells and, consequently, their cytotoxic capacity. The small size, spherical shape and monodisperse nature of these NPs promotes their internalization and intracellular bioavailability. In fact, spherical NPs have been shown to have a higher internalization than asymmetrically

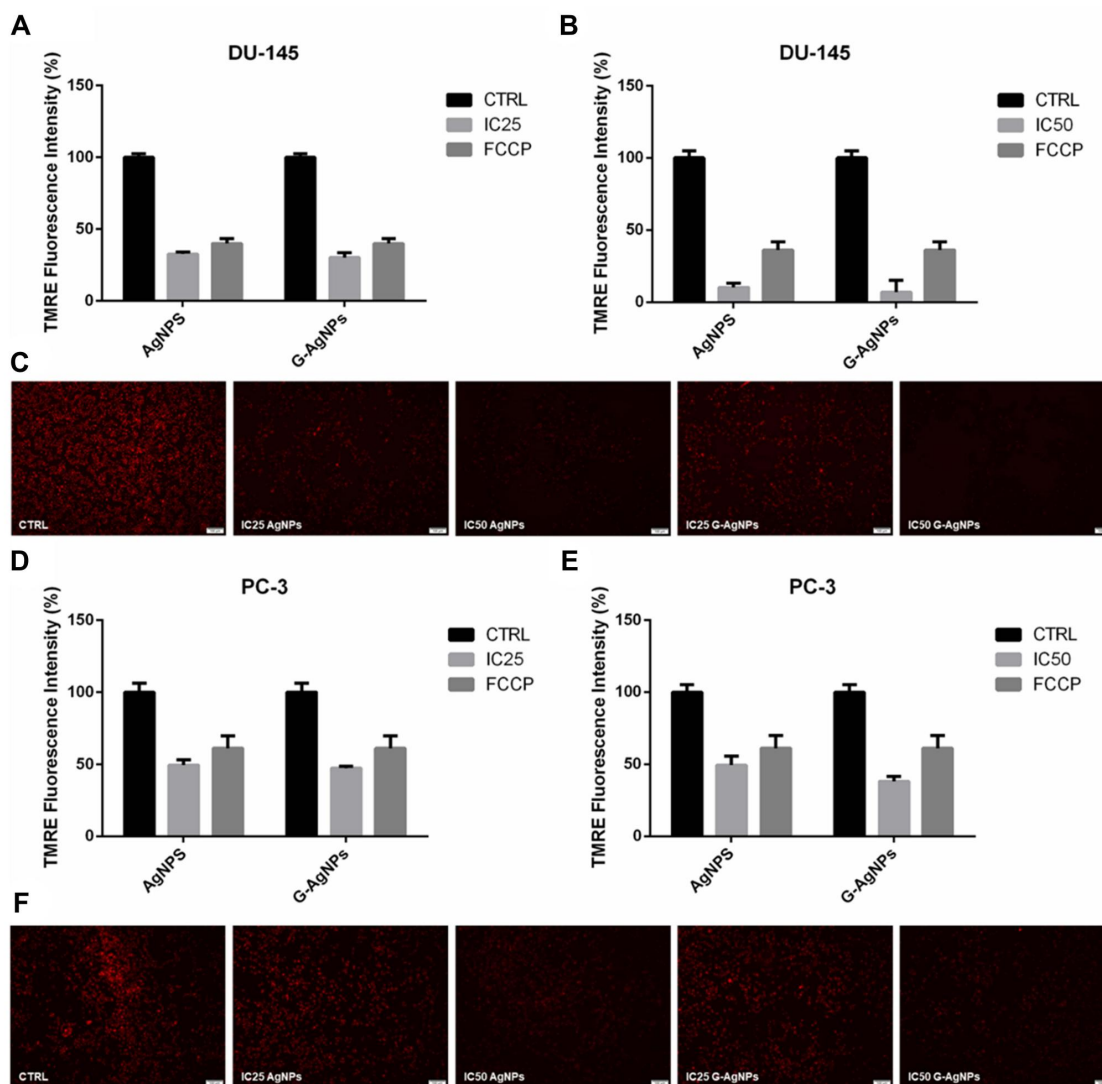


Figure 8 Evaluation of mitochondria membrane depolarization, by TMRE Assay, upon treatment with AgNPs and G-AgNPs in the Du-145 cell line (C) using the determined IC₂₅ (A) and the determined IC₅₀ (B); and in the PC-3 cell line (F) using the determined IC₂₅ (D) and the determined IC₅₀ (E). Representative images (10×) of TMRE labeled mitochondria (Photographs taken using an Olympus IX51 microscope). Results are expressed as percentage of control (untreated cells), as mean ± SEM.

shaped ones, and smaller-sized AgNPs have shown a higher uptake and cytotoxicity because of their increased surface area and particle number for the same mass/volume dose.³⁹ Additionally, the demonstrated stability of these NPs in cell culture medium is of great advantage because, contrary to small-molecule drugs, nanoscale drugs slowly leak from blood vessels after intravenous induction, thus their accumulation into the tumor would not be efficient if they were unstable in serum.⁴⁰

Both AgNPs and G-AgNPs had cytotoxic effects only on the hormone-resistant cells Du-145 and PC-3 and not to the hormone-sensitive cell line LNCaP, even when high concentrations of the NPs were administered. The different molecular profile of these cell lines apart from the absence of AR signal in both Du-145 and PC-3 can be the explanation behind this selective effect. Interestingly, Alvin Y. Liu has shown that the LNCaP cell line displays a very different cell

surface molecule profile from Du-145 and PC-3 cells (which are much more similar between them), which may lead to different internalization of the AgNPs.⁴¹ In fact, LNCaP cells do not express both caveolin-1 and caveolin-2, contrarily to Du-145 and PC-3.⁴² These molecules mediate the independent-clathrin pathway of endocytosis via caveolae, a ligand-triggered mechanism that leads to a more selective uptake and that may be behind the uptake of the synthesized nanoparticles and the absence of effect in the LNCaP cell line.⁴³

We also reported a higher cytotoxic effect and proliferation blockage of G-AgNPs in both PC-3 and Du-145, which show a lower IC₂₅ and IC₅₀ when compared with AgNPs. This higher effect can be explained by the different metabolic nature of hormone-resistant cells (Du-145 and PC-3). Previous studies have shown that these two cell lines show a metabolic shift towards the Warburg Effect, displaying lower consumption of O₂, higher rates of lactate production and higher glucose consumption rates which may favor the uptake of the NPs.^{44,45} In fact, Effert et al have already shown that GLUT-1's mRNA expression is higher in Du-145 and PC3, when compared with LNCaP cell line.⁴⁶ Interestingly, calveolin-1 has been shown to be involved in glucose metabolism, namely in the Warburg Effect and to enhance glucose uptake in prostate cancer cells.⁴⁷ This can explain the higher effect of G-AgNPs and may suggest that they are preferably uptaken by cells with high glucose consumption and calveolin expression. This would be an advantage in a systemic administration of the nanoparticles such as the CRPC context, since they would be preferably uptaken by these cells, which can allow a more directed effect.

To date, there are several studies reporting the effect of AgNPs synthesized by different methods and with a variety of cappings and their cytotoxic effect in PCa cell lines.^{48–63} Among these studies, the mechanism of action is, most of the times, common to the one we found in the present study, with an increase in ROS production, mitochondrial damage, nuclear fragmentation and cell apoptosis.^{50,52,60,62,63} However, most of them do not compare AgNPs' effect in hormone-sensitive and hormone-resistant cell lines and, to the best of our knowledge, the present study is the first one reporting the effect of AgNPs with a glucose functionalization in PCa and with a selective effect towards hormone resistance in this tumor model.

To better understand the observed cytotoxic effect, we performed a TEM analysis of the NPs location in the cells and several phenotypic assays. Firstly, we confirmed that the NPs enter the cells through endocytosis and targeted to lysosomes. In fact, lysosomes have in their composition a variety of hydrolytic enzymes and an acidic environment which will degrade AgNPs capping and release Ag⁺, a mechanism called “lysosome-enhanced Trojan horse effect”.⁶⁴ Moreover, it was already reported that AgNPs, when interacting with the acidic lysosomal compartment, induce the production of several ROS (superoxide anions (O₂^{•−}), hydroxyl radicals (•OH), and hydrogen peroxide (H₂O₂), which explains the increase in ROS production observed after treatment with NPs.³⁹ The diffusion of these ROS into cytoplasm leads to oxidative damage in different organelles such as mitochondria. In the present study, a depolarization of the mitochondrial membrane of Du-145 and PC-3 was observed when treated with both NPs. We have also observed a S-Phase cell cycle arrest after treatment with AgNPs and G-AgNPs. The successful progression through cell cycle is mediated by different proteins, cyclins, cyclin-dependent kinases and their inhibitors, being this strict regulation responsible for guaranteeing the safe progression through DNA replication and division.⁶⁵ The S-phase checkpoint checks the fidelity of DNA replication confirming there are no areas of unrepaired DNA damage from earlier cell cycle points.⁶⁶ Thus, the S-phase arrest shown in our study can be indicative of DNA damage and, along with the increased ROS production and mitochondrial damage can support the blockage of cellular proliferation observed and lead to the apoptotic state of the cells which we observed through FITC annexin V and PI staining.

Conclusions

In this study, we successfully designed AgNPs with a glucose functionalization that selectively induce damage in CRPC cells (as opposed to hormone-sensitive ones). The effect of the NPs was higher after functionalization, showing the potential of glucose to favor the uptake of the AgNPs by cancer cells. To the best of our knowledge, this was the first study reporting a selective effect of AgNPs directed to CRPC and the effect of glucose functionalization of AgNPs in PCa. This study shows the potential of these AgNPs as a new therapeutic approach to castration-resistant prostate cancer that needs to be validated in 3D cell cultures to understand the penetration ability of the NPs and in vivo to confirm the results and study the delivery and excretion pathways' safety. Additionally, exploring the molecular profile, including the

expression of calveolins, of the three cell lines could be of use to explain the differences of AgNP's effect. Nevertheless, the present results are very promising and these NPs could be a potential therapeutic strategy and improve the management of patients with PCa resistant to hormone therapy or with the presence of metastasis.

Abbreviations

ADT, androgen deprivation therapy; AgNPs, silver nanoparticles; AR, androgen receptor; BrdU, bromodeoxyuridine; CRPC, castration-resistant prostate cancer; DLS, dynamic light scattering; EMA, European Medicines Agency; FTIR, Fourier transform infrared spectroscopy; G-AgNPs, AgNPs functionalized with glucose; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; IC, inhibitory concentration; MQ, MilliQ; NPs, nanoparticles; OS, overall survival; PCa, prostate cancer; PI, propidium iodide; ROS, reactive oxygen species; SEM, standard error of mean; TEM, transmission electron microscopy; TMRE, tetramethylrhodamine, ethyl ester.

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Disclosure

The authors report no conflicts of interest in this work.

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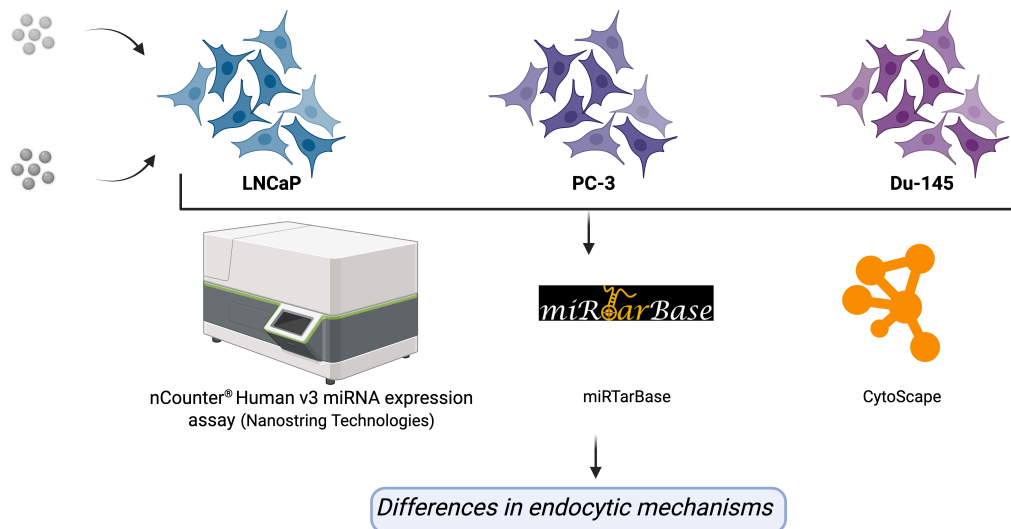
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2. Glucose-functionalized Silver Nanoparticles (G-AgNPs) uptake by caveolae-dependent endocytosis is responsible for their selective effect towards Castration Resistant Prostate Cancer

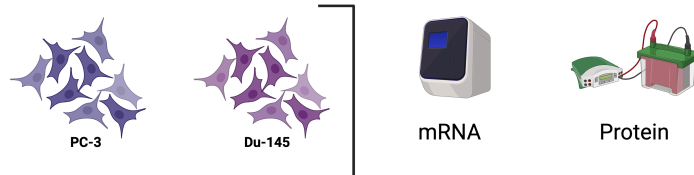
2.1. Study design

Phase I: Molecular mechanism behind lack of uptake of AgNPs and G-AgNPs by LNCaP cells

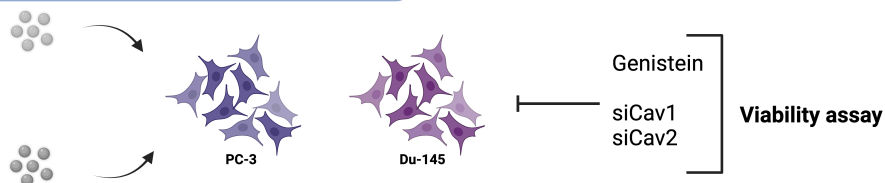


Phase II: Impact of modulation of Caveolae-dependent Endocytosis in cytotoxic effect of AgNPs and G-AgNPs

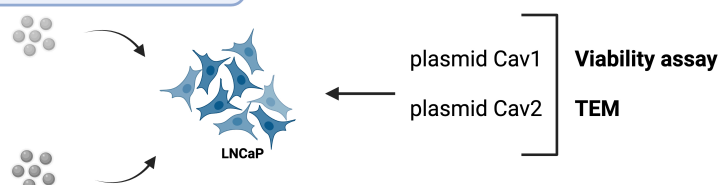
Cav1 and Cav2 quantification



Caveolae-dependent endocytosis inhibition



Caveolae-dependent endocytosis induction



Glucose-functionalized Silver Nanoparticles (G-AgNPs) uptake by caveolae-dependent endocytosis is responsible for their selective effect towards Castration Resistant Prostate Cancer

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Abstract

Castration Resistant Prostate Cancer (CRPC) is characterized by a poor prognosis and limited therapeutic options. AgNPs functionalized with glucose (G-AgNPs) were observed cytotoxic to two CRPC cell lines (PC-3 and Du-145) and not to LNCaP. This study aims to evaluate the uptake mechanisms of AgNPs and G-AgNPs by these three cell lines and understand their possible role in the selective effect against CRPC cells. Uptake of AgNPs and G-AgNPs was assessed through transmission electron microscopy (TEM). A microRNA (miRNAs) analysis approach was used to uncover the main molecular differences responsible for the regulation of endocytic mechanisms. Caveolin (Cav) 1 and 2 mRNA and protein levels were assessed in the three cell lines. Caveolae-dependent endocytosis was inhibited with genistein or siCav1⁻ and siCav2⁻ in PC-3 and Du-145 and resazurin assay was used to evaluate viability after AgNPs and G-AgNPs administration. Caveolae-dependent endocytosis was induced with Cav1⁺ and Cav2⁺ plasmids in LNCaP cells, resazurin assay was used to evaluate viability after AgNPs and G-AgNPs administration and TEM to assess their location. AgNPs and G-AgNPs were not uptaken by LNCaP cells. The miRNA analysis revealed 37 upregulated and 90 downregulated miRNAs. Functional enrichment analysis of the miRNAs' targets resulted in enrichment of terms related to endocytosis and caveolae. We observed that Cav1 and Cav2 are not expressed in LNCaP cells. Inhibiting caveolae-dependent endocytosis in Du-145 and PC-3 cells led to a significative reduction of cytotoxic capacity of AgNPs and G-AgNPs and induction of caveolae-dependent endocytosis in LNCaP cells lead to a significative increase as well as their uptake by cells. This study shows the potential of these AgNPs as a new therapeutic approach directed to CRPC patients, uncovers caveolae-dependent endocytosis as the mechanism of uptake of these AgNPs and highlights deregulation of Cav1 and Cav2 expression as a key difference in hormone sensitive and resistant PCa cells which may be responsible for drug resistance.

Keywords: Prostate Cancer Resistant to Castration, Silver Nanoparticles, uptake mechan

1. Introduction

In the last decades, there have been breakthrough advances in the oncology field, but therapy resistance is still the main concern in the success of cancer cell control [1]. In prostate cancer (PCa), androgen deprivation therapy (ADT) is the most frequent approach in advanced disease, affecting the androgen receptor (AR) signaling pathway, with high early response rates [2]. However, patients develop resistance and progress to castration resistant PCa (CRPC) within a period of 2 to 3 years [3]. This form of the disease is characterized by a poor prognosis, impaired quality of life and limited therapeutic options, making the development of new therapies an urgent need [4, 5].

When designing a new therapeutic agent, there are several aspects to be considered to achieve the highest efficacy of the potential treatment. One of the most important is the efficiency of the uptake of such drug by the targeted cells [6].

Nanomedicine has arrived as a promising solution regarding the development of new therapeutic approaches in different biomedical fields, including cancer medicine [7]. Among the different nanomaterials' formulation, metallic nanoparticles, such as silver nanoparticles (AgNPs), have brought special interest due to their apparent tissue-specificity, low side-effects, promising antitumoral effects and low production cost [8].

Regarding drug delivery, AgNPs can both increase drug concentration in the tumor tissue and improve cellular uptake [9]. On one hand, due to the pathophysiological differences of tumor and normal tissues, tumor tissues represent the primary accumulation target of AgNPs. This is called the enhanced permeability and retention (EPR) effect and represents the selective leakage of AgNPs from the vascular lumen and accumulation in the tumor tissue, because of its blood vessels' high permeability and impaired lymphatic drainage [10]. On the other hand, AgNPs need, not only to enter the tissue, but to translocate through cell membranes and target sub-cellular organelles, being endocytosis, which is commonly deregulated in cancer, the main pathway to achieve it [11]. When in the extracellular environment, AgNPs interact with cell surface through electrostatic and hydrophobic interactions or via specific ligand-receptor interactions. This will lead to the formation of invaginations from the cell membrane around the AgNPs (budding) and consequent pinch off, forming different endocytic vesicles (early endosomes). The early endosomes will then work as sorting machine carrying the AgNPs either to the different organelles, to recycling endosomes or even evolve as lysosomes [9]. Endocytosis can be classified as phagocytosis or pinocytosis, depending on the proteins involved. AgNPs are

mainly incorporated through pinocytosis, which can be clathrin-mediated, caveolae-mediated or clathrin and caveolae-independent [12]. In PCa, it is known that both clathrin and caveolins' expression, the major players in clathrin-mediated and caveolae-mediated endocytosis, is androgen regulated, varying according to the stage of the tumor and resistance to therapy, which can influence the success of the uptake of AgNPs through these pathways [13-17].

We have previously studied the effect of AgNPs functionalized with glucose (G-AgNPs) in two PCa AR⁻ cell lines (PC-3 and Du-145) and one PCa AR⁺ cell line (LNCaP) and have observed that they were only cytotoxic to the hormone resistant cell lines and not to the hormone sensitive one [18]. Therefore, and considering the importance of the uptake mechanisms for the cytotoxic effect, the aim of the current study is to evaluate the uptake mechanisms of AgNPs and G-AgNPs by these three cell lines and understand its possible role in the selective effect against CRPC cells. To do so, we applied a microRNA (miRNAs) analysis approach to uncover the main differences in the molecular pathways responsible for the regulation of the endocytic mechanisms. miRNAs are a class of short non-coding RNAs that negatively regulate gene expression at a post-transcriptional level through the binding to complementary sequences in the 3' untranslated region (3' UTR) of messenger ribonucleic acids (mRNAs) [19]. Having such a clear influence in the expression patterns of mRNAs and consequent activation and suppression of different signaling pathways, the levels of miRNAs are different in different pathophysiologic situations, varying between normal and tumor cells, and even among different states of the disease [20]. Due to their stability and facility to isolate and quantify, they are an easy approach to screen the molecular state of the cells and find transcriptomic differences that can explain the different cytotoxic effect of G-AgNPs.

2. Material and Methods

Cell Culture and treatments conditions

PC-3 (ATCC[®] CRL-1435[™] RRID:CVCL_0035) and LNCaP (ATCC[®] CRL-1740[™] RRID:CVCL_1379) cells were purchased at ATCC and Du-145 cells were kindly provided by Professor C  rmen Jer  nimo from IPO-Porto Research Center (Portugal). All cells were kept in culture in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator at 37  C, 5% CO₂ and 95% humidity. Cells were grown in tissue culture flasks and, when at 80-90% confluence, trypsinized with trypsin-EDTA 0.05% (Gibco[®], Gaithersburg, MD, USA) and counted using trypan blue solution (VWR[™]) and an automatic cell counter (EVETM – NanoEntek). All cells

were weekly tested for mycoplasma presence and were found to be free from contamination.

Synthesis of AgNPs

AgNPs were synthesized and characterized as described by Morais et al [18]. Briefly, an aqueous solution of 100 mL containing sodium citrate (5 mM) was mixed with tannic acid (5 mM) and heated at 100 °C, for 15 min under vigorous stirring. After boiling, 1 mL of AgNO₃ (25 mM) was added. AgNPs were purified through centrifugation at 20,000 g for 15 min and redispersed in MilliQ (MQ) water. To functionalize the AgNPs, 1mg was redispersed with 2 mL of 2 mM sodium citrate containing 5 mM glucosamine hydrochloride and stirred for 24h at room temperature. The resulting G-AgNPs were washed, redispersed in MQ-water, and stored at 4 °C.

Determination of cellular localization of AgNPs and G-AgNPs by Transmission Electron Microscopy (TEM)

LNCaP cells were cultured in 12-multi-well plates at a density of 2×10^5 cells per mL and treated with 2000µg/mL of either AgNPs or G-AgNPs for 24 h. After, cells were harvested with a solution of trypsin-EDTA and fixed. Cells were then further processed, and TEM images were collected by the Histology and Electron Microscopy platform from I3S Porto using a Transmission Electron Microscope Jeol JEM 1400.

RNA extraction

Two million Du-145, PC-3 and LNCaP cells were counted and centrifuged to form a pellet. miRNA isolation and purification was done using the Plasma/Serum RNA Purification Mini Kit from NORGEN (Norgen Biotek Corporation, Thermo, ON, Canada) according to the manufacturer protocol. RNA concentration and purity were measured using the NanoDrop Lite spectrophotometer (Thermo Scientific®, Waltham, MA, USA).

miRNA profiling through NanoString Analysis

The miRNA profiling of the three cell lines was performed using the nCounter® Human v3 miRNA expression assay (NanoString Technologies), using an input of 100ng of sample, according to manufacturer instructions. Through a NanoString's amplification-free technology the assay does expression profiling by direct quantification of individual RNA molecules, without the use of reverse transcription or amplification by using molecular barcodes. This panel allows the analysis of 799 miRNAs, 5 mRNAs and contains 25 internal reference controls. Data was then analyzed through ROSALIND®

(<https://rosalind.onramp.bio/>), with a HyperScale architecture developed by ROSALIND, Inc. (San Diego, CA). Read Distribution percentages, violin plots, identity heatmaps, and sample MDS plots were generated as part of the QC step. The criteria given by Nanostring (nSolver 4.0 Analysis Software User Manual) was used to calculate normalization, fold changes and p-values. After background subtraction based on POS_A probe correction factors, normalization was performed in two steps: positive control normalization and codeset normalization. During both steps the geometric mean of each probeset is used to create a normalization factor. Differential expression of the hormone resistant cell lines (PC-3 and Du-145) against hormone sensitive cell line (LNCaP) was then assessed through linear models, using the ROSALIND software, and miRNAs were classified as differentially expressed according to a fold-change <-1.5 or >1.5 and $p < 0.05$.

In silico analysis

miRTarBase (version 9), the largest known online database of validated miRNA:mRNA target interactions, was used to establish a network of the target mRNAs of the deregulated miRNAs [21]. A total of 699 validated targets for the downregulated miRNAs and 965 validated targets for the upregulated miRNAs were retrieved. The Search Tool for the Retrieval of Interacting Genes (STRINGapp) in the Cytoscape software (v3.9.1.) was used to build the protein interaction relationship of the selected target genes encoding proteins, considering as significant the ones with a combined score of >0.4 . To better understand the resulting PPI network, proteins were grouped according to their cellular compartment location using the compartment filters of cytoscape focusing on three different categories: lysosome, plasma membrane and endosome. Following, the functional enrichment analysis of GO, Reactome, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Compartments was performed with the enrichment analysis tool of the STRINGapp, with a False Discovery Rate (FDR) of $p < 0.01$ as significance threshold. The enrichment results were filtered to remove redundant terms, using the redundancy filtering and the Jaccard index.

Caveolins mRNA quantification

Two million cells were counted and centrifuged to form a pellet. mRNA extraction was performed using the GRS Total-Blood & Cultured Cells kit (Grisp Research Solutions[®], Porto, Portugal). Concentration and purity of the isolated mRNA was assessed by absorbance measurement at 260/280 nm using NanoDrop Lite Spectrophotometer (Thermo fisher[®], Waltham, MA, USA). mRNA samples served as templates for complementary DNA (cDNA) synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied

Biosystems®, Waltham, MA, USA). The thermal conditions for PCR amplification were optimized to 25 °C for 10min, followed by 37 °C for 120min and 85 °C for 5min. mRNA expression was assessed by quantitative real-time PCR using StepOne™qPCR Real-Time PCR machine. The reaction was performed using 1X Master mix (Applied Biosystems), with 1X probes (TaqMan mRNA Expression Assays CAV1 Hs00971716_m1, CAV2 Hs00184597_m1, CAV2 Hs00971711_m1 and B2M Hs00187842_m1) and cDNA sample. The amplification conditions were as follows: holding stage 95 °C for 20sec, followed by 45 cycles of 95 °C for 1sec and 60 °C for 20sec. Three technical replicates were made for each sample. StepOne Software v2.2 (Applied Biosystems®, Waltham, MA, USA) was used for data analysis and the baseline and thresholds were set for each plate to create the threshold cycle (Ct) values. All quantifications were performed in duplicate, and each plate had a negative control.

Caveolins protein expression

Two million PC-3, Du-145 and LNCaP cells were counted and centrifuged to form a pellet. Pellet was incubated for 15sec with 150 µL of RIPA buffer (Radioimmunoprecipitation Assay Buffer) (Santa Cruz Biotechnology®, Dallas, TX, USA) supplemented with 1.5 µL of phosphatase inhibitor cocktail (Thermo Fisher Scientific®) and then centrifuged for 15 min at 14,000× g, at 4 °C. The supernatant was recovered for protein quantification using a DC Protein Assay (BioRad Laboratories®, Hercules, CA, USA), measuring the solution's absorbance at 750 nm. The electrophoretic separation of proteins (20 µg) was performed in Mini-Protean TGX Gels (4–20%) (BioRad Laboratories®) and transferred onto a nitrocellulose membrane in 25 mM Tris-base/glycine buffer. Membranes were blocked using 5% BSA (Albumin BovineFraction V) (Enzytech®, Lisbon, Portugal) in Tris-buffered saline with Tween20 (TBS-T). Membranes were then incubated with primary antibodies (CAV1 (7C8) (1:1000) (Thermofisher®), CAV2 (ZC013) (1:1000) (Thermofisher®), B2M (1:2500) (Thermofisher®)), overnight at 4°C. Membranes were then incubated with conjugated secondary antibodies (anti-mouse (1:70,000) (62-6520) and anti-rabbit (65-6120) (1:60 000) (Thermofisher®)) for 30' at room temperature. The chemiluminescence was evaluated using ECL™ Prime Western Blotting System (Cytiva™, Amersham, UK), according to manufacturer's instructions.

Endocytosis inhibition through genistein

Genistein is a highly specific, dose-dependent inhibitor of tyrosine kinases and is known to disrupt the arrangements of actin and prevent the mobilization of dynamin for plasma membranes, two mechanisms vital for Caveolae-dependent endocytosis [22]. To

inhibit caveolae-dependent endocytosis, Du-145 and PC-3 cells were plated at a concentration of 2.0×10^5 cells/mL in 96-well plates at a final volume of 100 μ L/well. The following day, genistein was added at a concentration of 25 μ M for 1 h and 20 μ M for 2 h, respectively.

siRNA Cav1 and Cav2 transfection

2.5×10^5 cells were plated per well in 6-well plates at a final volume of 2.2 mL of antibiotics-free RPMI medium supplemented with 10% FBS. After 24 h, transfection mix was incubated for 15' in a final volume of 300 μ L/well of Opti-mem (ThermoFisher®), using 40 nM siCav1 (ThermoFisher®, Assay ID 10297) or siCav2 (ThermoFisher®, Assay ID 145681) alone and in combination with 6 μ L (or 9 μ L, when co-transfected Cav1 and Cav2) of Lipofectamine RNAiMax (ThermoFisher®). Transfection mix was then added to the plated cells for 24 h, after which cells were collected for RNA or protein extraction. Silencer™ Negative Control No. 1 siRNA (#4404021) was used as a negative control of transfection, BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control (ThermoFisher®) was used as a positive control of transfection and cells were imaged with filter set appropriate for Tetramethylrhodamine (TRITC) using a fluorescence microscope OLYMPUS IX51 (Tokyo, Japan). For resazurin assay, 2.0×10^5 cells/mL were plated in 96-well plates at a final volume of 100 μ L/well in antibiotics-free RPMI medium supplemented with 10% FBS. After 24 h, transfection mix was incubated for 15 min in a final volume of 50 μ L/well of Opti-mem, using 40 nM siCav1 or siCav2 alone and in combination with 1 μ L (or 1.5 μ L when co-transfected Cav1 and Cav2) of Lipofectamine RNAiMax. Transfection mix was added to the plated cells for 24 hours, after which AgNPs and G-AgNPs were added, and the assay was performed.

Plasmid transfection

pCMV6-CAV1 (RefSeq: NM_001753.3, Origene, Cat#RC210274L3V) and pCMV6-CAV2 (RefSeq: NM_198212.3, Origene, Cat#RC217364) were transformed in E. coli DH5 α strain, by heat shock method (Rahimzadeh et al., 2016; Li et al., 2007), using 100 ng of plasmid. Transformed E. coli DH5 α cells were cultured overnight at 37°C in 20 mL of Luria Bertani (LB) broth with shaking (200 rpm) to obtain a large quantity of the desired plasmid. Plasmid extraction was performed using NZYMiniprep kit (NZYTech, MB01002) following manufacturer's instructions and final DNA plasmid was quantified using the NanoDrop One (ThermoFisher). 5×10^5 cells were plated per well in 6-well plates at a final volume of 2.25 mL of antibiotics-free RPMI medium supplemented with 10% FBS. After 24 h, transfection mix was incubated for 10' in a final volume of 250 μ L/well of Opti-mem, using 3 μ g of each plasmid, 5.75 μ L of Lipofectamine3000 (ThermoFisher®) and 5 μ L of P3000 (ThermoFisher®).

Transfection mix was added to the plated cells for 24 hours, after which cells were collected for RNA or protein extraction. For resazurin assay, 2.0×10^5 cells/mL were plated in 96-well plates at a final volume of 100 μ L/well in antibiotics-free RPMI medium supplemented with 10% FBS. After 24 h, transfection mix was incubated for 10' in a final volume of 10 μ L/well of Opti-mem, using 150ng of each plasmid, 0.4 μ L of Lipfectamine3000 and 0.2 μ L of P3000. Transfection mix was added to the plated cells for 24 hours, after which AgNPs and G-AgNPs were added, and the assay was performed.

Resazurin Assay

After transfection of Cav1 or/and Cav2, AgNPs and G-AgNPs were administrated at a concentration range of 600 μ g/mL-1000 μ g/mL for PC-3 cells, 600-25000 μ g/mL for Du-145 and 500-25000 μ g/mL for LNCaP cells, for 24 h. After, resazurin Sodium Salt (ACROS Organic™ – Fisher Scientific®, MA, USA) was used to assess cells' viability. Cell's viability after AgNPs and G-AgNPs administration was compared to cells administrated with genistein, siRNA or plasmid, alone.

Statistical Analysis. Using the sample T test, the Mann-Whitney and the Kruskal Wallis tests (depending on whether the results followed or not a normal distribution), the results were analyzed using SPSS28 software (release 28, SPSS Inc., Chicago, IL/USA). p-values less than <0.05 were considered as statistically significant.

3. Results

3.1. AgNPs and G-AgNPs are not uptake by LNCaP cells

We have previously demonstrated that AgNPs and G-AgNPs were cytotoxic to PC-3 and Du-145 cells, being uptaken through an apparent endocytic mechanism and being located in different subcellular locations of the cell. However, they were found not cytotoxic to LNCaP cells [18]. Through the TEM analysis, we can observe that both AgNPs (Figure 1 A-C) and G-AgNPs (Figure D-F) are mainly located outside the cell, which indicates there is no significant uptake by LNCaP cells.

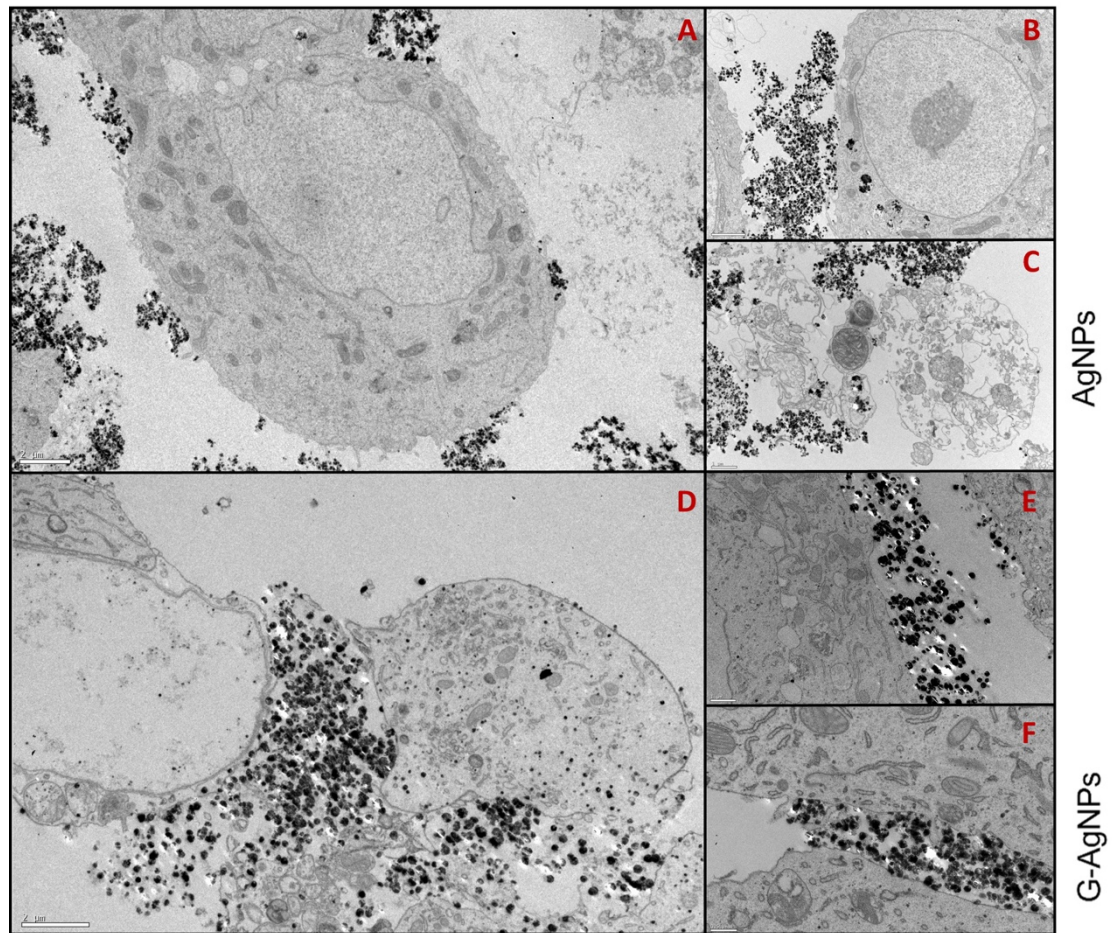


Figure 1 - TEM images of LNCaP cells treated with 2000 μ g/mL of AgNPs (A–C) or G-AgNPs (D–F). From each sample, three pictures were taken with different ampliations. Thus, scale bars in A, B and D are 2 μ m, in C and E are 1 μ m, and in F are 0.5 μ m. NPs are the black dots presented in the figures.

3.2. LNCaP cells have a different miRNA-profile compared with PC-3 and Du-145

To better understand the differences in toxicity of AgNPs and G-AgNPs, we compared the molecular profile of PC-3 and Du-145 with LNCaP cells, through a microRNA expression profile approach. After expression analysis of 799 hsa-miRNAs, using the Nanostring[®] technology, 37 downregulated miRNAs and 90 upregulated miRNAs were found (Figure 2-A and Table-S1-2). To explore the biological impact of these deregulated miRNAs, the 699 target genes of the downregulated miRNAs were analyzed using the STRINGapp from Cytoscape software (v3.7.2). To assess their relevance in the uptake of the NPs, we focused on the following cellular compartments: lysosome, plasma membrane and endosome. We then filtered the target genes to a PPI network of 40 nodes and 183 edges that presented significant enrichment ($p = 1.0 \times 10^{-16}$) (Figure 2-B). The functional enrichment analysis was performed with an FDR threshold of 1%, and the redundant terms

were eliminated using a redundancy cutoff of 0.5, resulting in a total of 239 enriched terms among the Reactome, KEGG, and GO categories (Tables S3–S8). The top 20 enriched terms for each category are represented in Figure 2-D. Among the enriched terms, we highlight the terms “plasma membrane signaling complex”, “cytoplasm vesicle membrane”, “endocytic vesicle membrane”, “membrane trafficking”, “ABC transporters”, “endocytosis” and “caveolae”.

Similarly, we analyzed the 965 target genes of the upregulated miRNAs. Once again, their relevance in the uptake of the NPs was assessed, focusing on lysosome, plasma membrane and endosome. This filtered the target genes to a PPI network of 45 nodes and 252 edges that presented significant enrichment ($p = 1.0 \times 10^{-16}$) (Figure 2-C). The functional enrichment analysis was performed with an FDR threshold of 1%, and the redundant terms were eliminated using a redundancy cutoff of 0.5, resulting in a total of 281 enriched terms among the Reactome, KEGG, and GO categories (Tables S9–S14). The top 20 enriched terms for each category are represented in Figure 2-E. Among the enriched terms, we highlight the terms “endolysosome”, “phagocytosis”, “regulation of vesicle-mediated transport”, “clathrin-coated vesicle” and “lysosomal membrane”.

Considering these results, which relate the caveolae-dependent endocytosis with the downregulated miRNAs and upregulated proteins and, the clathrin-dependent endocytosis with the upregulated miRNAs and downregulated proteins, we further studied the caveolae-dependent endocytosis in the three cell lines and their role in the cytotoxic effect.

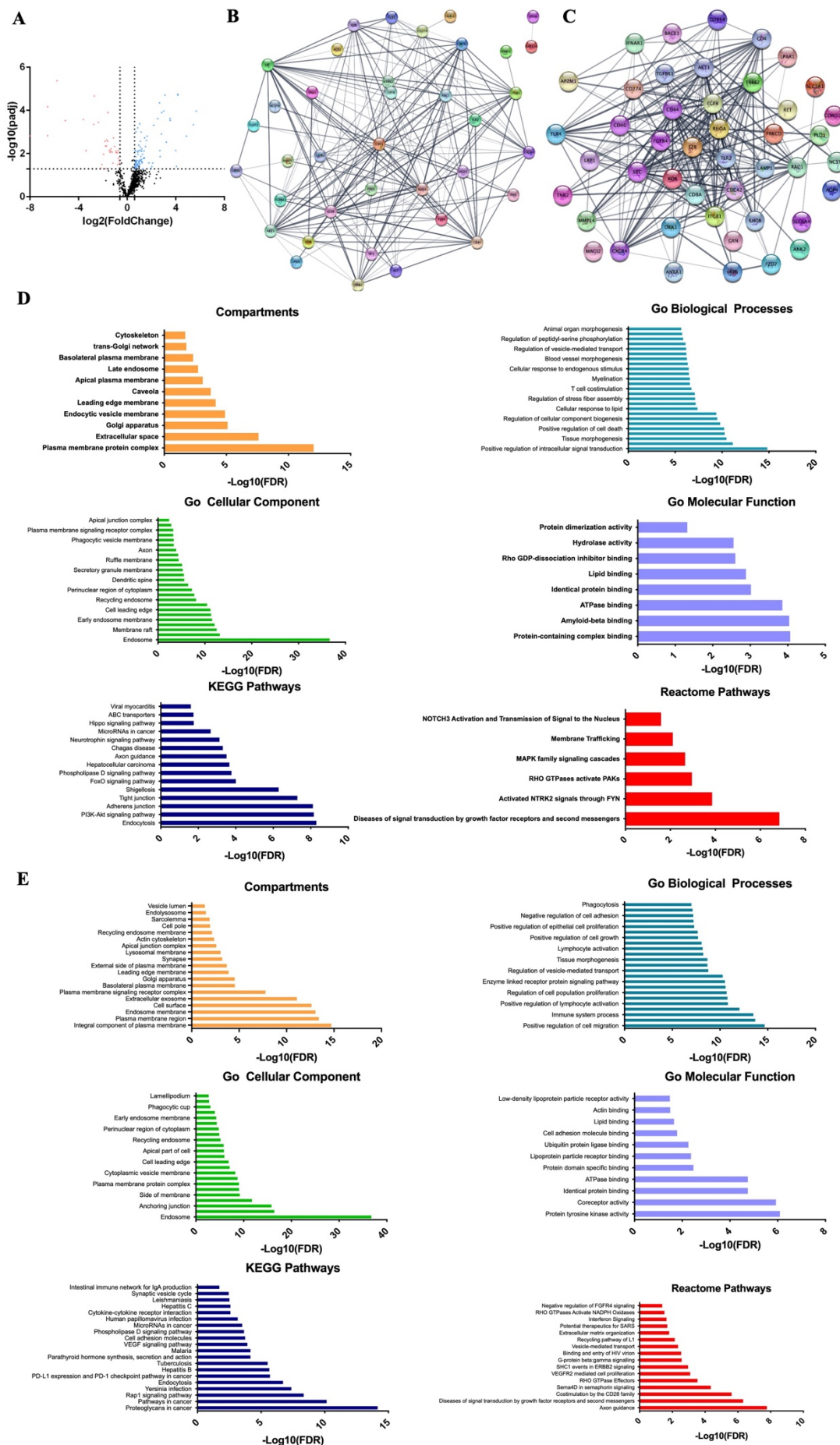


Figure 2 - Molecular profile comparison of PC-3 and Du-145 with LNCaP cells. A - Volcano plots of the differentially expressed miRNAs when comparing PC-3 and Du-145 cells with LNCaP cells. The y-axis indicates the Log10 of the p values and the x-axis is the Log2 transformed ratio of the expression between the experimental groups. B – PPI network of the 40 proteins derived from the target genes of the upregulated miRNAs performed using Cytoscape. C – PPI network of the 45 proteins derived from the target genes of the downregulated miRNAs performed using Cytoscape. D – Compartments, GO, KEGG and Reactome analysis of the 40 selected genes derived from the upregulated miRNAs. The functional enrichment analysis was made with the STRINGapp from Cytoscape. E – Compartments, GO, KEGG and Reactome analysis of the 45 selected genes derived from the downregulated miRNAs. The functional enrichment analysis was made with the STRINGapp from Cytoscape.

3.3. Caveolin 1 and Caveolin 2 are expressed differently among LNCaP, PC-3 and Du-145 cells

To study the caveolar-dependent endocytosis pathway in LNCaP, PC-3 and Du-145 cells, the mRNA expression levels and the protein levels of both Cav1 and Cav2 were assessed in the three cells lines. In fact, the mRNA levels of *Cav1* are significantly higher in PC-3 and Du-145 cells when compared to LNCaP (fold-change=3104.2, $p<0.001$; fold-change=4871.0, $p<0.001$, respectively) (Figure 3-A). Similarly, the mRNA levels of *Cav2* are significantly higher in PC-3 and Du-145 cells when compared to LNCaP (fold-change=891.0, $p<0.001$; fold-change=3492.4, $p<0.001$, respectively) (Figure 3-A). Regarding the protein levels of both Cav1 and Cav2, one can observe that there is no expression in the LNCaP cell line (Figure 3-B).

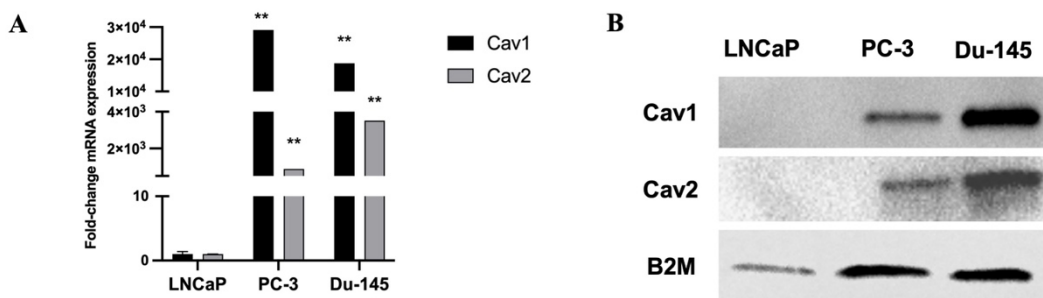


Figure 3 – A – Variation of the relative expression levels of Cav1 and Cav2 in LNCaP, PC-3 and Du-145 cells (mean $\pm$ SE); B – Protein levels of Cav1 and Cav2 in LNCaP, PC-3 and Du-145 cells according to Western Blot analysis.

3.4. The modulation of the Caveolae-dependent endocytosis pathway inhibits AgNPs and G-AgNPs cytotoxic effect in PC-3 and in Du-145

To confirm that the caveolae-dependent endocytosis is a key player in the AgNPs and G-AgNPs cytotoxic effect in PC-3 and in Du-145 cell line, we inhibited this pathway either through genistein or inhibition of Cav1 and Cav2 using siRNA technology, confirming this transfection using both positive and negative control (FigS1 A-D). In fact, inhibition of this endocytic pathway with genistein 20 μ M in PC-3, led to a reduction of the inhibitory effect of both AgNPs and G-AgNPs (Figure 4-A and B, respectively). After administration of 1500 μ g/mL of AgNPs and G-AgNPs, PC-3 cells showed a viability of 70.61% ($p < 0.001$) and 70.35% ($p < 0.001$), respectively, when genistein was previously administrated, compared with 29.78% and 29.08%, respectively, without genistein administration. Similarly, inhibition of Cav1 led to a reduction of the inhibitory effect of both AgNPs and G-AgNPs (Figure 4-C and D, respectively). After administration of 1500 μ g/mL of AgNPs and G-AgNPs, PC-3 cells showed a viability of 61.25% ($p < 0.001$) and 58.02% ($p < 0.001$), respectively, when Cav1 was inhibited, compared with 29.78% and 29.08%, respectively, without inhibition. Inhibition of Cav2 led to a reduction of the inhibitory effect of both AgNPs and G-AgNPs (Figure 4-E and F, respectively). After administration of 1500 μ g/mL of AgNPs and G-AgNPs, PC-3 cells showed a viability of 59.32% ($p < 0.001$) and 59.06% ($p < 0.001$), respectively, when Cav2 was inhibited, compared with 29.78% and 29.08%, respectively, without inhibition. Inhibition of Cav2, combined, led to a reduction of the inhibitory effect of both AgNPs and G-AgNPs (Figure 4-G and H, respectively). After administration of 1500 μ g/mL of AgNPs and G-AgNPs, PC-3 cells showed a viability of 54.76% ($p < 0.001$) and 59.84% ($p < 0.001$), respectively, when Cav1 and Cav2 were inhibited, compared with 29.78% and 29.08%, respectively, without inhibition. In a similar way, inhibition of the endocytic pathway in Du-145 cells, using genistein 25 μ M, led to a reduction of the inhibitory effect of both AgNPs and G-AgNPs (Figure 5-A and B, respectively). After administration of 2500 μ g/mL of AgNPs and G-AgNPs, Du-145 cells showed a viability of 83.78% ($p < 0.001$) and 42.54% ($p < 0.001$), respectively, when genistein was previously administrated, compared with 26.99% and 23.56%, respectively, without genistein administration. Inhibition of Cav1 led to a reduction of the inhibitory effect of both AgNPs and G-AgNPs (Figure 5-C and D, respectively). After administration of 2500 μ g/mL of AgNPs and G-AgNPs, Du-145 cells showed a viability of 93.79 % ($p < 0.001$) and 64.32% ($p < 0.001$), respectively, when Cav1 was inhibited, compared with 26.99% and 23.56%, respectively, without inhibition. Inhibition of Cav2 led to a reduction of the inhibitory effect of both AgNPs and G-AgNPs (Figure 5-E and F,

respectively). After administration of 2500 μ g/mL of AgNPs and G-AgNPs, Du-145 cells showed a viability of 100% ($p < 0.001$) and 91.97% ($p < 0.001$), respectively, when Cav2 was inhibited, compared with 26.99% and 23.56%, respectively, without inhibition. Finally, inhibition of Cav1 and Cav2, combined, led to a reduction of the inhibitory effect of both AgNPs and G-AgNPs (Figure 5-G and H, respectively). After administration of 2500 μ g/mL of AgNPs and G-AgNPs, Du-145 cells showed a viability of 50.77% ($p < 0.001$) and 53.15% ($p < 0.001$), respectively, when Cav1 and Cav2 were inhibited, compared with 26.99% and 23.56%, respectively, without inhibition.

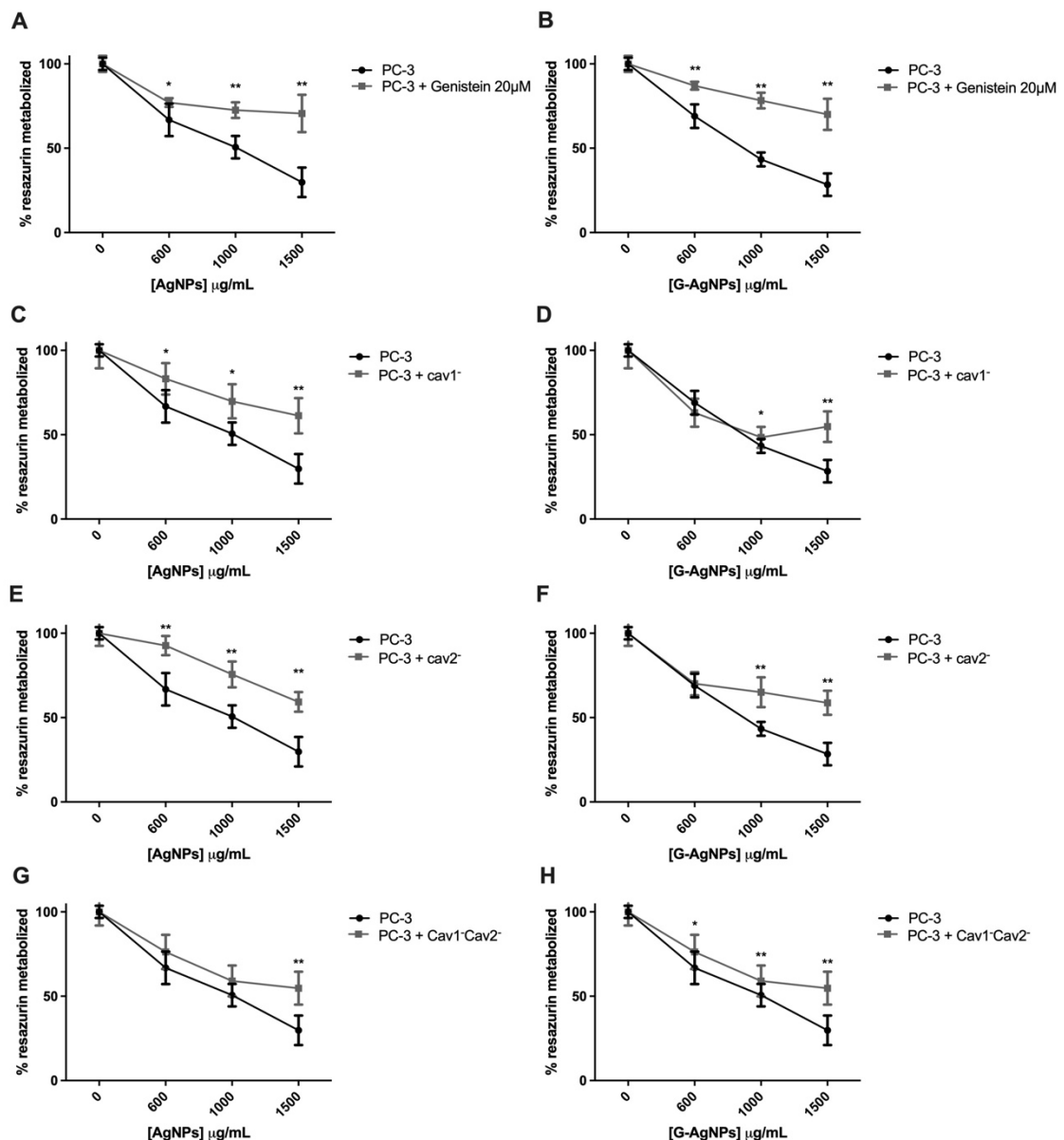


Figure 4 – Comparison of cell viability, by Resazurin Assay, upon treatment with AgNPs and G-AgNPs for 24 hours in PC-3 cell line with or without previous inhibition using Genistein 20 μ M (A,B), using siCav1(Cav1⁻) (C,D), using siCav2(Cav2⁻) (E,F), and both siCav1 and siCav2

(Cav¹-Cav²-) (G,H). Results are expressed as percentage of control (untreated cells), as mean $\pm$ SEM.

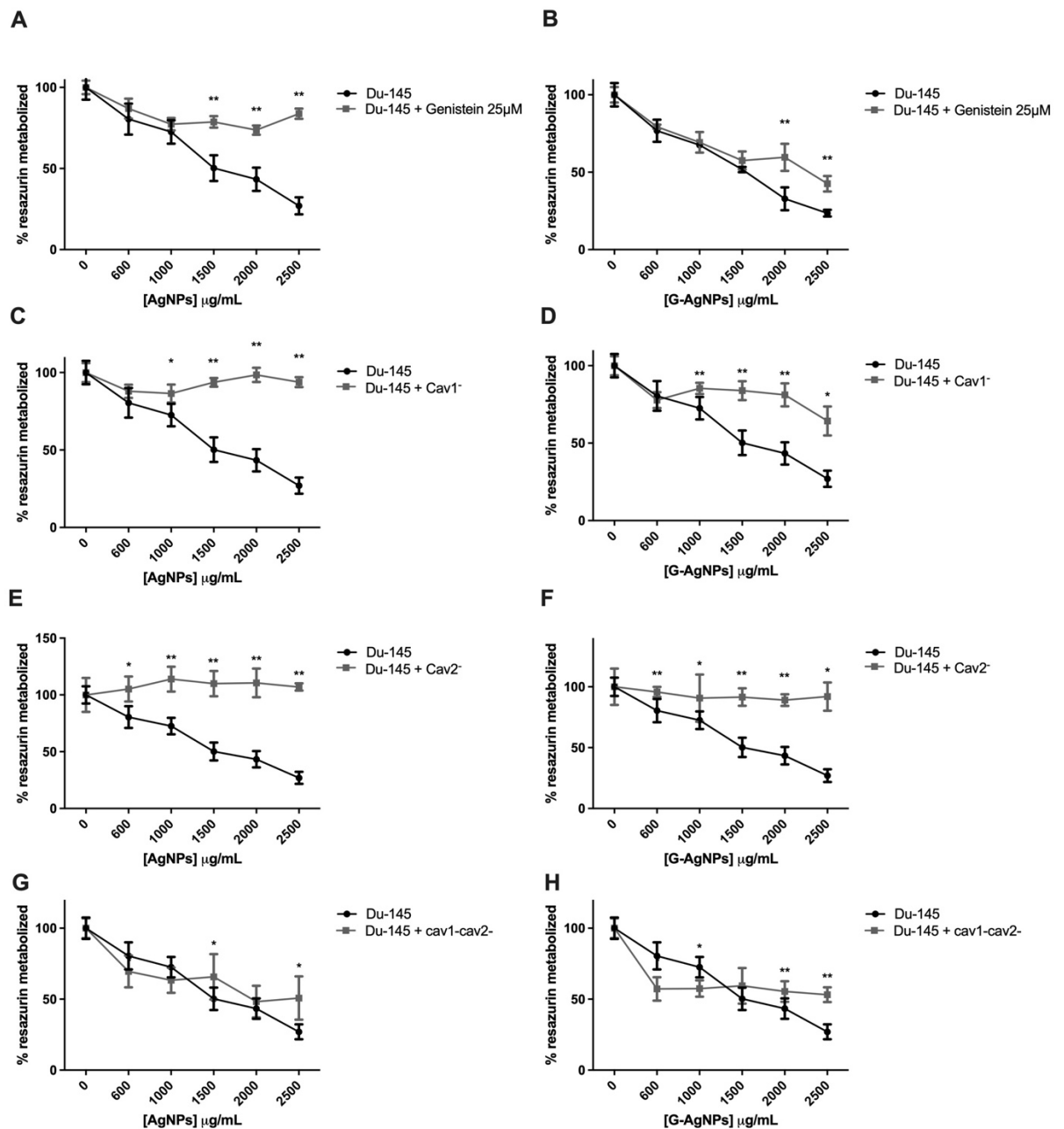


Figure 5 - Comparison of cell viability, by Resazurin Assay, upon treatment with AgNPs and G-AgNPs for 24 hours in Du-145 cell line with or without previous inhibition using Genistein 20 μ M (A,B), using siCav1(Cav1⁻) (C,D), using siCav2(Cav2⁻) (E,F), and both siCav1 and siCav2 (Cav1⁻Cav2⁻) (G,H). Results are expressed as percentage of control (untreated cells), as mean $\pm$ SEM.

3.5. The modulation of the Caveolae-dependent endocytosis pathway induces AgNPs and G-AgNPs cytotoxic effect in LNCaP

To further confirm caveolae-dependent endocytosis pathway as the key factor in AgNPs and G-AgNPs entrance in cells, Cav1 and Cav2's expression was induced in LNCaP cell line, and the NPs cytotoxic effect was evaluated. Cav1 and Cav2 mRNA expression were further confirmed (Figure S1E). Induction of Cav1 led to an increased inhibitory effect of both AgNPs and G-AgNPs (Figure 6-A and B, respectively). After administration of 2500µg/mL of AgNPs and G-AgNPs, LNCaP cells showed a viability of 42.27% ($p<0.001$) and 53.39% ($p=0.002$), respectively, when Cav1 was induced, compared with 108.18% and 95.82%, respectively, without induction. Similarly, induction of Cav2 led to an augmented inhibitory effect of both AgNPs and G-AgNPs (Figure 5-C and D, respectively). After administration of 2500µg/mL of AgNPs and G-AgNPs, LNCaP cells showed a viability of 40.29% ($p<0.001$) and 88.75% ($p=0.507$), respectively, when Cav2 was induced, compared with 108.18% and 95.82%, respectively, without induction. Finally, induction of Cav1 and Cav2, combined, led to an inhibitory effect of both AgNPs and G-AgNPs (Figure 6-E and F, respectively). After administration of 2500µg/mL of AgNPs and G-AgNPs, LNCaP cells showed a viability of 45.80% ($p<0.001$) and 57.02% ($p<0.001$), respectively, when Cav1 and Cav2 were induced, compared with 108.18% and 95.82%, respectively, without inhibition.

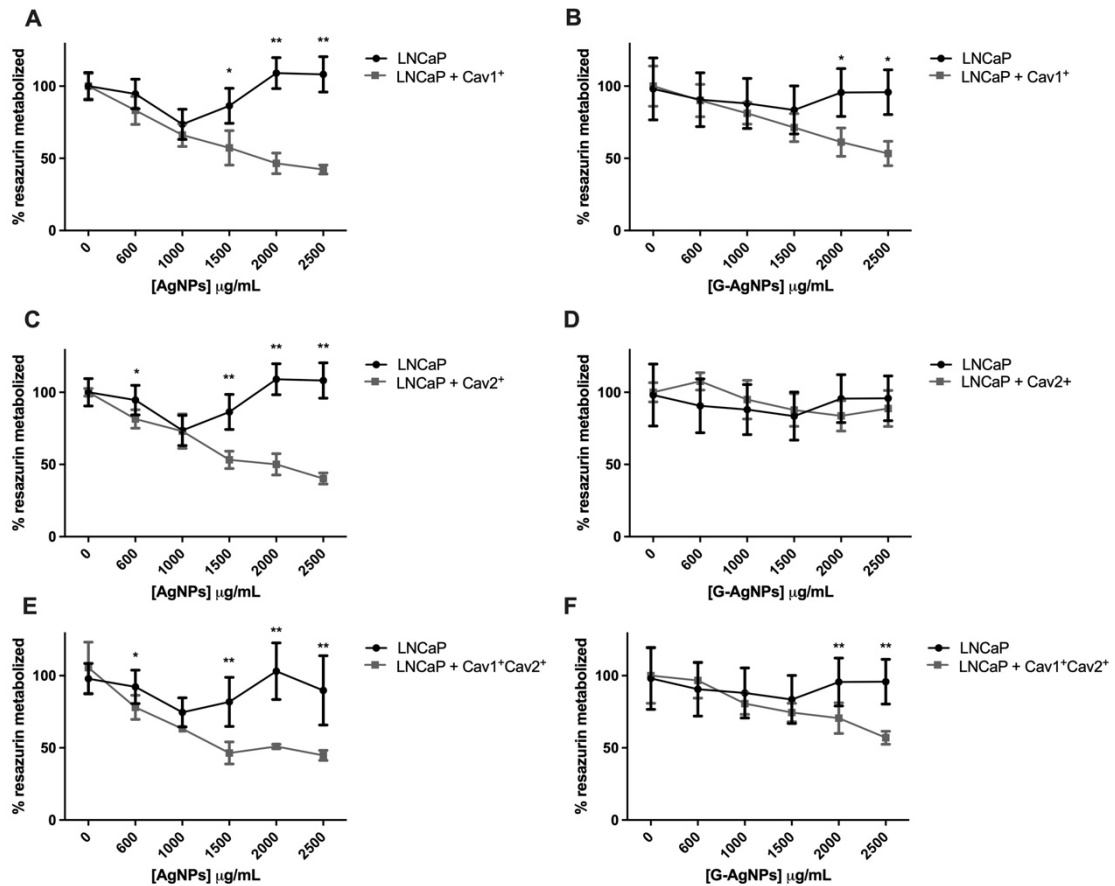


Figure 6 - Comparison of cell viability, by Resazurin Assay, upon treatment with AgNPs and G-AgNPs for 24 hours in PC-3 cell line with or without previous induction of Cav1(Cav¹⁺) (A,B), Cav2(Cav²⁺) (C,D), and both Cav1 and Cav2 (Cav¹⁺Cav²⁺) (E,F). Results are expressed as percentage of control (untreated cells), as mean $\pm$ SEM

3.6. AgNPs and G-AgNPs are uptaken by LNCaP cells after induction of Cav1 and Cav2's expression

To prove that the differences in the cytotoxic effect of AgNPs and G-AgNPs after induction of Cav1 and Cav2 expression was due to the uptake of the NPs by the cells, we conducted a TEM analysis (Figure 7). One can see that, in the different conditions (Cav1⁺, Cav2⁺ and Cav1⁺Cav2⁺), for both AgNPs and G-AgNPs, the NPs are located inside the cells. They seem to be uptaken by endocytosis (Figure 7-R), being trapped inside lysosomes or caveosomes (Figure 7-E). Inside the cell, they are located in different organelles, such as: mitochondria (Figure 7-D), nucleus (Figure 7-A) and cytoplasm (Figure 7-G). Moreover, the cells display apoptotic features such as membrane blebbing (Figure 7-B) which are in accordance with the viability studies performed.

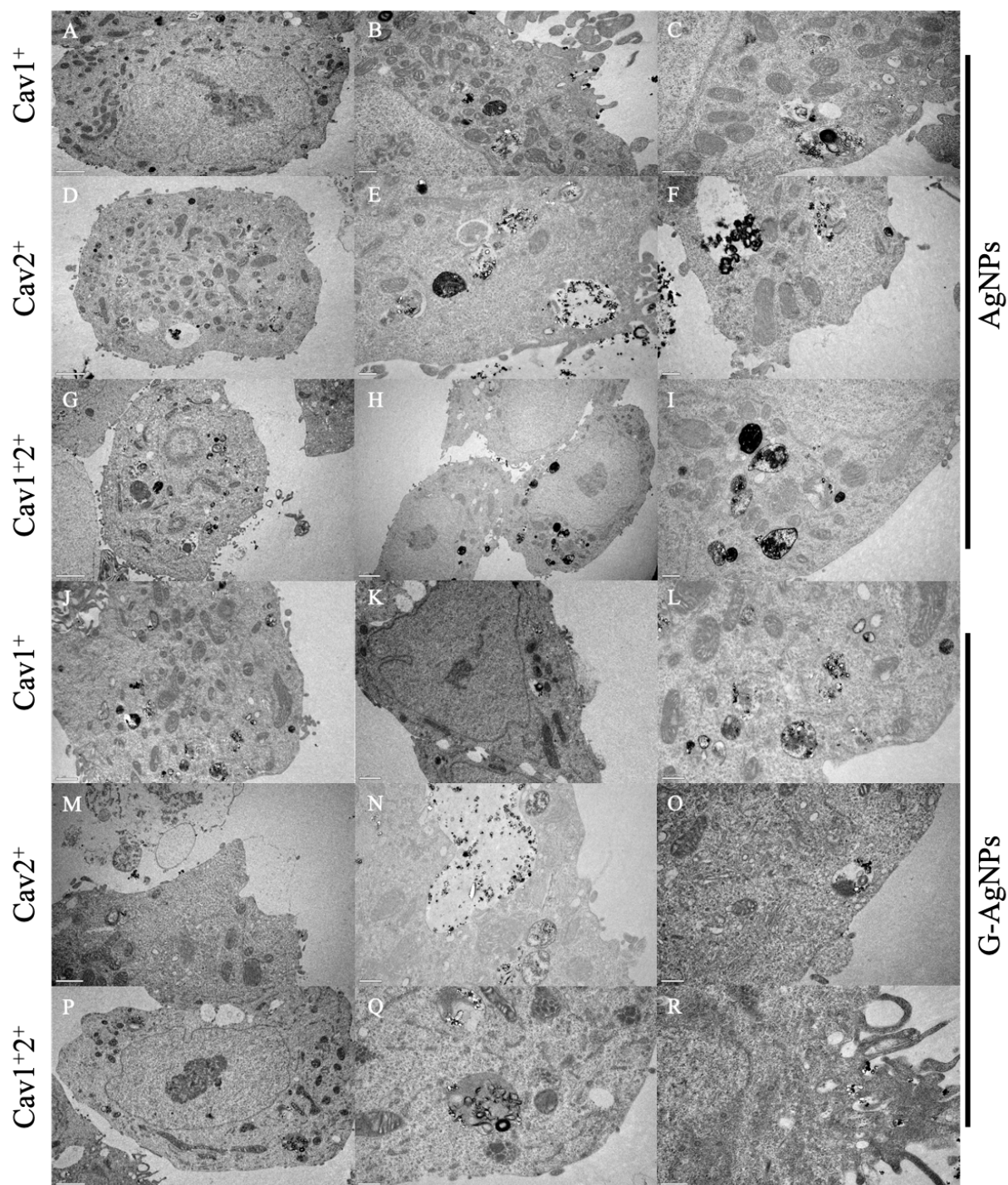


Figure 7 - TEM images of LNCaP cells transfected with Cav1⁺ (A-C and J-L), Cav2⁺ (D-F and M-O), Cav1⁺Cav2⁺ (G-I and P-R) and treated with 2000 μ g/mL of AgNPs (A-I) or G-AgNPs (J-R). From each sample, three pictures were taken with different ampliations. Thus, scale bars

in A, D, G and H are 2µm, in J, K and M are 1µm, and in B, C, E, F, L, N, O, Q and R are 0.5 µm.

Discussion

Despite the development and introduction of new drugs such as enzalutamide and abiraterone acetate in the management of the most lethal form of PCa – CRPC, this form of the disease remains non curative, and there is an urgent need for new therapies in the clinical management of these patients [2]. With the burst of nanomedicine research, the opportunity to use AgNPs as anticancer agents has been widely studied [8]. Indeed, we have previously designed AgNPs functionalized with glucose that were able to target PCa cells, more specifically Androgen Receptor (AR)⁻ cells (PC-3 and Du-145). However, they were shown not cytotoxic to the AR⁺ ones (LNCaP) [18]. In this study, we focused on understanding what was the mechanism responsible for the selective cytotoxic effect of these NPs in PC cell lines.

After a TEM analysis of LNCaP cells previously incubated with either AgNPs or G-AgNPs, we could observe that there was no uptake by the cells. In fact, previous studies have reported that the entry pathway and intracellular localization of NPs are determinant for the induction of cytotoxicity [23]. Yusuf and collaborators, for example, showed that by encapsulating AgNPs in a dipalmitoyl-phosphatidyl choline (DPPC) liposome, there was an increase in the uptake of the AgNPs by macrophages and that directly correlated with an increased cytotoxic capacity [24].

Due to the observed lack of uptake by LNCaP cells in the present study, we sought any molecular differences, through a definition of a deregulated-miRNA profile. In the present study we determined 37 downregulated and 90 upregulated miRNAs when comparing PC-3 and Du-145 with LNCaP cells. We further performed a functional annotation analysis on the validated target genes of the downregulated and upregulated miRNAs, focusing on the cellular compartments lysosome, plasma membrane and endosome and we observed that most enriched terms were related to endocytosis, cytoplasm vesicle, endocytic vesicle and lysosomal membrane, membrane trafficking and regulation of vesicle mediated transport. These results seem to indicate that the deregulated miRNAs may play a role in the expression of genes related to the endocytic pathways. In the past, Johnson and colleagues had already shown that endosome biogenesis and function was altered in PCa, with a significant increase in gene and protein expression for early endosomal proteins and an altered endosomal traffic and signaling of the transferrin receptors in PC cells [25]. Moreover, in another study, Xu and colleagues have reported that dynamin 2 (Dyn2), essential for intracellular vesicle formation and trafficking, cytokinesis, and receptor

endocytosis, was significantly increased across advanced stages of PCa and was associated with poor prognosis [26].

In the present study, the potentially upregulated protein network related with the downregulated miRNAs revealed the term caveolae and the downregulated protein network related with the upregulated miRNAs revealed the term clathrin-coated vesicle. The different expression of caveolae related proteins in the different phases of PCa has been already described [27-30]. On one hand, Xie and collaborators reported an overexpression of clathrin and downregulation of Cav1 in PCa samples compared with normal prostatic tissue [27]. However, Cav1 and Cav2, two key proteins in caveolae-dependent endocytosis, have been consistently described as overexpressed in CRPC tissues, plasma, and serum samples by different authors, being related with cell motility and migration promotion in CRPC [28-31]. Moreover, Cav1 downregulation has been shown to increase sensitivity to AR targeting in PCa cells [30]. Our study showed similar results, since we found that LNCaP cells did not express Cav1 and Cav2, contrarily to PC-3 and Du-145. Considering these results, we hypothesized that the lack of caveolins and consequent blockage of the caveolae-dependent endocytosis could be responsible for the failure of uptake of the NPs by the LNCaP cells.

To further confirm this hypothesis, we blocked the caveolae-dependent endocytosis in both PC-3 and Du-145 cells using two different approaches. Genistein is a tyrosine inhibitor that inhibits caveolae-dependent endocytosis both by depolymerizing the local cortical cytoskeleton (which precedes the internalization of caveolar vesicles) and by recruiting dynamin 2 which is needed to split the endocytic vesicle from the cellular membrane [32]. In the present study, we incubated cells with genistein previously to the administration of the NPs and we consistently observed a loss of cytotoxic effect, even at higher doses. These results seem to suggest that by blocking endocytosis, we are able to reduce NPs cellular uptake, which translates in loss of cytotoxic effect. Moreover, we inhibited Cav1 and Cav2, alone and in combination, through a siRNA technology, and confirmed the previous results. It is important to notice that, genistein is considered not to be a specific inhibitor of the caveolae-dependent endocytosis pathway, since some researchers defend it also inhibits exosome secretion from cells and other endocytic pathways such as macropinocytosis and clathrin-dependent endocytosis [22, 33]. Moreover, genistein has been proved cytotoxic against PCa, both *in vitro* and *in vivo* [34, 35]. On the other hand, the usage of genetic approaches that change the expression of specific proteins, such as the siRNAs used in this study, have been employed to overcome the non-specificity of chemical inhibitors such as genistein. Some limitations such as the upcoming of compensatory mechanisms in the cell when inhibiting protein expression or the fact that,

depending on the required time to silence a gene, the cell may adapt and change gene expression have been reported using this technology [36]. Nevertheless, Guggenheim et al. have shown the importance of the caveolae-dependent endocytosis in the uptake of Superparamagnetic Iron Oxide Nanoparticles through cav1 inhibition [37]. Similarly, Sago et al used an *in vivo* Cav1 knockout mouse model to show that lipid NPs bio-distribution was affected by Cav1 in a cell type specific manner [38]. All in all, in the present study, we observed a loss of cytotoxic effect of both AgNPs and G-AgNPs by the two methods, strengthening the hypothesis of caveolae-dependent endocytosis as the mechanism of uptake of these NPs.

Besides, we induced Cav1 and Cav2 expression in LNCaP cells which previously had no expression of both proteins. After induction, the NPs were shown cytotoxic to these cells. Moreover, TEM analysis of LNCaP expressing Cav1 and Cav2 shows NPs both entering the cell and located inside the cell, namely in the nucleus, mitochondria and cytoplasm. All in all, this data confirmed the hypothesis that the uptake of the NPs in PCa cells was performed through caveolae-dependent endocytosis.

AgNPs and G-AgNPs were proven, in the present study, to enter PCa cells through caveolae-dependent endocytosis, which is a crucial factor for their cytotoxic power. In fact, the pathway by which NPs enter cells depends on different factors such as particle size, shape and surface coating [39]. Although the caveolae-dependent endocytosis' mechanism is not yet completely understood, studies report that the size of the vesicle involved is about 60–80 nm and that anionic NPs are more likely to use this pathway [40]. Indeed, the NPs of the present study have an average size of circa 60 nm and a negative surface charge, which reinforces these data [18].

Nevertheless, it is important to notice that the differences in the cytotoxic effect of G-AgNPs were of a smaller magnitude compared with AgNPs. G-AgNPs result of a functionalization of glucosamine with AgNPs, so we hypothesize that GLUT1 facilitates the uptake of these NPs in the cells. Since we were not able to completely shut down the caveolae-dependent pathway, the high levels of GLUT1 in Du-145 and PC-3 cells were able to ease the uptake of G-AgNPs. Interestingly, Komuro and collaborators have previously demonstrated something similar regarding the uptake of hydroxyapatite NPs modified with glucose. Through genistein inhibition, they showed that the uptake of these NPs was significantly inhibited and, when inhibiting GLUT1, there was also a significant inhibition of the NPs uptake. This suggested that the NPs were taken up through the caveolae-mediated endocytosis process with an involvement of GLUT1 in the internalization process [41].

Conclusion

In this study, we successfully proved that AgNPs enter PCa cells through caveolae-dependent endocytosis. Moreover, we uncovered the reason behind the selective effect of the AgNPs towards CRPC cells since the lack of expression of Cav1 and Cav2 in LNCaP cells was shown to be the main responsible for the absence of cytotoxic effect of the NPs in this cell line. In the future, the role of GLUT-1 in the G-AgNPs entry in cells should be further studied and *in vivo* studies can help confirm the role of caveolae-dependent endocytosis in the uptake of these NPs preferable by CRPC tissues.

Given the current landscape in PCa treatment, this study reinforces, not only, the potential of these AGNPs as a new therapeutic approach directed to CRPC patients, but it is also an opportunity to better understand key features, such as the deregulation of Cav1 and Cav2 expression, that distinguish hormone sensitive and resistant PCa cells and may be responsible for drug resistance, opening the doors for the definition of new therapeutic agents.

List of Abbreviations

ADT, Androgen Deprivation Therapy; AgNPs, Silver Nanoparticles; AR, Androgen Receptor; ATCC, American Type Culture Collection; BSA, Bovine Serum Albumin; Cav, Caveolin; cDNA, complementary DNA; CRPC, Castration Resistant Prostate Cancer; Ct, threshold cycle; DC, Detergent compatible; DNA, Deoxyribonucleic acid; DPPC, Dipalmitoyl-phosphatidyl choline; Dyn2, Dynamin 2; EDTA, Ethylenediamine tetra acetic acid; EPR, Enhanced Permeability and Retention; FBS, Fetal bovine serum FDR, False Discovery Rate; G-AgNPs, glucose functionalized silver nanoparticles; GLUT1, Glucose transporter 1; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA; mRNA, messenger RNA; NPs, nanoparticles; PCa, Prostate Cancer; PPI, Protein-protein interaction; RIPA, Radioimmunoprecipitation Assay; RNA, Ribonucleic acid; rpm, rotations per minute; RPMI, Roswell Pearl Memorial Institute; siRNA, small interfering RNA; STRINGapp, Search Tool for the Retrieval of Interacting Genes; TBS-T, Tris-buffered saline with Tween; TEM, Transmission Electron Microscopy; TGX, Tris-Glycine extended; TRITC, Tetramethylrhodamine; 3'UTR - 3' Untranslated Region

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing Interests

The authors declare that they have no competing interests.

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Supplementary Tables and Figures

Supplementary Table 1 - Downregulated miRNAs in PC-3 and Du-145 compared to LNCaP cells

Downregulated miRNAs	log2FoldChange	-log10(padj)
hsa-miR-99a-5p	-7.971	2.824
hsa-miR-200c-3p	-6.556	2.826
hsa-let-7c-5p	-6.547	4.173
hsa-miR-148a-3p	-5.777	5.377
hsa-miR-141-3p	-5.094	3.538
hsa-miR-34a-5p	-3.611	3.582
hsa-miR-4454+hsa-miR-7975	-3.390	2.140
hsa-miR-203a-3p	-2.763	3.356
hsa-miR-497-5p	-2.707	4.667
hsa-miR-29c-3p	-2.379	3.457
hsa-miR-1973	-2.168	1.330
hsa-miR-539-5p	-2.086	1.495
hsa-let-7b-5p	-1.950	1.968
hsa-miR-193b-3p	-1.825	2.576
hsa-miR-663a	-1.800	2.352
hsa-miR-493-3p	-1.751	1.306
hsa-miR-1260a	-1.696	2.359
hsa-miR-324-5p	-1.691	2.642
hsa-miR-135a-5p	-1.614	2.576
hsa-miR-340-5p	-1.593	3.900
hsa-miR-1246	-1.474	2.140
hsa-miR-195-5p	-1.406	2.058
hsa-miR-105-5p	-1.356	2.113
hsa-miR-1269b	-1.343	2.225
hsa-miR-769-5p	-1.237	2.066
hsa-miR-26a-5p	-1.180	1.560
hsa-miR-200b-3p	-1.131	2.735
hsa-miR-425-5p	-1.040	2.330
hsa-miR-429	-0.926	2.122
hsa-miR-664a-3p	-0.912	2.053
hsa-miR-132-3p	-0.832	2.037
hsa-miR-874-5p	-0.799	1.487
hsa-miR-148b-3p	-0.739	2.140
hsa-miR-186-5p	-0.735	1.784
hsa-miR-362-5p	-0.681	1.684
hsa-miR-18a-5p	-0.646	1.560

Supplementary Table 2 - Upregulated miRNAs in PC-3 and Du-145 compared to LNCaP cells

Upregulated miRNAs	log2FoldChange	-log10(padj)
hsa-miR-1266-5p	0.600	1.442
hsa-miR-210-5p	0.611	1.667
hsa-miR-450b-3p	0.639	1.376
hsa-miR-1206	0.643	2.141
hsa-miR-301b-5p	0.665	1.306
hsa-miR-196a-3p	0.672	1.611
hsa-miR-521	0.677	1.412
hsa-miR-942-5p	0.679	2.122
hsa-miR-302d-3p	0.688	1.563
hsa-miR-577	0.697	1.862
hsa-miR-573	0.708	1.684
hsa-miR-517a-3p	0.708	1.575
hsa-miR-2116-5p	0.709	1.330
hsa-miR-122-5p	0.717	3.013

hsa-miR-933	0.722	1.330
hsa-miR-519c-3p	0.723	1.524
hsa-miR-412-3p	0.729	1.441
hsa-miR-548v	0.735	1.609
hsa-miR-512-5p	0.736	1.662
hsa-miR-514b-5p	0.759	1.306
hsa-miR-369-3p	0.762	1.349
hsa-miR-370-3p	0.777	1.441
hsa-miR-3144-3p	0.787	1.684
hsa-miR-127-3p	0.790	1.641
hsa-miR-802	0.796	1.325
hsa-miR-1255b-5p	0.810	1.330
hsa-miR-612	0.831	1.523
hsa-miR-1234-3p	0.858	1.495
hsa-miR-183-5p	0.859	3.187
hsa-miR-876-3p	0.860	2.115
hsa-miR-151a-5p	0.860	2.122
hsa-miR-302e	0.864	1.434
hsa-miR-1909-3p	0.873	2.066
hsa-miR-526a+hsa-miR-518c-5p+hsa-miR-518d-5p	0.883	1.968
hsa-miR-1178-3p	0.896	1.441
hsa-miR-224-5p	0.920	1.575
hsa-miR-25-5p	0.943	1.666
hsa-miR-608	0.956	2.391
hsa-miR-548ah-5p	0.965	1.370
hsa-miR-1236-3p	0.975	2.141
hsa-miR-4755-5p	0.977	1.392
hsa-miR-486-3p	0.980	1.621
hsa-miR-96-5p	0.995	1.611
hsa-miR-616-3p	1.012	1.478
hsa-miR-548d-5p	1.018	1.524
hsa-miR-503-3p	1.018	3.383
hsa-miR-151a-3p	1.052	1.703
hsa-miR-381-3p	1.066	2.308
hsa-miR-595	1.082	2.576
hsa-miR-4536-5p	1.110	2.141
hsa-miR-449c-5p	1.112	1.868
hsa-miR-1268b	1.135	1.661
hsa-miR-208b-3p	1.135	3.003
hsa-miR-655-3p	1.158	1.727
hsa-miR-338-5p	1.193	2.107
hsa-miR-770-5p	1.198	2.225
hsa-miR-542-3p	1.214	1.621
hsa-miR-193a-5p+hsa-miR-193b-5p	1.223	1.571
hsa-miR-487b-5p	1.262	1.968
hsa-miR-549a	1.337	1.783
hsa-miR-376a-2-5p	1.382	2.122
hsa-miR-766-5p	1.391	1.817
hsa-miR-377-3p	1.417	1.789
hsa-miR-181a-3p	1.432	2.735
hsa-miR-1277-3p	1.494	2.330
hsa-miR-548m	1.570	2.617
hsa-miR-221-5p	1.636	2.812
hsa-miR-455-5p	1.782	1.575
hsa-miR-21-5p	1.959	1.330
hsa-miR-34c-3p	2.023	1.409
hsa-miR-181c-5p	2.101	1.560
hsa-miR-625-5p	2.179	3.314
hsa-let-7i-5p	2.504	3.622
hsa-miR-24-3p	2.650	4.358
hsa-miR-196a-5p	2.692	2.141
hsa-miR-450a-5p	2.702	1.864
hsa-miR-196b-5p	2.816	2.912
hsa-miR-181b-5p+hsa-miR-181d-5p	2.831	3.900
hsa-miR-29b-3p	2.998	3.979
hsa-miR-29a-3p	3.099	4.710
hsa-miR-10a-5p	3.210	2.534
hsa-miR-181a-5p	3.529	3.622
hsa-miR-31-5p	3.537	3.539
hsa-miR-221-3p	3.994	2.147
hsa-miR-503-5p	4.047	1.409

hsa-miR-23a-3p	4.155	4.752
hsa-miR-424-5p	4.166	4.710
hsa-miR-222-3p	4.880	2.812
hsa-miR-135b-5p	5.437	3.337
hsa-miR-130a-3p	5.673	3.979

Supplementary Table 3 - Compartments data enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the downregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
17	GOCC:0098797	Plasma membrane protein complex	9.53E-13
5	GOCC:0016324	Apical plasma membrane	8.0E-4
12	GOCC:0005794	Golgi apparatus	8.06E-6
5	GOCC:0031256	Leading edge membrane	7.35E-5
15	GOCC:0005615	Extracellular space	2.59E-8
4	GOCC:0005901	Caveola	1.8E-4
6	GOCC:0030666	Endocytic vesicle membrane	1.3E-5
10	GOCC:0005856	Cytoskeleton	0.0206
4	GOCC:0005802	trans-Golgi network	0.0166
4	GOCC:0016323	Basolateral plasma membrane	0.0048
5	GOCC:0005770	Late endosome	0.0019

Supplementary Table 4 – Gene Ontology (GO) Biological Processes data enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the downregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
23	GO:1902533	Positive regulation of intracellular signal transduction	1.47E-15
12	GO:0051896	Regulation of protein kinase b signaling	6.99E-12
15	GO:0048729	Tissue morphogenesis	3.33E-11
16	GO:0030155	Regulation of cell adhesion	5.2E-11
16	GO:0010942	Positive regulation of cell death	5.86E-11
17	GO:0051050	Positive regulation of transport	1.57E-10
17	GO:0044087	Regulation of cellular component biogenesis	3.18E-10
17	GO:0007417	Central nervous system development	4.09E-10
12	GO:0071396	Cellular response to lipid	4.14E-8
10	GO:0050900	Leukocyte migration	6.53E-8
7	GO:0051492	Regulation of stress fiber assembly	7.37E-8
8	GO:0051701	Interaction with host	8.02E-8
6	GO:0031295	T cell costimulation	1.83E-7
11	GO:0022604	Regulation of cell morphogenesis	2.67E-7
7	GO:0042552	Myelination	2.67E-7
8	GO:2000377	Regulation of reactive oxygen species metabolic process	3.46E-7
15	GO:0071495	Cellular response to endogenous stimulus	3.56E-7
14	GO:0032101	Regulation of response to external stimulus	4.59E-7
10	GO:0048514	Blood vessel morphogenesis	5.67E-7
17	GO:0042592	Homeostatic process	6.33E-7
11	GO:0060627	Regulation of vesicle-mediated transport	6.65E-7
8	GO:0009612	Response to mechanical stimulus	7.87E-7
7	GO:0033135	Regulation of peptidyl-serine phosphorylation	1.4E-6
12	GO:0051345	Positive regulation of hydrolase activity	1.84E-6
13	GO:0009887	Animal organ morphogenesis	2.24E-6
14	GO:0030030	Cell projection organization	2.43E-6
7	GO:0050714	Positive regulation of protein secretion	3.14E-6
9	GO:0032147	Activation of protein kinase activity	3.53E-6
9	GO:0008015	Blood circulation	4.99E-6
4	GO:2000811	Negative regulation of anoikis	5.62E-6
7	GO:0050679	Positive regulation of epithelial cell proliferation	7.29E-6
7	GO:0071219	Cellular response to molecule of bacterial origin	7.98E-6
16	GO:0016192	Vesicle-mediated transport	1.03E-5
14	GO:0050877	Nervous system process	1.19E-5
8	GO:0043010	Camera-type eye development	1.25E-5
5	GO:0043542	Endothelial cell migration	1.64E-5
8	GO:1901342	Regulation of vasculature development	1.84E-5
12	GO:0051093	Negative regulation of developmental process	1.91E-5
9	GO:1901652	Response to peptide	2.06E-5
9	GO:0050769	Positive regulation of neurogenesis	2.11E-5
5	GO:0060193	Positive regulation of lipase activity	2.61E-5
13	GO:0051726	Regulation of cell cycle	2.67E-5
10	GO:0002520	Immune system development	2.69E-5

7	GO:0050730	Regulation of peptidyl-tyrosine phosphorylation	4.07E-5
6	GO:2001236	Regulation of extrinsic apoptotic signaling pathway	4.35E-5
10	GO:0008285	Negative regulation of cell population proliferation	4.5E-5
5	GO:0048013	Ephrin receptor signaling pathway	4.55E-5
12	GO:0051248	Negative regulation of protein metabolic process	5.11E-5
5	GO:0003158	Endothelium development	5.23E-5
7	GO:0007162	Negative regulation of cell adhesion	6.41E-5
11	GO:0022610	Biological adhesion	7.26E-5
5	GO:1901216	Positive regulation of neuron death	7.82E-5
5	GO:0022612	Gland morphogenesis	8.13E-5
6	GO:0046328	Regulation of jnk cascade	8.13E-5
7	GO:0070372	Regulation of erk1 and erk2 cascade	8.13E-5
5	GO:1901890	Positive regulation of cell junction assembly	9.2E-5
5	GO:1903076	Regulation of protein localization to plasma membrane	9.99E-5
8	GO:0042391	Regulation of membrane potential	1.0E-4
8	GO:0050804	Modulation of chemical synaptic transmission	1.1E-4
14	GO:1903508	Positive regulation of nucleic acid-templated transcription	1.1E-4
6	GO:1901654	Response to ketone	1.2E-4
11	GO:0071345	Cellular response to cytokine stimulus	1.5E-4
4	GO:0030301	Cholesterol transport	1.9E-4
4	GO:0010676	Positive regulation of cellular carbohydrate metabolic process	2.5E-4
8	GO:0010035	Response to inorganic substance	3.7E-4
5	GO:0007612	Learning	4.0E-4
5	GO:0050921	Positive regulation of chemotaxis	4.2E-4
7	GO:0022411	Cellular component disassembly	4.5E-4
3	GO:1904886	Beta-catenin destruction complex disassembly	4.6E-4
5	GO:0071346	Cellular response to interferon-gamma	6.0E-4
6	GO:0014706	Striated muscle tissue development	6.6E-4
7	GO:0010256	Endomembrane system organization	6.7E-4
7	GO:0019216	Regulation of lipid metabolic process	6.7E-4
2	GO:0032707	Negative regulation of interleukin-23 production	6.8E-4
4	GO:0048015	Phosphatidylinositol-mediated signaling	6.8E-4
6	GO:0043270	Positive regulation of ion transport	7.1E-4
5	GO:0043409	Negative regulation of mapk cascade	7.1E-4
7	GO:0006897	Endocytosis	7.4E-4
5	GO:0021543	Pallium development	7.7E-4
6	GO:0007204	Positive regulation of cytosolic calcium ion concentration	8.7E-4
5	GO:0008361	Regulation of cell size	0.001
6	GO:0060562	Epithelial tube morphogenesis	0.001
2	GO:1903598	Positive regulation of gap junction assembly	0.0011
3	GO:1901889	Negative regulation of cell junction assembly	0.0013
3	GO:0030032	Lamellipodium assembly	0.0014
5	GO:0071695	Anatomical structure maturation	0.0014
7	GO:0008283	Cell population proliferation	0.0015
3	GO:0043552	Positive regulation of phosphatidylinositol 3-kinase activity	0.0015
4	GO:0048259	Regulation of receptor-mediated endocytosis	0.0015
5	GO:0006909	Phagocytosis	0.0017
3	GO:0033619	Membrane protein proteolysis	0.002
2	GO:0090244	Wnt signaling pathway involved in somitogenesis	0.002
6	GO:0032386	Regulation of intracellular transport	0.0021
3	GO:0001937	Negative regulation of endothelial cell proliferation	0.0025
2	GO:0008354	Germ cell migration	0.0025
3	GO:0045933	Positive regulation of muscle contraction	0.0026
5	GO:0097305	Response to alcohol	0.0026
3	GO:0014003	Oligodendrocyte development	0.0028
3	GO:0035272	Exocrine system development	0.0029
3	GO:0051898	Negative regulation of protein kinase b signaling	0.0029
6	GO:0046649	Lymphocyte activation	0.003
14	GO:0022607	Cellular component assembly	0.0032
3	GO:0031952	Regulation of protein autophosphorylation	0.0034
4	GO:1900180	Regulation of protein localization to nucleus	0.0034
3	GO:1905207	Regulation of cardiocyte differentiation	0.0034
10	GO:0007186	G protein-coupled receptor signaling pathway	0.0036
3	GO:0002011	Morphogenesis of an epithelial sheet	0.0037
4	GO:0035637	Multicellular organismal signaling	0.0039
3	GO:0006968	Cellular defense response	0.0043
2	GO:0002645	Positive regulation of tolerance induction	0.0044
3	GO:0022029	Telencephalon cell migration	0.0047
2	GO:0060907	Positive regulation of macrophage cytokine production	0.0051
4	GO:0071560	Cellular response to transforming growth factor beta stimulus	0.0051
3	GO:0090303	Positive regulation of wound healing	0.0052

5	GO:0050808	Synapse organization	0.0054
4	GO:0030902	Hindbrain development	0.0055
2	GO:1903961	Positive regulation of anion transmembrane transport	0.0057
2	GO:2000392	Regulation of lamellipodium morphogenesis	0.0057
4	GO:0051092	Positive regulation of nf-kappab transcription factor activity	0.0059
4	GO:0061138	Morphogenesis of a branching epithelium	0.006
2	GO:0042532	Negative regulation of tyrosine phosphorylation of stat protein	0.0065
4	GO:0031960	Response to corticosteroid	0.0066
2	GO:0007494	Midgut development	0.0072
2	GO:0048548	Regulation of pinocytosis	0.0072
2	GO:0050966	Detection of mechanical stimulus involved in sensory perception of pain	0.0081
3	GO:0032024	Positive regulation of insulin secretion	0.0087
3	GO:0007422	Peripheral nervous system development	0.0089
2	GO:0044764	Multi-organism cellular process	0.0089
5	GO:0048638	Regulation of developmental growth	0.0096
2	GO:1902992	Negative regulation of amyloid precursor protein catabolic process	0.0098
5	GO:0030198	Extracellular matrix organization	0.0101
5	GO:0022412	Cellular process involved in reproduction in multicellular organism	0.011
4	GO:0010821	Regulation of mitochondrion organization	0.0114
4	GO:0051302	Regulation of cell division	0.0115
4	GO:0010952	Positive regulation of peptidase activity	0.0117
4	GO:0060348	Bone development	0.0118
2	GO:2000641	Regulation of early endosome to late endosome transport	0.0128
3	GO:0110110	Positive regulation of animal organ morphogenesis	0.0133
2	GO:0031998	Regulation of fatty acid beta-oxidation	0.0139
13	GO:0033036	Macromolecule localization	0.0139
8	GO:0009894	Regulation of catabolic process	0.0155
2	GO:0051957	Positive regulation of amino acid transport	0.0158
4	GO:0017157	Regulation of exocytosis	0.0163
2	GO:0048714	Positive regulation of oligodendrocyte differentiation	0.0169
2	GO:0051894	Positive regulation of focal adhesion assembly	0.0193
2	GO:0030225	Macrophage differentiation	0.0204
2	GO:0050927	Positive regulation of positive chemotaxis	0.0204
2	GO:0070723	Response to cholesterol	0.0218
2	GO:0033137	Negative regulation of peptidyl-serine phosphorylation	0.023
2	GO:0001964	Startle response	0.0242
2	GO:0051590	Positive regulation of neurotransmitter transport	0.0242
4	GO:0030336	Negative regulation of cell migration	0.0267
5	GO:0051051	Negative regulation of transport	0.0267
2	GO:0035025	Positive regulation of rho protein signal transduction	0.0294
2	GO:0008045	Motor neuron axon guidance	0.0309
2	GO:0061384	Heart trabecula morphogenesis	0.0324
2	GO:0090049	Regulation of cell migration involved in sprouting angiogenesis	0.0366
2	GO:0014911	Positive regulation of smooth muscle cell migration	0.0381
2	GO:1904646	Cellular response to amyloid-beta	0.0381
3	GO:0060041	Retina development in camera-type eye	0.0383
2	GO:0061900	Glial cell activation	0.0413
2	GO:1901998	Toxin transport	0.0413
8	GO:0006508	Proteolysis	0.0416
3	GO:0045580	Regulation of t cell differentiation	0.0416
5	GO:0006518	Peptide metabolic process	0.042
3	GO:0010977	Negative regulation of neuron projection development	0.0444
2	GO:0045806	Negative regulation of endocytosis	0.0444
3	GO:0010970	Transport along microtubule	0.0469
3	GO:0016236	Macroautophagy	0.0476

Supplementary Table 5 - Gene Ontology (GO) Cellular Components data enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the downregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
35	GO:0005768	Endosome	2.33E-37
19	GO:0009986	Cell surface	6.5E-14
14	GO:0045121	Membrane raft	2.94E-13
18	GO:0070161	Anchoring junction	8.36E-13
11	GO:0031901	Early endosome membrane	2.88E-12
17	GO:0030659	Cytoplasmic vesicle membrane	4.43E-12
14	GO:0031252	Cell leading edge	6.54E-12
19	GO:0098590	Plasma membrane region	3.37E-11
9	GO:0055037	Recycling endosome	7.01E-9
17	GO:0045202	Synapse	1.79E-8

13	GO:0048471	Perinuclear region of cytoplasm	5.8E-8
9	GO:0030139	Endocytic vesicle	3.78E-7
7	GO:0043197	Dendritic spine	2.45E-6
10	GO:0098552	Side of membrane	3.04E-6
8	GO:0030667	Secretory granule membrane	5.2E-6
10	GO:0044297	Cell body	7.28E-6
5	GO:0032587	Ruffle membrane	3.89E-5
8	GO:0045177	Apical part of cell	5.03E-5
9	GO:0030424	Axon	1.3E-4
9	GO:0000139	Golgi membrane	4.3E-4
4	GO:0030670	Phagocytic vesicle membrane	4.4E-4
9	GO:0005773	Vacuole	5.6E-4
5	GO:0098802	Plasma membrane signaling receptor complex	5.6E-4
4	GO:0030175	Filopodium	0.0015
4	GO:0043296	Apical junction complex	0.0042
4	GO:0042734	Presynaptic membrane	0.006
3	GO:0044291	Cell-cell contact zone	0.0084
5	GO:0098984	Neuron to neuron synapse	0.016
2	GO:0043198	Dendritic shaft	0.0445

Supplementary Table 6 - Gene Ontology (GO) Molecular Function data enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the downregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
13	GO:0044877	Protein-containing complex binding	8.58E-5
5	GO:0001540	Amyloid-beta binding	9.18E-5
5	GO:0051117	ATPase binding	1.4E-4
14	GO:0042802	Identical protein binding	9.6E-4
9	GO:0008289	Lipid binding	0.0013
2	GO:0051022	Rho GDP-dissociation inhibitor binding	0.0025
15	GO:0016787	Hydrolase activity	0.0028
8	GO:0046983	Protein dimerization activity	0.0477

Supplementary Table 7 – KEGG pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the downregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
10	hsa04144	Endocytosis	5.02E-9
11	hsa04151	PI3K-Akt signaling pathway	6.83E-9
7	hsa04520	Adherens junction	7.71E-9
8	hsa04530	Tight junction	5.19E-8
8	hsa05131	Shigellosis	5.18E-7
5	hsa04068	FoxO signaling pathway	1.0E-4
5	hsa04072	Phospholipase D signaling pathway	1.7E-4
5	hsa05225	Hepatocellular carcinoma	2.2E-4
5	hsa04360	Axon guidance	3.1E-4
4	hsa05142	Chagas disease	4.9E-4
4	hsa04722	Neurotrophin signaling pathway	7.4E-4
4	hsa05206	MicroRNAs in cancer	0.0022
3	hsa04390	Hippo signaling pathway	0.0174
2	hsa02010	ABC transporters	0.0181
2	hsa05416	Viral myocarditis	0.025

Supplementary Table 8 – Reactome Pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the downregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
11	HSA-5663202	Diseases of signal transduction by growth factor receptors and second messengers	1.42E-7
3	HSA-9032500	Activated NTRK2 signals through FYN	1.4E-4
3	HSA-5627123	RHO GTPases activate PAKs	0.0011
6	HSA-5683057	MAPK family signaling cascades	0.0022
7	HSA-199991	Membrane Trafficking	0.0078
2	HSA-9013507	NOTCH3 Activation and Transmission of Signal to the Nucleus	0.0261

Supplementary Table 9 - Compartments data enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
21	GOCC:0005887	Integral component of plasma membrane	1.92E-15
19	GOCC:0098590	Plasma membrane region	3.95E-14
15	GOCC:0010008	Endosome membrane	8.81E-14
16	GOCC:0009986	Cell surface	2.37E-13
14	GOCC:0070062	Extracellular exosome	8.30E-12
10	GOCC:0098802	Plasma membrane signaling receptor complex	1.67E-08
6	GOCC:0016323	Basolateral plasma membrane	2.83E-05
12	GOCC:0005794	Golgi apparatus	2.96E-05
5	GOCC:0031256	Leading edge membrane	1.30E-04
5	GOCC:0009897	External side of plasma membrane	2.00E-04
8	GOCC:0045202	Synapse	6.10E-04
6	GOCC:0005765	Lysosomal membrane	9.20E-04
4	GOCC:0043296	Apical junction complex	0.0026
6	GOCC:0015629	Actin cytoskeleton	0.0043
3	GOCC:0055038	Recycling endosome membrane	0.0072
2	GOCC:0060187	Cell pole	0.0116
3	GOCC:0042383	Sarcolemma	0.0132
2	GOCC:0036019	Endolysosome	0.0322
4	GOCC:0031983	Vesicle lumen	0.0412

Supplementary Table 10 - Gene Ontology (GO) Biological Processes data enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
19	GO:0030335	Positive regulation of cell migration	2.14E-15
20	GO:0043408	Regulation of mapk cascade	2.03E-14
30	GO:0002376	Immune system process	3.18E-14
24	GO:1901700	Response to oxygen-containing compound	9.24E-13
13	GO:0051251	Positive regulation of lymphocyte activation	1.5E-11
17	GO:0120035	Regulation of plasma membrane bounded cell projection organization	1.65E-11
23	GO:0042127	Regulation of cell population proliferation	2.04E-11
12	GO:0042063	Gliogenesis	2.58E-11
17	GO:0007167	Enzyme linked receptor protein signaling pathway	3.02E-11
19	GO:0060341	Regulation of cellular localization	5.15E-11
14	GO:0060627	Regulation of vesicle-mediated transport	1.7E-09
21	GO:0042592	Homeostatic process	2.09E-09
14	GO:0048729	Tissue morphogenesis	2.1E-09
10	GO:0009612	Response to mechanical stimulus	5.76E-09
12	GO:0046649	Lymphocyte activation	6.56E-09
16	GO:0014070	Response to organic cyclic compound	8.55E-09
9	GO:0030307	Positive regulation of cell growth	2.03E-08
12	GO:0042060	Wound healing	2.17E-08
9	GO:0050679	Positive regulation of epithelial cell proliferation	5.02E-08
15	GO:0043066	Negative regulation of apoptotic process	5.94E-08
10	GO:0007162	Negative regulation of cell adhesion	6.32E-08
18	GO:0080134	Regulation of response to stress	7.41E-08
9	GO:0006909	Phagocytosis	9.79E-08
12	GO:0032103	Positive regulation of response to external stimulus	0.0000001
12	GO:0090066	Regulation of anatomical structure size	0.00000011
7	GO:1903076	Regulation of protein localization to plasma membrane	0.000000359
8	GO:0051897	Positive regulation of protein kinase b signaling	0.00000039
10	GO:0051222	Positive regulation of protein transport	0.000000442
7	GO:0042552	Myelination	0.000000541
7	GO:0090175	Regulation of establishment of planar polarity	0.000000541
6	GO:0051893	Regulation of focal adhesion assembly	0.000000655
12	GO:0048534	Hematopoietic or lymphoid organ development	0.000000702
14	GO:0007155	Cell adhesion	0.000000728
16	GO:0051336	Regulation of hydrolase activity	0.000000754
12	GO:1903530	Regulation of secretion by cell	0.000000831
6	GO:1903036	Positive regulation of response to wounding	0.00000102
11	GO:0006954	Inflammatory response	0.00000113
5	GO:1901889	Negative regulation of cell junction assembly	0.00000113
8	GO:0070374	Positive regulation of erk1 and erk2 cascade	0.00000159
9	GO:0045765	Regulation of angiogenesis	0.00000165
9	GO:1901214	Regulation of neuron death	0.00000232
7	GO:0010634	Positive regulation of epithelial cell migration	0.00000283

6	GO:0048013	Ephrin receptor signaling pathway	0.00000315
12	GO:0045596	Negative regulation of cell differentiation	0.00000345
7	GO:0008360	Regulation of cell shape	0.00000362
12	GO:0007420	Brain development	0.00000425
10	GO:1901652	Response to peptide	0.00000548
12	GO:0016032	Viral process	0.00000636
11	GO:0002366	Leukocyte activation involved in immune response	0.00000655
9	GO:0032147	Activation of protein kinase activity	0.00000821
11	GO:1901699	Cellular response to nitrogen compound	0.00000849
10	GO:1902532	Negative regulation of intracellular signal transduction	0.00000912
5	GO:0051496	Positive regulation of stress fiber assembly	0.00000913
7	GO:0034341	Response to interferon-gamma	0.00000976
9	GO:0048514	Blood vessel morphogenesis	0.0000155
9	GO:0006897	Endocytosis	0.0000232
7	GO:0043393	Regulation of protein binding	0.000024
5	GO:0043542	Endothelial cell migration	0.0000242
6	GO:0034614	Cellular response to reactive oxygen species	0.0000278
7	GO:0050670	Regulation of lymphocyte proliferation	0.000028
9	GO:0001819	Positive regulation of cytokine production	0.000036
7	GO:0032872	Regulation of stress-activated mapk cascade	0.000036
9	GO:0050769	Positive regulation of neurogenesis	0.0000477
11	GO:0061024	Membrane organization	0.0000521
10	GO:0009617	Response to bacterium	0.0000527
5	GO:0048017	Inositol lipid-mediated signaling	0.0000585
7	GO:0050730	Regulation of peptidyl-tyrosine phosphorylation	0.0000737
4	GO:0014002	Astrocyte development	0.0000756
5	GO:0003158	Endothelium development	0.0000778
8	GO:0070482	Response to oxygen levels	0.000079
8	GO:0007409	Axonogenesis	0.0000863
10	GO:0019221	Cytokine-mediated signaling pathway	0.0000887
8	GO:0045787	Positive regulation of cell cycle	0.0000918
5	GO:0060191	Regulation of lipase activity	0.00012
6	GO:0060491	Regulation of cell projection assembly	0.00013
10	GO:0098771	Inorganic ion homeostasis	0.00015
4	GO:1902743	Regulation of lamellipodium organization	0.00016
6	GO:0043123	Positive regulation of i-kappa kinase/nf-kappa signaling	0.00018
4	GO:0034446	Substrate adhesion-dependent cell spreading	0.00019
6	GO:0060326	Cell chemotaxis	0.00023
4	GO:0043525	Positive regulation of neuron apoptotic process	0.00034
7	GO:0048871	Multicellular organismal homeostasis	0.00044
9	GO:0030855	Epithelial cell differentiation	0.00055
5	GO:0030217	T cell differentiation	0.00057
3	GO:1905208	Negative regulation of cardiocyte differentiation	0.00059
6	GO:0002833	Positive regulation of response to biotic stimulus	0.00061
5	GO:0033135	Regulation of peptidyl-serine phosphorylation	0.00063
7	GO:0045862	Positive regulation of proteolysis	0.00065
9	GO:0043269	Regulation of ion transport	0.00068
6	GO:0007611	Learning or memory	0.00077
2	GO:0032707	Negative regulation of interleukin-23 production	0.00078
4	GO:0032677	Regulation of interleukin-8 production	0.00084
3	GO:0030225	Macrophage differentiation	0.00098
13	GO:0045893	Positive regulation of transcription, dna-templated	0.001
6	GO:0001818	Negative regulation of cytokine production	0.0011
4	GO:0046651	Lymphocyte proliferation	0.0011
4	GO:1901224	Positive regulation of nik/nf-kappa signaling	0.0011
3	GO:0038179	Neurotrophin signaling pathway	0.0012
6	GO:1902105	Regulation of leukocyte differentiation	0.0012
3	GO:0050690	Regulation of defense response to virus by virus	0.0013
5	GO:0031345	Negative regulation of cell projection organization	0.0015
7	GO:0002683	Negative regulation of immune system process	0.0017
2	GO:0033590	Response to cobalamin	0.0018
4	GO:0048771	Tissue remodeling	0.0018
6	GO:0007264	Small gtpase mediated signal transduction	0.002
4	GO:0003015	Heart process	0.0022
4	GO:0048259	Regulation of receptor-mediated endocytosis	0.0022
4	GO:0007041	Lysosomal transport	0.0023
2	GO:0032613	interleukin-10 production	0.0023
2	GO:0090244	Wnt signaling pathway involved in somitogenesis	0.0023
3	GO:0043491	Protein kinase b signaling	0.0024
3	GO:0090049	Regulation of cell migration involved in sprouting angiogenesis	0.0024
6	GO:0048638	Regulation of developmental growth	0.0025

3	GO:0014911	Positive regulation of smooth muscle cell migration	0.0026
3	GO:0033619	Membrane protein proteolysis	0.0026
3	GO:1904646	Cellular response to amyloid-beta	0.0026
4	GO:0032526	Response to retinoic acid	0.0027
7	GO:0034330	Cell junction organization	0.0027
3	GO:0045740	Positive regulation of dna replication	0.0027
2	GO:0008354	Germ cell migration	0.003
10	GO:0008219	Cell death	0.0032
6	GO:0050727	Regulation of inflammatory response	0.0032
3	GO:0043277	Apoptotic cell clearance	0.0033
3	GO:0045746	Negative regulation of notch signaling pathway	0.0033
3	GO:0014003	Oligodendrocyte development	0.0036
6	GO:0032386	Regulation of intracellular transport	0.0036
14	GO:0008104	Protein localization	0.0043
2	GO:0051964	Negative regulation of synapse assembly	0.0045
2	GO:0023035	CD40 signaling pathway	0.0053
6	GO:0040007	Growth	0.0054
3	GO:0022029	Telencephalon cell migration	0.0061
3	GO:0032663	Regulation of interleukin-2 production	0.0061
5	GO:0030336	Negative regulation of cell migration	0.0064
6	GO:0002697	Regulation of immune effector process	0.007
4	GO:0050921	Positive regulation of chemotaxis	0.007
2	GO:0097011	Cellular response to granulocyte macrophage colony-stimulating factor stimulus	0.007
4	GO:0043524	Negative regulation of neuron apoptotic process	0.0071
4	GO:0030902	Hindbrain development	0.008
4	GO:0051147	Regulation of muscle cell differentiation	0.008
10	GO:0007186	G protein-coupled receptor signaling pathway	0.0082
4	GO:0000187	Activation of mapk activity	0.0085
2	GO:0007494	Midgut development	0.009
2	GO:0032530	Regulation of microvillus organization	0.009
9	GO:0032269	Negative regulation of cellular protein metabolic process	0.0092
4	GO:2001236	Regulation of extrinsic apoptotic signaling pathway	0.0092
5	GO:1903522	Regulation of blood circulation	0.0095
6	GO:0051346	Negative regulation of hydrolase activity	0.0096
4	GO:0019722	Calcium-mediated signaling	0.0099
2	GO:0050966	Detection of mechanical stimulus involved in sensory perception of pain	0.0099
3	GO:0014015	Positive regulation of gliogenesis	0.0109
3	GO:0018107	Peptidyl-threonine phosphorylation	0.0109
2	GO:0060263	Regulation of respiratory burst	0.011
5	GO:0010769	Regulation of cell morphogenesis involved in differentiation	0.0117
4	GO:0010469	Regulation of signaling receptor activity	0.0119
2	GO:0010715	Regulation of extracellular matrix disassembly	0.012
2	GO:0043217	Myelin maintenance	0.012
2	GO:0046209	Nitric oxide metabolic process	0.0132
6	GO:0001501	Skeletal system development	0.0147
4	GO:0071248	Cellular response to metal ion	0.0158
3	GO:0050871	Positive regulation of b cell activation	0.016
4	GO:0046890	Regulation of lipid biosynthetic process	0.0169
3	GO:0035023	Regulation of rho protein signal transduction	0.0174
3	GO:0110110	Positive regulation of animal organ morphogenesis	0.0177
6	GO:0007507	Heart development	0.0178
2	GO:0032288	Myelin assembly	0.0182
2	GO:0048799	Animal organ maturation	0.0182
4	GO:0048872	Homeostasis of number of cells	0.0186
2	GO:0036473	Cell death in response to oxidative stress	0.0196
3	GO:0010522	Regulation of calcium ion transport into cytosol	0.0238
3	GO:0045807	Positive regulation of endocytosis	0.0249
2	GO:0003401	Axis elongation	0.0252
2	GO:0044319	Wound healing, spreading of cells	0.0252
6	GO:0048598	Embryonic morphogenesis	0.0258
2	GO:0070723	Response to cholesterol	0.0265
5	GO:0031331	Positive regulation of cellular catabolic process	0.0287
2	GO:0042310	Vasoconstriction	0.0317
3	GO:0098693	Regulation of synaptic vesicle cycle	0.0317
2	GO:2000108	Positive regulation of leukocyte apoptotic process	0.0317
2	GO:0001774	Microglial cell activation	0.0349
2	GO:0007202	Activation of phospholipase c activity	0.0383
2	GO:0043403	Skeletal muscle tissue regeneration	0.0383
4	GO:0042493	Response to drug	0.0481
2	GO:0045058	T cell selection	0.0492

Supplementary Table 11 - Gene Ontology (GO) Cellular Components data enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
37	GO:0005768	Endosome	1.69E-37
18	GO:0005769	Early endosome	3.65E-17
22	GO:0070161	Anchoring junction	1.49E-16
14	GO:0045121	Membrane raft	1.82E-12
14	GO:0098552	Side of membrane	6.61E-10
24	GO:0042995	Cell projection	8.76E-10
14	GO:0098797	Plasma membrane protein complex	9.05E-10
11	GO:0005770	Late endosome	1.94E-09
15	GO:0030659	Cytoplasmic vesicle membrane	5.67E-09
14	GO:0005773	Vacuole	8.44E-08
11	GO:0031252	Cell leading edge	0.00000137
7	GO:0042383	Sarcolemma	0.00000106
10	GO:0045177	Apical part of cell	0.00000134
13	GO:0036477	Somatodendritic compartment	0.00000152
7	GO:0055037	Recycling endosome	0.00000675
8	GO:0030139	Endocytic vesicle	0.0000124
11	GO:0048471	Perinuclear region of cytoplasm	0.0000166
4	GO:0097386	Glial cell projection	0.0000428
6	GO:0031901	Early endosome membrane	0.000055
5	GO:0042470	Melanosome	0.00011
3	GO:0001891	Phagocytic cup	0.00089
5	GO:0030136	Clathrin-coated vesicle	0.0017
5	GO:0030027	Lamellipodium	0.0021
3	GO:0032154	Cleavage furrow	0.0054
4	GO:0031902	Late endosome membrane	0.0058
3	GO:0101003	ficolin-1-rich granule membrane	0.0077
3	GO:0005771	Multivesicular body	0.0079
4	GO:0042582	Azurophil granule	0.0084
4	GO:0009898	Cytoplasmic side of plasma membrane	0.0086
7	GO:0030424	Axon	0.0104
3	GO:0030670	Phagocytic vesicle membrane	0.0119
2	GO:0001772	Immunological synapse	0.0499

Supplementary Table 12 - Gene Ontology (GO) Molecular Function data enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
8	GO:0004713	Protein tyrosine kinase activity	0.000000814
6	GO:0015026	Coreceptor activity	0.00000118
18	GO:0042802	Identical protein binding	0.0000178
6	GO:0051117	ATPase binding	0.0000178
9	GO:0019904	Protein domain specific binding	0.0034
3	GO:0070325	Lipoprotein particle receptor binding	0.0043
6	GO:0031625	Ubiquitin protein ligase binding	0.0055
7	GO:0050839	Cell adhesion molecule binding	0.0164
8	GO:0008289	Lipid binding	0.0222
6	GO:0003779	Actin binding	0.0319
2	GO:0005041	Low-density lipoprotein particle receptor activity	0.033

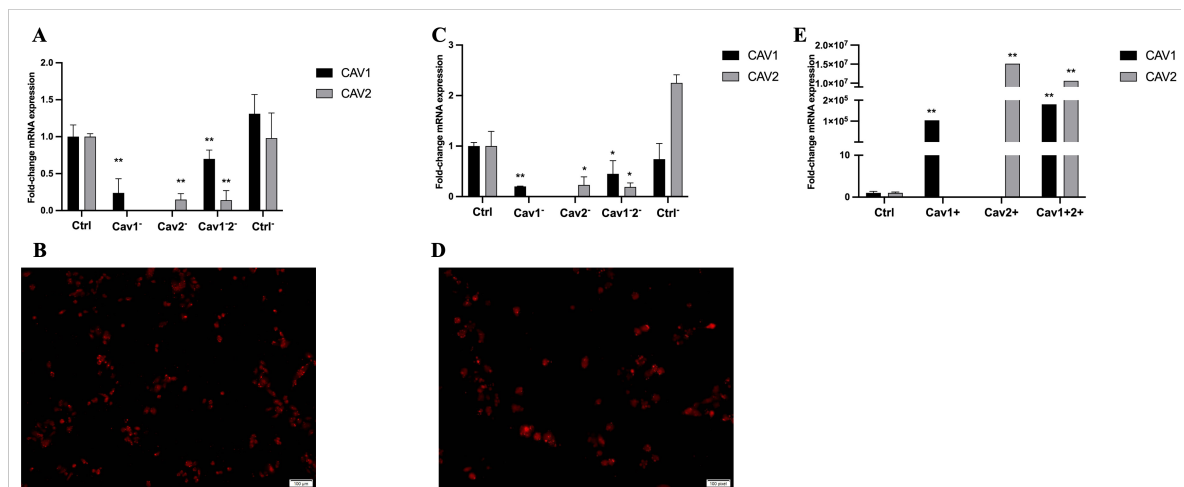
Supplementary Table 13 - KEGG pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
14	hsa05205	Proteoglycans in cancer	7.05E-15
15	hsa05200	Pathways in cancer	7.14E-11
10	hsa04015	Rap1 signaling pathway	4.53E-09
8	hsa05135	Yersinia infection	4.24E-08
9	hsa04144	Endocytosis	0.000000192
6	hsa05235	PD-L1 expression and PD-1 checkpoint pathway in cancer	0.00000202
7	hsa05161	Hepatitis B	0.00000228
7	hsa05152	Tuberculosis	0.00000305
5	hsa04928	Parathyroid hormone synthesis, secretion and action	0.0000691
4	hsa05144	Malaria	0.0000691
4	hsa04370	VEGF signaling pathway	0.00012
5	hsa04514	Cell adhesion molecules	0.00018

5	hsa04072	Phospholipase D signaling pathway	0.00022
5	hsa05206	MicroRNAs in cancer	0.00031
6	hsa05165	Human papillomavirus infection	0.00069
5	hsa04060	Cytokine-cytokine receptor interaction	0.0026
4	hsa05160	Hepatitis C	0.0026
3	hsa05140	Leishmaniasis	0.0031
3	hsa04721	Synaptic vesicle cycle	0.0035
2	hsa04672	Intestinal immune network for IgA production	0.0191

Supplementary Table 14 - Reactome Pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs.

Number of Genes	Term Name	Description	FDR value
14	HSA-422475	Axon guidance	1.68E-08
11	HSA-5663202	Diseases of signal transduction by growth factor receptors and second messengers	0.000000473
6	HSA-388841	Costimulation by the CD28 family	0.00000239
4	HSA-400685	Sema4D in semaphorin signaling	0.0000449
7	HSA-195258	RHO GTPase Effectors	0.00029
3	HSA-5218921	VEGFR2 mediated cell proliferation	0.00081
3	HSA-1250196	SHC1 events in ERBB2 signaling	0.0011
3	HSA-397795	G-protein beta:gamma signalling	0.0027
2	HSA-173107	Binding and entry of HIV virion	0.0029
8	HSA-5653656	Vesicle-mediated transport	0.0045
3	HSA-437239	Recycling pathway of L1	0.0071
5	HSA-1474244	Extracellular matrix organization	0.0155
3	HSA-9679191	Potential therapeutics for SARS	0.0203
4	HSA-913531	Interferon Signaling	0.0229
2	HSA-5668599	RHO GTPases Activate NADPH Oxidases	0.0308
2	HSA-5654733	Negative regulation of FGFR4 signaling	0.0418

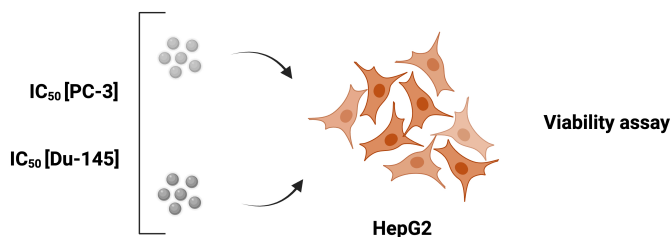


Supplementary Figure 1 – Inhibition and induction of cav1 and cav2 expression in PCa cell lines. A – mRNA levels of Cav1 and Cav2 after transfection with siCav1, siCav2, both siCav1 and siCav2 and Ctrl⁻ in PC-3 cells. B – Fluorescence microscopy image of PC-3 cells after incubation with BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control used as a positive control of transfection (OLIMPUS IX51, 10x). C – mRNA levels of Cav1 and Cav2 after transfection with siCav1, siCav2, both siCav1 and siCav2 and Ctrl⁻ in Du-145 cells. D – Fluorescence microscopy image of Du-145 cells after incubation with BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control used as a positive control of transfection in PC-3 cells (OLIMPUS IX51, 10x). E - mRNA levels of Cav1 and Cav2 after transfection with Cav1 plasmid, Cav2 plasmid, and both Cav1 and Cav2 plasmid in LNCaP cells.

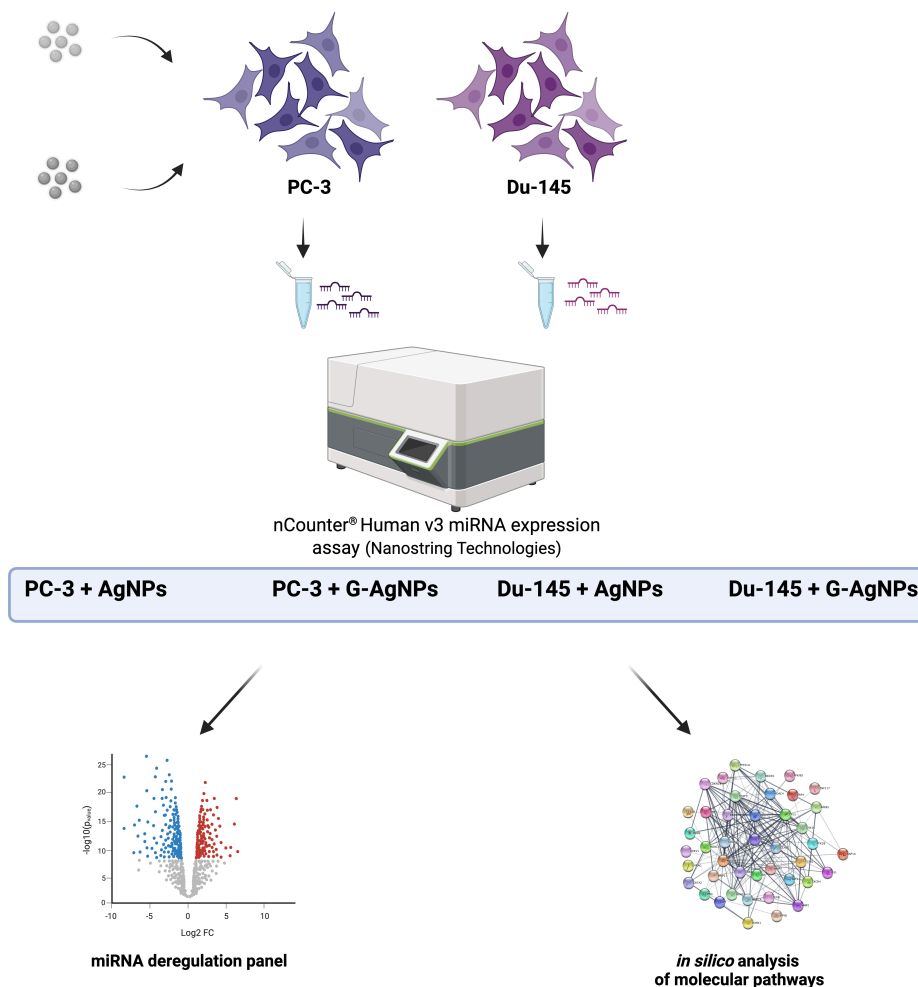
3. Do we have evidence for the multi-targeted therapy effectiveness against Castration Resistant Prostate Cancer (CRPC)? - a miRNA-profile definition approach and its potential as biomarker of response against Silver Nanoparticles (AgNPs) functionalized with Glucose (G-AgNPs)

3.1 Study Design

Phase 1: Hepatotoxicity *in vitro* study



Phase 2: miRNA and gene expression deregulation of PC-3 and Du-145 cells by AgNPs and G-AgNPs



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)

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Abstract

Background: Castration Resistant Prostate Cancer (CRPC) is the most advanced form of prostate cancer (PCa) being characterized by poor prognosis and impaired life quality. Current therapies are not curative and have many side effects, making the development of new therapies one of the priorities in research of CRPC. AgNPs functionalized with glucosamine (G-AgNPs) were shown to be cytotoxic to two CRPC cell lines (PC-3 and Du-145). The aim of this study was to *in vitro* study the hepatotoxicity of the nanoparticles and evaluate the effect of AgNPs and G-AgNPs in PC-3 and Du-145 cells regarding the miRNA expression pattern and the consequent affected molecular pathways.

Material and Methods: Cytotoxicity against HepG2 was assessed through resazurin assay. RNA was extracted from Du-145 and PC-3 cells, after AgNPs and G-AgNPs administration. miRNA profiling was performed using the nCounter[®] Human v3 miRNA. An *in silico* analysis and functional enrichment analysis was performed using *miRTarBase* and STRINGapp in Cytoscape software to uncover the deregulated molecular pathways in response to AgNPs and G-AgNPs.

Results: Low toxicity against AgNPs and G-AgNPs was observed in HepG2 cells. 799 miRNAs were screened regarding the miRNA deregulation pattern of PC-3 and Du-145 in response to AgNPs and G-AgNPs. Both AgNPs and G-AgNPs target multiple molecular pathways commonly deregulated in CRPC. Response to oxidative stress, DNA damage as well as deregulation of metabolism and modulation of tumor microenvironment through cytokines and interleukines come together with common pathways that promote proliferation, invasion and angiogenesis in the establishment of the damage by AgNPs and G-AgNPs that ultimately leads to apoptosis. We propose a miRNA profile as a response biomarker for AgNPs and G-AgNPs administration in CRPC composed by 2 downregulated and 16 upregulated miRNAs.

Conclusions: With this study we further proved the potential application of AgNPs and G-AgNPs in CRPC and showed that they target multiple pathways commonly deregulated in CRPC, which may help surpass resistance to treatment. The proposed miRNA profile can be used as a biomarker of response to this possible future therapeutic approach. Future studies must include replication of the study in 3D spheroid models and *in vivo*.

Keywords: Prostate Cancer Resistant to Castration, Silver Nanoparticles, biocompatibility, HepG2, miRNAs, biomarkers, molecular pathways, *in silico*

4. Background

With an estimate of 1.4 million new cases and 375 000 deaths by 2020, prostate cancer (PCa) is the second most frequent and the fifth leading cause of cancer death among men worldwide [1]. Although localized disease is successfully managed with surgery and/or radiotherapy, locally advanced and metastatic PCa treatment usually included androgen deprivation therapy (ADT) [2]. However, most patients develop resistance to ADT and progress to Castration Resistant Prostate Cancer (CRPC) which is characterized by a poor prognosis, an impaired life quality, with limited therapeutic options [3].

While most primary PCa can be molecularly classified in one of seven subtypes (defined by specific gene fusions – ERG, ETV1, ETV4 and FLI1; or mutations – SPOP, FOXA1, IDH1), the genomic profile of metastatic CRPC shows some differences [4, 5]. In a study involving 150 patients with metastatic CRPC (mCRPC), 63% of patients showed mutations in *AR*, 57% of patients had *ETS* gene fusions, 53% of patients had mutations in *TP53* and 41% in *PTEN* gene [6]. Approximately 89% had clinically actionable genomic aberration, 71% in the *AR* signaling pathway, 49% in the PI3K signaling pathway, 18% in Wnt signaling pathway and 23% in DNA repair pathways [6]. Currently, the CRPC patients are treated

with second-generation antiandrogens (enzalutamide and abiraterone acetate), chemotherapy agents (docetaxel and cabazitaxel), PARPinhibitors (when mutations in BRCA are present), bisphosphonate, denosumab and 223RA (which is directed to bone metastasis) [7]. However, these therapies do not have a curative intent and significantly impair life quality while an increase of overall survival of only 2.8-4.8 months [8]. Therefore, there is an urgent need of new therapies for the clinical management of these patients.

Despite the advances in the knowledge of cancer biology in the last years, and the consequent identification of several potential therapeutic targets, many of them are difficult to approach using conventional drug development strategies due to the difficulty in targeting protein–protein interactions or protein–nucleic acid interactions [9].

The advent of nanomedicine has shown promising results in the oncology field [10]. In the last years, silver nanoparticles (AgNPs) have been studied as anticancer agents with encouraging results in different cancer models [11]. On one hand, AgNPs have been shown to damage different cellular organelles, since they increase the production of Reactive Oxygen Species (ROS), induce damage in the DNA and in the cellular membrane and are able to interfere with the cell cycle [12]. Moreover, they affect several molecular pathways, interfering with the expression of genes involved in carcinogenesis, such as the vascular endothelial growth factor (VEGF) [12]. On the other hand, biocompatibility of AgNPs has already been shown in some *in vivo* studies in healthy mice, which concluded that the body weight, serum markers, and oxidative stress markers in liver of this mice returned to normal after the washout period [13]. We have previously shown the cytotoxic effect of AgNPs functionalized with glucosamine (G-AgNPs) in two CRPC cell lines (PC-3 and Du-145) [14]. AgNPs and G-AgNPs enter cells through endocytosis and are encapsulated in lysosomes, increasing the reactive oxygen species generation, which in turns induces mitochondrial damage, and arrests cell cycle in S Phase, therefore blocking proliferation, and inducing apoptosis [14]. Nevertheless, it is important to explore the molecular pathways that are deregulated behind these phenotypic effects. Moreover, one of the main challenges in the clinical setting of drug development and implementation is its cost-effectiveness. The application of a biomarker-driven patient sub selection strategy can help reduce costs and improve success rate and the screening of potential biomarkers can start in a preclinical setting [9]. Therefore, the establishment of a biomarker profile of therapy response in the early stages, can help fine tune the whole process of drug development. microRNA (miRNA) have been shown to be one of the major biomarker candidates for drug response studies in the past years, due to their stability and easy quantification in tissues and body fluids [15].

5. Material and Methods

Aim of the study

The present study aims to define a miRNA profile of cellular response to AgNPs and G-AgNPs, using the PCa cell lines PC-3 and Du-145, that can also provide insights regarding the molecular pathways of AgNPs and G-AgNPs action. Moreover, we also intend to explore their biocompatibility, studying their cytotoxic effect in a liver cell line.

Cell Culture and treatments conditions

PC-3 cells (ATCC® CRL-1435™ RRID:CVCL_0035) were obtained from ATCC. Du-145 (ACC®HTB-81 187 RRID:CVCL_0105) and HepG-2 cell lines were kindly granted by Professor Cármen Jerónimo from IPO-Porto Research Center (Portugal) and the Basic and Clinical Research on Iron Biology Group from I3S, respectively. PC-3 and Du-145 cells were kept in culture in RPMI-1640 medium (Sigma-Aldrich®) while HepG-2 was kept in culture in DMEM medium (Sigma-Aldrich®), both supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator at 37°C, 5% CO₂ and 95% humidity. Cells were grown in tissue culture flasks and, trypsinized at 80-90% confluence using trypsin-EDTA 0.05% (Gibco®, Gaithersburg, MD, USA), counted using trypan blue (VWR™) and an automatic cell counter (EVETM – NanoEntek). All cells were weekly tested for mycoplasma presence and were found to be free from contamination.

Synthesis of AgNPs

AgNPs and G-AgNPs were synthesized and characterized as described by Morais *et al* [14]. Briefly, 100 mL of an aqueous solution of sodium citrate (5 mM) was mixed with tannic acid (5 mM) and heated at 100 °C, for 15 min under vigorous stirring. After boiling, 1 mL of AgNO₃ (25 mM) was added. AgNPs were purified through centrifugation at 20,000 g for 15 min and redispersed in MilliQ (MQ) water. AgNPs were then functionalized: 1mg was redispersed with 2 mL of 2 mM sodium citrate containing 5 mM glucosamine hydrochloride and stirred for 24h at room temperature. The functionalized G-AgNPs were washed, redispersed in MQ-water, and stored at 4 °C.

HepG-2 toxicity evaluation - Resazurin Assay

HepG-2 has been described as a good predictor of liver toxicity [16, 17]. HepG-2 cells were plated in 96well-plates at a concentration of 2.0×10^5 cells/mL at a final volume of 100µL. AgNPs and G-AgNPs were administrated at the determined IC₅₀ of PC-3 and Du-145 cells to the HepG-2 cell line for 24 hours, to assess their biocompatibility [14]. After, resazurin Sodium Salt (ACROS Organic™ – Fisher Scientific®, MA, USA) was used to assess cells' viability.

RNA extraction

Du-145 and PC-3 cells were plated in 6-well plates at 2.0×10^5 cells/mL at a final volume of 1.5 mL. AgNPs and G-AgNPs were added to the cells at the determined IC_{50} [14]. After 24 hours incubation, cells were trypsinized and centrifuged to form a pellet. MiRNA isolation and purification was done using the Plasma/Serum RNA Purification Mini Kit from NORGEN (Norgen Biotek Corporation, Thorold, ON, Canada) according to the manufacturer instructions. RNA concentration and purity were measured using the NanoDrop Lite spectrophotometer (Thermo Scientific®, Waltham, MA, USA).

miRNA profiling through NanoString Analysis

The miRNA profiling of the two cell lines with and without AgNPs and G-AgNPs stimulus was performed using the nCounter® Human v3 miRNA expression assay (NanoString Technologies), using an input of 100 ng of sample, according to manufacturer instructions. Data was analyzed by ROSALIND® (<https://rosalind.bio/>), with a HyperScale architecture developed by ROSALIND, Inc. (San Diego, CA). Read Distribution percentages, violin plots, identity heatmaps, and sample MDS plots were generated as part of the QC step. The criteria given by Nanostring (nSolver 4.0 Analysis Software User Manual) was used to calculate normalization, fold changes and p-values. After background subtraction based on POS_A probe correction factors, normalization was performed in two steps: positive control normalization and codeset normalization. During both steps the geometric mean of each probeset is used to create a normalization factor. Differential expression of the PC-3 and Du-145 cells without and with incubation of AgNPs or G-AgNPs was then assessed using the t-test method. P-value adjustment is performed using the Benjamini-Hochberg method of estimating false discovery rates (FDR) and miRNAs were classified as differentially expressed according to a fold-change <-10 or >10 and $p < 0.001$. Clustering of miRNA for the final heatmap of differentially expressed miRNA was done using the PAM (Partitioning Around Medoids) method using the fpc R library (Hennig, C. Cran-package fpc. <https://cran.r-project.org/web/packages/fpc/index.html>) that takes into consideration the direction and type of all signals on a pathway, the position, role and type of every miRNA, etc. The top targeted validated genes were analyzed using the multiMiR R library [18, 19].

***In silico* analysis**

miRTarBase (version 9), the largest known online database of validated miRNA:mRNA target interactions, was used to establish a network of the validated target mRNAs of the top deregulated miRNAs [20]. The Search Tool for the Retrieval of Interacting

Genes (STRINGapp) in the Cytoscape software (v3.9.1.) was used to build the protein interaction relationship of the selected target genes encoding proteins, considering as significant the ones with a combined score of >0.4 . To better understand the resulting protein protein interaction (PPI) network, proteins were grouped according to their cellular compartment location using the compartment filters of cytoscape focusing on three different categories: lysosome, plasma membrane and endosome. Following, the functional enrichment analysis of GO, Reactome, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Compartments was performed with the enrichment analysis tool of the STRINGapp, with a False Discovery Rate (FDR) of $p < 0.01$ as significance threshold.

6. Results

6.1. AgNPs and G-AgNPs show low cytotoxicity against HepG-2 cell line

As a first screening of liver toxicity of AgNPs and G-AgNPs, HepG-2 cell line was used. The previously determined IC_{50} of PC-3 for AgNPs ($911\mu\text{g/mL}$) and G-AgNPs ($870\mu\text{g/mL}$) and of Du-145 for AgNPs ($1579\mu\text{g/mL}$) and G-AgNPs ($1528\mu\text{g/mL}$) by Morais and co-workers, were the administrated concentrations to the HepG-2 cells [14]. It is possible to observe that, in the HepG-2 cells, the AgNPs at a concentration of $911\mu\text{g/mL}$ reduced the viability of cells to 85.20% against the 50% observed in PC-3 cells. Similarly, G-AgNPs at a concentration of $870\mu\text{g/mL}$ reduced the viability of cells to 80.86% against the 50% in PC-3 cells. Comparing to the 50% of viability in the Du-145 cell line, AgNPs at a concentration of $1579\mu\text{g/mL}$ reduced the viability of cells to 73.68% in HepG-2 cells and G-AgNPs at a concentration of $1528\mu\text{g/mL}$ reduces the viability of cells to 81.61% (Figure 1).

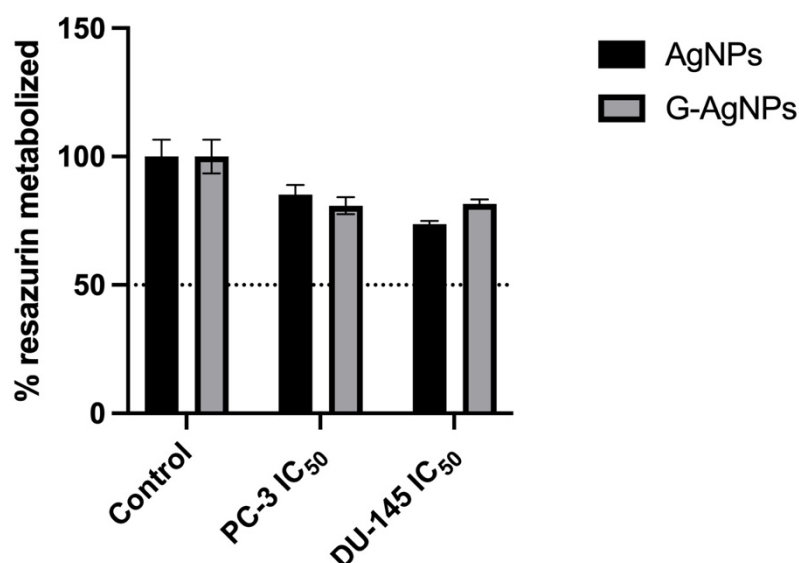


Figure 1 - Evaluation of cell viability in the HepG-2 cell line, by Resazurin Assay, upon treatment with the determined IC₅₀ of AgNPs and G-AgNPs of Du-145 and PC-3 cells. Results are expressed as a percentage of control (untreated cells), as mean $\pm$ SEM.

6.2. AgNPs and G-AgNPs deregulate the miRNA and gene expression pattern in PC-3 and Du-145 cell lines

To evaluate the impact of AgNPs or G-AgNPs administration to PC-3 and Du-145 cells, Nanostring® technology through the nCounter® Human v3 miRNA expression assay was used. After administration of AgNPs, PC-3 cells displayed 312 upregulated miRNAs and 2 downregulated miRNAs (Figure 2-A, Supplementary Table 1-2). After administration of G-AgNPs, PC-3 cells displayed 294 upregulated miRNAs and 3 downregulated miRNAs (Figure 2-C, Supplementary Table 1-2). Regarding Du-145, after administration of AgNPs, they displayed 156 upregulated miRNAs and 2 downregulated miRNAs (Figure 2-E, Supplementary Table 1-2) and after administration of G-AgNPs, they displayed 241 upregulated miRNAs and 2 downregulated miRNAs (Figure 2-G, Supplementary Table 1-2).

Through the multiMiR R library, the targeted validated genes were identified, and the 20 genes targeted by the higher number of deregulated miRNAs were selected (Figure 2-B, D, F and H). Interestingly, most of the top 20 targeted validated genes are common to the different miRNA panels obtained. Indeed, 11 genes (BTG2, CCND1, CDK6, CDKN1A, GIGYF1, KLHL15, MDM2, NUFIP2, SRRM2, TAOK1 and ZNF264) are common to the four panels of deregulated miRNAs, 5 genes to three of the panels (MCL1, WEE1, CELF1, TNRC6A and TNRC6A) and 3 to two of them (CCND2, ZNF460 and SOD2) and 14 exclusive to one panel (AHNAK, HNRNPU, LRRC58, SOX4, XIAP, MAP1B, MKNK2,

CDKN1B, IGF1R, TXNIP, DICER1, NACC1, SRCAP, TNPO1). Using all these 33 genes, an *in silico* analysis was made with the STRINGapp from Cytoscape. A total of 33 protein coding genes were filtered into a protein-protein interaction (PPI) network with 33 nodes and 73 edges that presented significant enrichment ($p = 1 \times 10^{-16}$). To further understand this protein cluster impact, a functional enrichment analysis was performed with an FDR threshold of 1%, which resulted in a total of 200 functional enriched terms among the Reactome, KEGG and GO categories (Supplementary tables 3). The top 20 enriched terms for each category are represented on Figure 2-I-N).

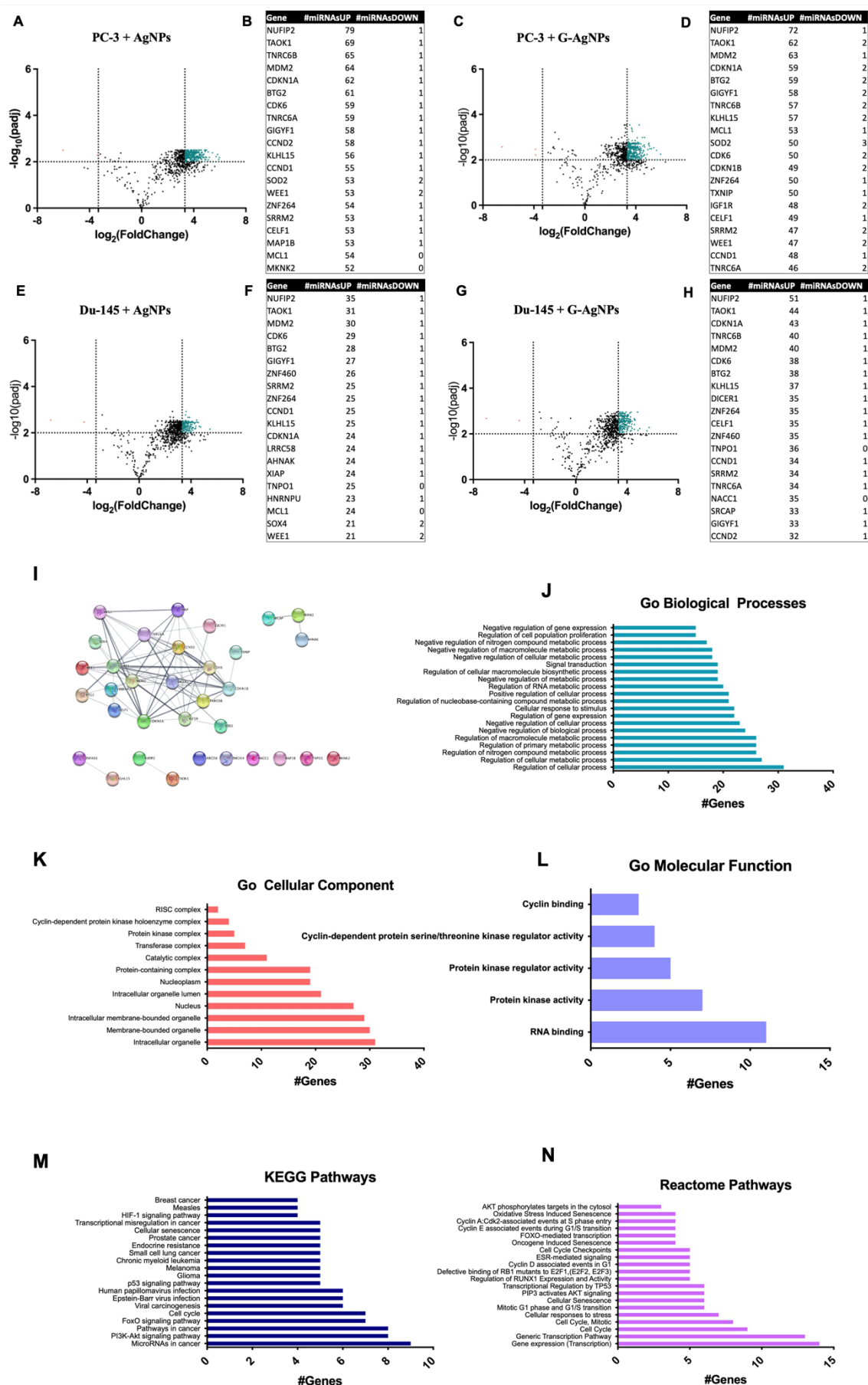


Figure 2 – Volcano plots of the differentially expressed miRNAs when comparing PC-3 before and after administration of AgNPs (A) or G-AgNPs (C) and Du-145 cells before and after administration of AgNPs (E) or G-AgNPs (G). The y-axis indicates the Log10 of the p values and the x-axis is the Log2 transformed ratio of the expression between the experimental groups; Top 20 targeted validated genes and the number of upregulated and downregulated miRNAs that target them (B, D, F, H). Each Top20 refers to the volcano plot on its left. Analysis was performed using the multiMiR R library; PPI network of the 33 proteins derived from the top targeted validated genes performed using Cytoscape (I). GO Biological Processes (J), Cellular Compartments (K) and Molecular Function (L), KEGG Pathways (M) and Reactome Pathways (N) analysis of the 33 selected genes. The functional enrichment analysis was made with the STRINGapp from Cytoscape.

Among the GO terms, we can observe that terms are mostly related to cellular metabolic processes, negative regulation of gene expression and signal transduction, cellular organelles complexes such as RISC Complex (involved in miRNAs biogenesis) and the activity of protein kinases, and cyclin binding. Among the functionally enriched terms in the Reactome and KEGG pathways, we can find HIF-1, p53, PI3K-Akt, FOXO, Cyclins, RB1 mutants, ESR and RUNX1 as well as regulation of cell cycle, oxidative stress induced senescence and prostate cancer.

6.3. Narrowing of the microRNA profile related with PC-3 and Du-145 response to AgNPs and G-AgNPs and insights on the modulated molecular pathways

To further explore the biomarker potential of the deregulated miRNAs, the 10 most upregulated (with highest Log10(p-value) and Log2(Fold-Change) values) and the 2 or 3 downregulated miRNAs were selected from each volcano plot. After retrieval of all validated target genes of these miRNAs, a PPI network was performed for each downregulated and upregulated miRNA profile.

Interestingly, the downregulated miRNAs (let7i-5p and hsa-miR-1283) were common between Du-145's response to AgNPs, G-AgNPs and PC-3's response to AgNPs (Supplementary Table 1). From the 15 targeted genes, an *in-silico* analysis was performed with the STRINGapp from Cytoscape. A total of 15 protein coding genes were filtered into a protein-protein interaction (PPI) network, after excluding 3 singletons, with 12 nodes and 19 edges that presented significant enrichment ($p = 3.36 \times 10^{-5}$) (ASCL1, BMP4, COPS6, COPS8, GPS1, IGF1, IL2, IL13, NEUROG1, NTRK3, SOCS1, TLR4; Figure 3-A). To further understand this protein cluster impact, a functional enrichment analysis was performed with an FDR threshold of 1%, which resulted in a total of 239 functional enriched terms among the Reactome, KEGG and GO categories (Figure 3- B-E; Supplementary table 4). Among

the GO terms, we can observe that terms are mostly related to cellular metabolic processes, regulation of gene expression and signal transduction, regulation of response to stimulus and COP9 signalosome. Among the functionally enriched terms in the Reactome and KEGG pathways, we can find PI3K-Akt, JAK-STAT, TC-NER and GG-NER.

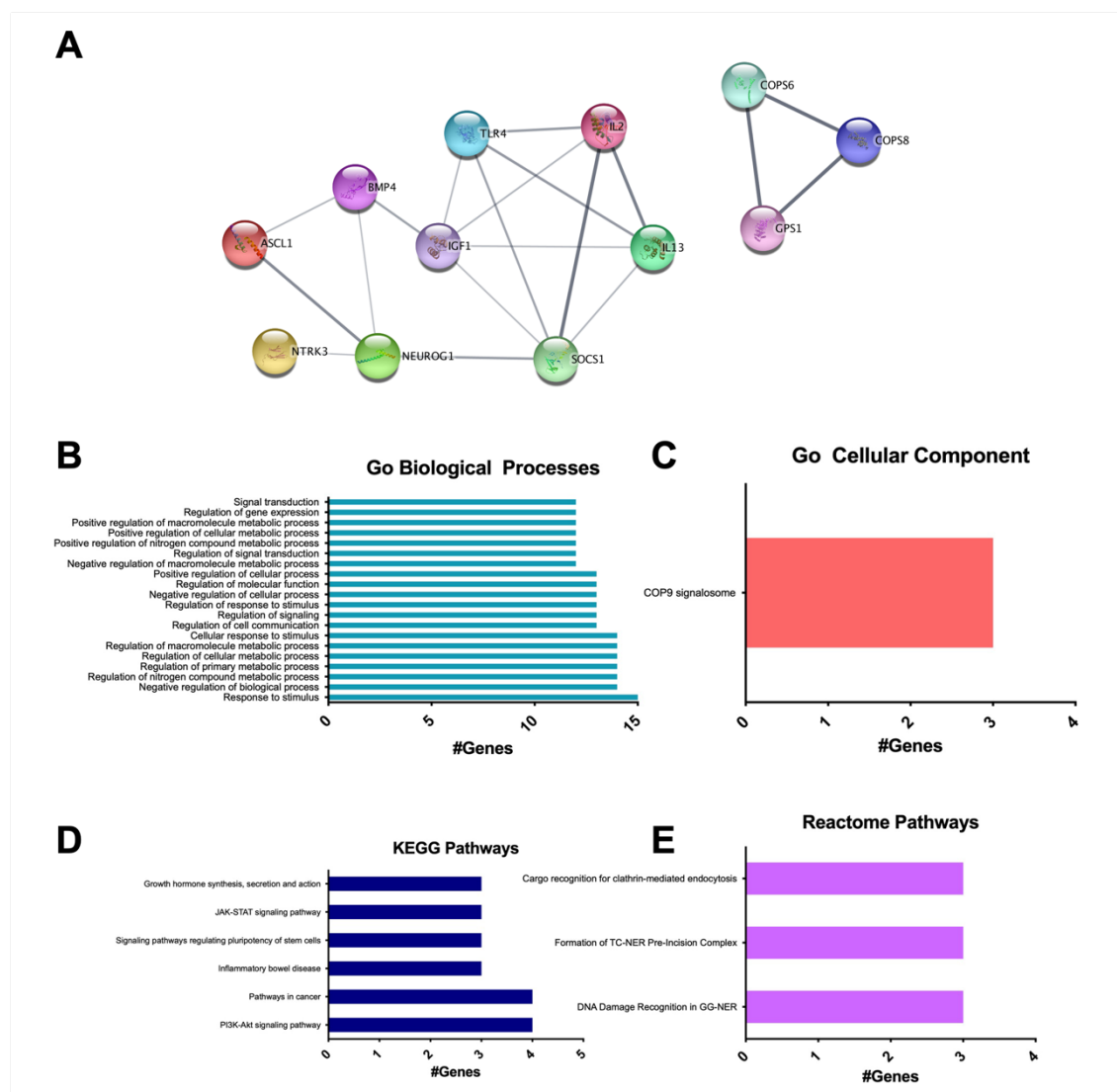
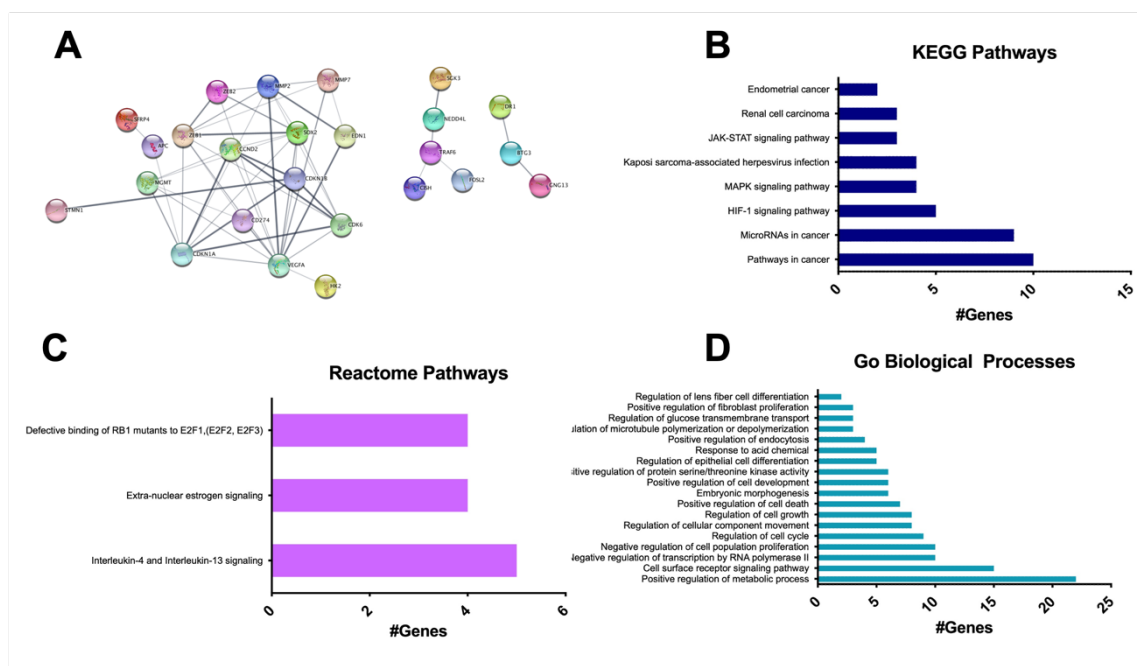


Figure 3 - PPI network of the 15 proteins derived from the top targeted validated genes of the downregulated miRNAs in Du-145 cells after AgNPs and G-AgNPs administration and PC-3 cells after AgNPs administration, using Cytoscape (A). GO Biological Processes (B), Cellular Compartments (C), KEGG Pathways (D) and Reactome Pathways (E) analysis of the 15 selected genes. The functional enrichment analysis was made with the STRINGapp from Cytoscape.

Using the 10 most upregulated miRNAs of Du-145's response to AgNPs (hsa-miR-590-3p, hsa-miR-1228-3p, hsa-miR-648, hsa-miR-5001-3p, hsa-miR-513a-5p, hsa-miR-548g-3p, hsa-miR-1200, hsa-miR-1272, hsa-miR-203a-5p, hsa-miR-572) (Figure 4) a total of 35 genes were retrieved. An *in silico* analysis was performed with the STRINGapp from Cytoscape and a total of 35 protein coding genes were filtered into a protein-protein



Using the 10 most upregulated miRNAs (hsa-miR-1-3p, hsa-miR-1200, hsa-miR-187-3p, hsa-miR-548g-3p, hsa-miR-495-5p, hsa-miR-1272, hsa-miR-675-5p, hsa-miR-203a-5p, hsa-miR-597-5p, hsa-miR-572) of Du-145's response to G- AgNPs (Figure 5) a total of 130 genes were retrieved. An *in silico* analysis was performed with the STRINGapp from Cytoscape and a total of 130 protein coding genes were filtered into a protein-protein

interaction (PPI) network with 130 nodes and 699 edges that presented significant enrichment ($p = 1.0 \times 10^{-16}$) (Figure 5-A). To further understand this protein cluster impact, a functional enrichment analysis was performed with an FDR threshold of 1% and the redundant terms were eliminated using a redundancy cutoff of 0.5, which resulted in a total of 1875 functional enriched terms among the Reactome, KEGG and GO categories (Figure 5-B-F, Supplementary table 6). Among the GO terms, we can observe that terms are mostly related to cellular metabolic processes, response to stimulus, different cellular complexes and cellular organelles such as cell surface, macropinosome and chromosome, histone, protein and kinase binding. Among the functionally enriched terms in the Reactome and KEGG pathways, we can find PI3K-Akt, FGFR, NTRK1, NOTCH, MAPK, RUNX2, mTOR, FoxO, p53, WNT, AGE-RAGE, immune factors such as interleukins, proteoglycans, receptor tyrosine kinases and microRNAs in cancer.

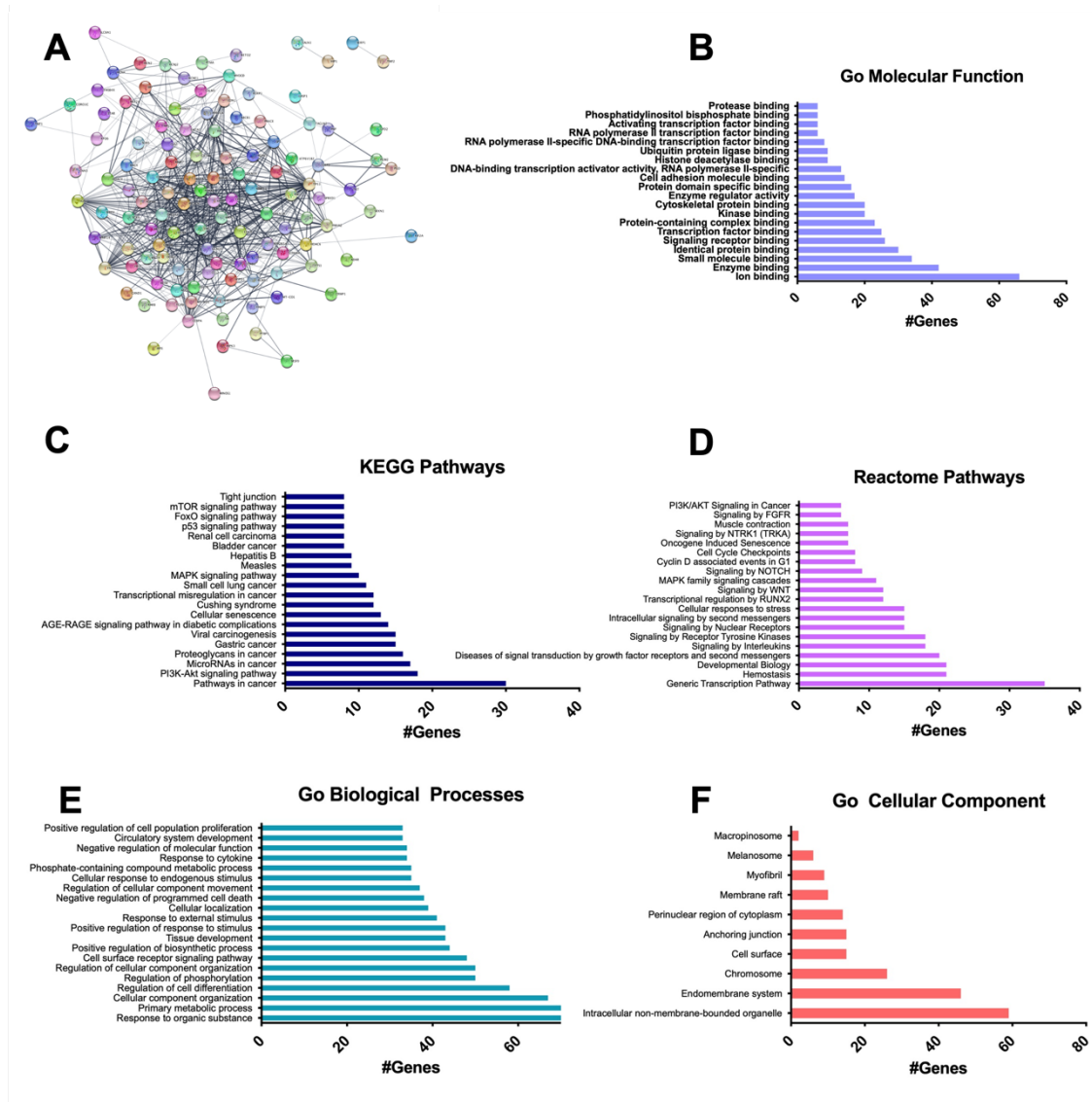


Figure 5 - PPI network of the 130 proteins derived from the top targeted validated genes of the upregulated miRNAs in Du-145 cells after AgNPs administration performed using Cytoscape (A). GO Molecular Function (B), KEGG Pathways (C) and Reactome Pathways (D), GO Biological Processes (E) and GO Cellular Component (F) analysis of the 130 selected genes. The functional enrichment analysis was made with the STRINGapp from Cytoscape.

Using the 10 most upregulated miRNAs (hsa-miR-494-5p, hsa-miR-518e-3p, hsa-miR-203a-5p, hsa-miR-517c-3p+hsa-miR-519a-3p, hsa-miR-637, hsa-miR-675-5p, hsa-miR-1228-3p, hsa-miR-572, hsa-miR-525-3p, hsa-miR-597-5p) of PC-3's response to AgNPs (Figure 6) a total of 52 genes were retrieved. An *in silico* analysis was performed with the STRINGapp from Cytoscape and a total of 52 protein coding genes were filtered into a protein-protein interaction (PPI) network with 52 nodes and 197 edges that presented significant enrichment ($p = 1.0 \times 10^{-16}$) (Figure 6-A). To further understand this protein cluster

impact, a functional enrichment analysis was performed with an FDR threshold of 1% and the redundant terms were eliminated using a redundancy cutoff of 0.5, which resulted in a total of 266 functional enriched terms among the Reactome, KEGG and GO categories (Figure 6-B-F, Supplementary table 7). Among the GO terms, we can observe that terms are mostly related to cellular metabolic processes, response to stimulus and membrane organization, nuclear body, cell junction, and cyclin, signaling receptor and protein binding. Among the functionally enriched terms in the Reactome and KEGG pathways, we can find RUNX2 and RUNX3, NOTCH, HIF1, FOXO, growth hormone receptor signaling, inhibition of replication initiation of damaged DNA, transcription of gene expression, Hedgehog signaling pathway, immune factors such as interleukins, microRNAs in cancer and pathways in cancer.

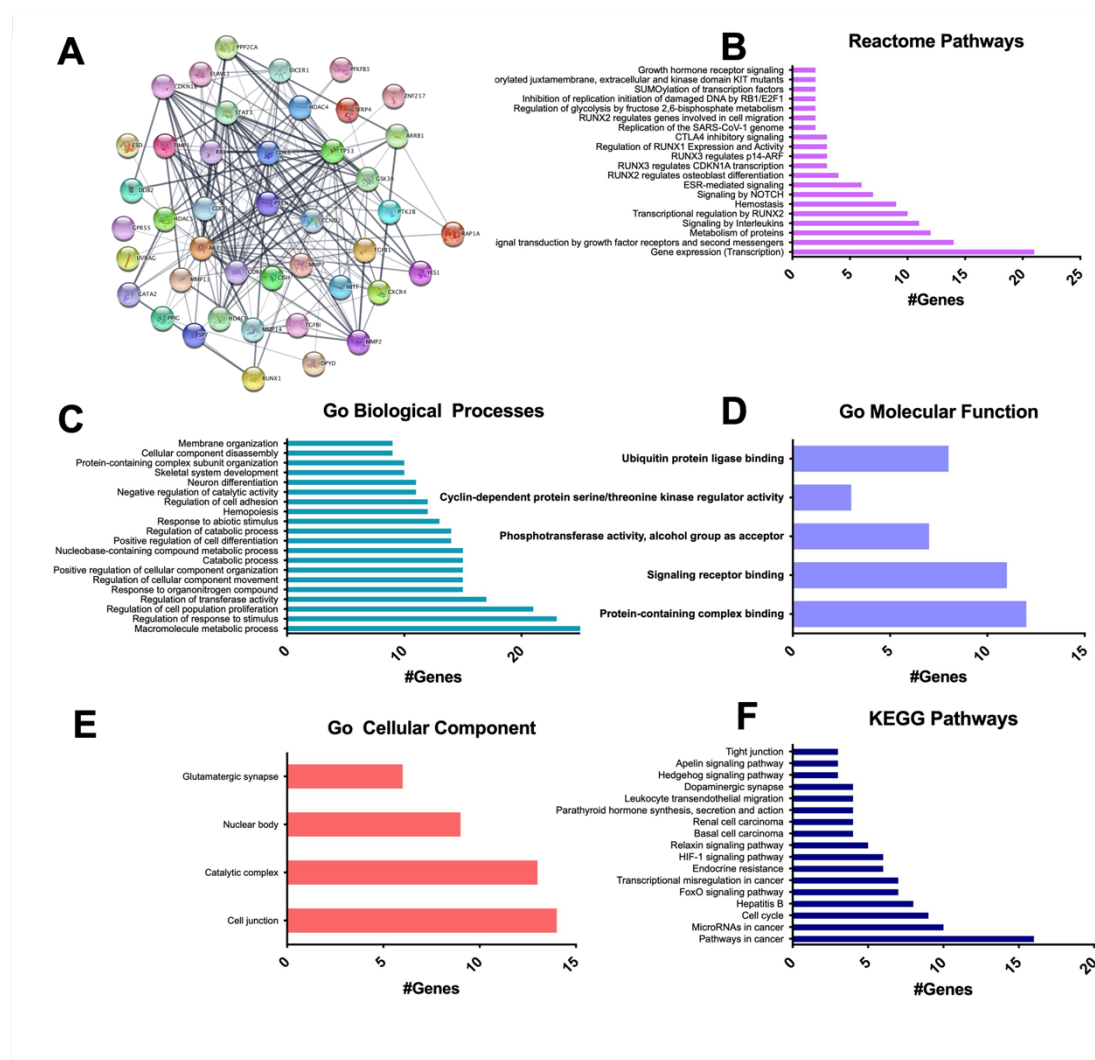


Figure 6 – PPI network of the 52 proteins derived from the top targeted validated genes of the upregulated miRNAs in PC-3 cells after AgNPs administration performed using Cytoscape (A). Reactome Pathways (B), GO Biological Processes (C) and GO Molecular Function (D), GO

Cellular Component (E) and KEGG Pathways (F) analysis of the 52 selected genes. The functional enrichment analysis was made with the STRINGapp from Cytoscape.

Using the 3 downregulated miRNAs (let7i-5p, has-miR125b-5p and hsa-miR-1283) of PC-3's response to G-AgNPs (Figure 7) a total of 142 genes were retrieved. An *in silico* analysis was performed with the STRINGapp from Cytoscape and a total of 142 protein coding genes were filtered into a protein-protein interaction (PPI) network with 142 nodes and 838 edges that presented significant enrichment ($p = 1.0 \times 10^{-16}$) followed by a Markov clustering (MCL), using the MCL algorithm in the clusterMaker2 Cytoscape app and an inflation value of 4.0. (Figure 7-A). To further understand this protein cluster impact, a functional enrichment analysis was performed with an FDR threshold of 1%, which resulted in a total of 1007 functional enriched terms among the Reactome, KEGG and GO categories (Figure 5-B-F, Supplementary table 8). Among the GO terms, we can observe that terms are mostly related to cellular metabolic processes, cellular response to stimulus, different cellular complexes and cellular organelles such as plasma membrane, cytosol, and nucleus, DNA, RNA and protein binding. Among the functionally enriched terms in the Reactome and KEGG pathways, we can find p53, Cyclins, oxidative stress, cytochrome c, immune factors such as cytokines and interleukins, cellular senescence and apoptosis, proteoglycans and microRNAs in cancer.

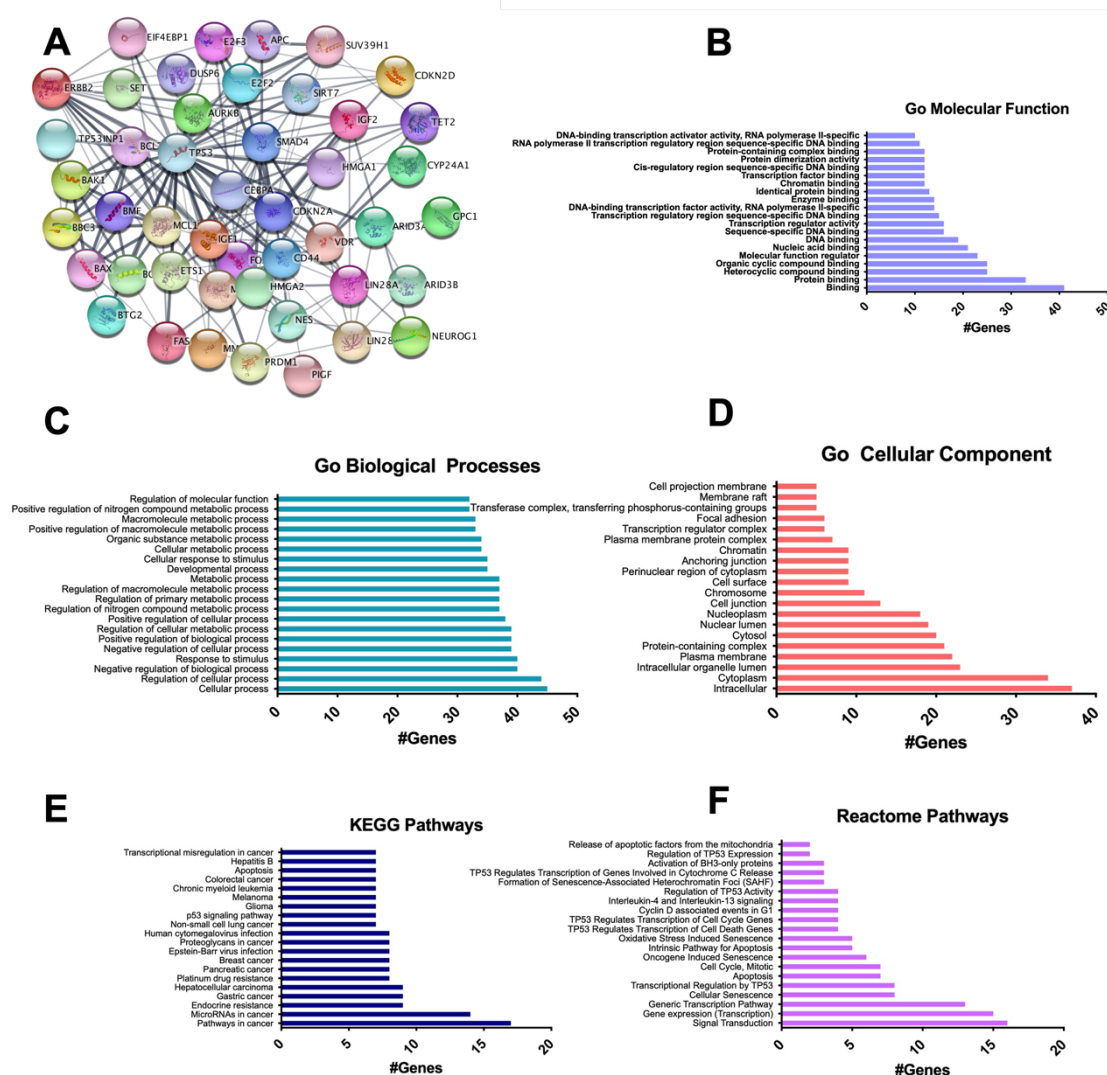


Figure 7 - PPI network of the 142 proteins derived from the top targeted validated genes of the downregulated miRNAs in PC-3 cells after G-AgNPs administration performed using Cytoscape (A). GO Biological Processes (B), Cellular Compartments (C) and Molecular Function (D), KEGG Pathways (E) and Reactome Pathways (F) analysis of the 142 selected genes. The functional enrichment analysis was made with the STRINGapp from Cytoscape.

Finally, using the 10 upregulated miRNAs (hsa-miR-208b-5p, hsa-miR-1248, hsa-miR-518e-3p, hsa-miR-3605-5p, hsa-miR-133b, hsa-miR-203a-5p, hsa-miR-572, hsa-miR-675-5p, hsa-miR-1228-3p, hsa-miR-597-5p) of PC-3's response to G-AgNPs (Figure 8) a total of 80 genes were retrieved. An *in silico* analysis was performed with the STRINGapp from Cytoscape and a total of 80 protein coding genes were filtered into a protein-protein interaction (PPI) network with 80 nodes and 394 edges that presented significant enrichment ($p = 1.0 \times 10^{-16}$). To further understand this protein cluster impact, a functional

enrichment analysis was performed with an FDR threshold of 1% and the redundant terms were eliminated using a redundancy cutoff of 0.5, which resulted in a total of 543 functional enriched terms among the Reactome, KEGG and GO categories (Figure 8-B-F, Supplementary table 9). Among the GO terms, we can observe that terms are mostly related to cellular metabolic processes, cellular response to stimulus and regulation of cell differentiation and proliferation, cellular complexes such as transcription repressor complex and catalytic complex, protein binding, kinase binding and histone binding. Among the functionally enriched terms in the Reactome and KEGG pathways, we can find cAMP, JAK-STAT, Hippo, FoxO, PI3K-AKT, RUNX1, RUNX2, PTK6, NOTCH, TP53, ESR, immune factors such as interleukins, cellular response to stress, glycolysis, proteoglycans, microRNAs in cancer and prostate cancer.

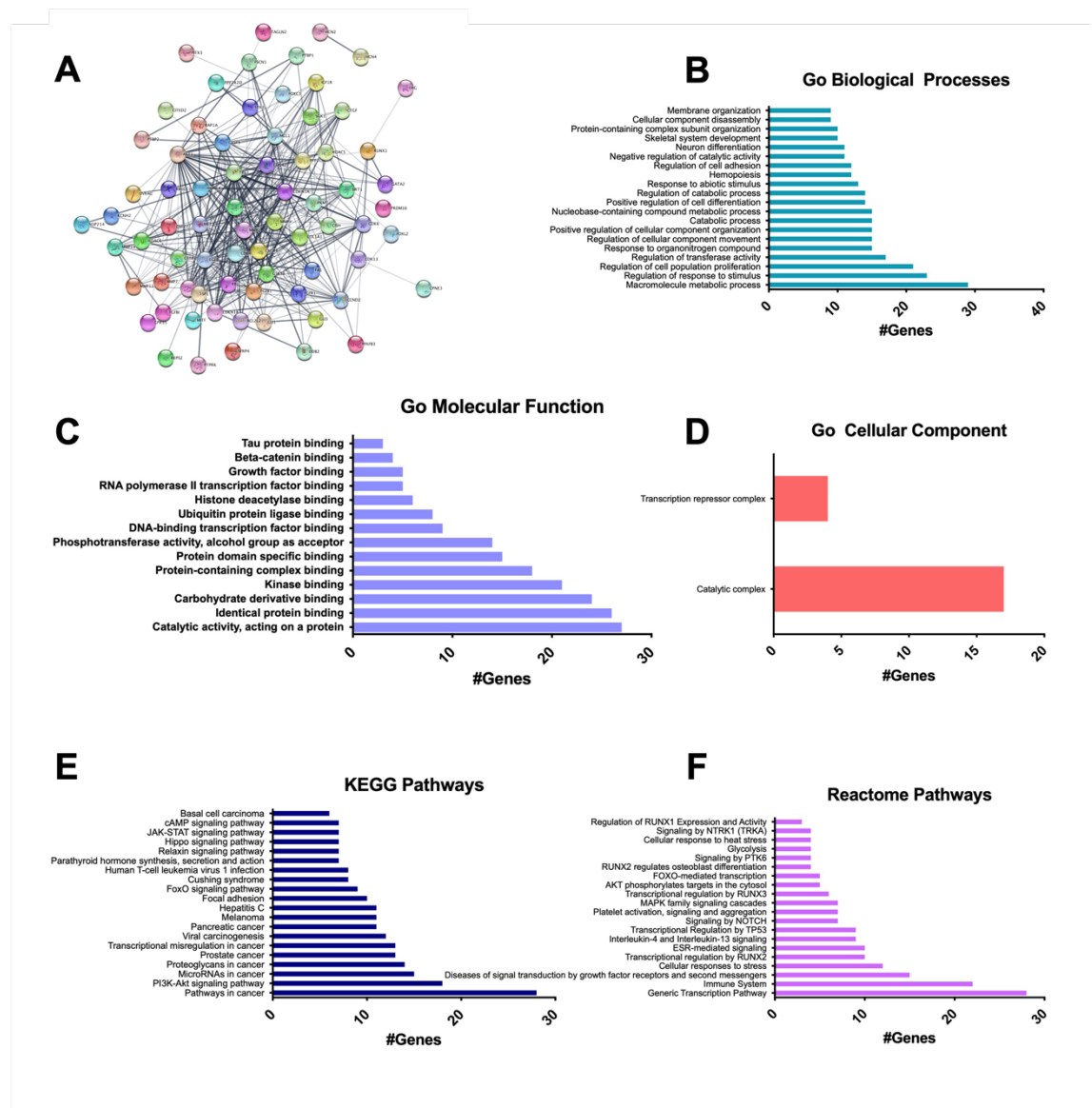


Figure 8 - PPI network of the 80 proteins derived from the top targeted validated genes of the upregulated miRNAs in PC-3 cells after G-AgNPs administration performed using

Cytoscape (A), GO Biological Processes (B), Molecular Function (C) and Cellular Component (D), KEGG Pathways (E) and Reactome Pathways (F) analysis of the 80 selected genes. The functional enrichment analysis was made with the STRINGapp from Cytoscape.

6.4. miRNA biomarker profile associated AgNPs and G-AgNPs' response in CRPC cells

To define a miRNA profile with potential as biomarker of response to AgNPs and G-AgNPs, we sought the common downregulated and upregulated miRNAs found in both cell lines submitted to AgNPs and G-AgNPs (Table 1).

Therefore, we report 2 downregulated miRNAs: hsa-miR-1283 and hsa-let-7i-5p. Moreover, we report 92 upregulated miRNAs, among which 16 are present in the top 10 most upregulated miRNAs of at least one of the conditions (hsa-miR-1-3p, hsa-miR-1200, hsa-miR-1228-3p, hsa-miR-1272, hsa-miR-187-3p, hsa-miR-203a-5p, hsa-miR-494-5p, hsa-miR-495-5p, hsa-miR-517c-3p, hsa-miR-519a-3p, hsa-miR-518e-3p, hsa-miR548g-3p, hsa-miR-572, hsa-miR-590-3p, hsa-miR-637 and hsa-miR-648). Thus, the proposed panel should include hsa-miR-1283 and hsa-let-7i-5p (downregulated) and hsa-miR-1-3p, hsa-miR-1200, hsa-miR-1228-3p, hsa-miR-1272, hsa-miR-187-3p, hsa-miR-203a-5p, hsa-miR-494-5p, hsa-miR-495-5p, hsa-miR-517c-3p, hsa-miR-519a-3p, hsa-miR-518e-3p, hsa-miR548g-3p, hsa-miR-572, hsa-miR-590-3p, hsa-miR-637 and hsa-miR-648 (upregulated).

Table 1 – Deregulated miRNAs common to the different conditions tested

Downregulated miRNAs			
hsa-miR-1283	hsa-let-7i-5p		
Upregulated miRNAs			
hsa-miR-1-3p	hsa-miR-301a-5p	hsa-miR-492	hsa-miR-585-3p
hsa-miR-1185-2-3p	hsa-miR-3168	hsa-miR-494-5p	hsa-miR-590-3p
hsa-miR-1185-5p	hsa-miR-3182	hsa-miR-495-5p	hsa-miR-607
hsa-miR-1200	hsa-miR-3185	hsa-miR-499a-5p	hsa-miR-612
hsa-miR-1228-3p	hsa-miR-328-5p	hsa-miR-5001-5p	hsa-miR-626
hsa-miR-1272	hsa-miR-337-3p	hsa-miR-5010-3p	hsa-miR-637
hsa-miR-1275	hsa-miR-338-5p	hsa-miR-510-3p	hsa-miR-639
hsa-miR-1281	hsa-miR-33b-5p	hsa-miR-512-3p	hsa-miR-643
hsa-miR-1295a	hsa-miR-345-3p	hsa-miR-512-5p	hsa-miR-648
hsa-miR-1302	hsa-miR-3614-5p	hsa-miR-513a-3p	hsa-miR-650
hsa-miR-1323	hsa-miR-363-3p	hsa-miR-516b-5p	hsa-miR-761
hsa-miR-133a-3p	hsa-miR-369-5p	hsa-miR-517c-3p+hsa-miR-519a-3p	hsa-miR-766-5p
hsa-miR-151b	hsa-miR-378d	hsa-miR-518e-3p	hsa-miR-767-5p
hsa-miR-182-3p	hsa-miR-381-5p	hsa-miR-522-3p	hsa-miR-802
hsa-miR-184	hsa-miR-433-5p	hsa-miR-532-3p	hsa-miR-873-3p
hsa-miR-187-3p	hsa-miR-4421	hsa-miR-548g-3p	hsa-miR-876-5p

hsa-miR-1910-3p	hsa-miR-4488	hsa-miR-548o-3p+hsa-miR-548ah-3p+hsa-miR-548av-3p	hsa-miR-887-5p
hsa-miR-197-5p	hsa-miR-449b-5p	hsa-miR-548v	hsa-miR-888-5p
hsa-miR-199a-3p+hsa-miR-199b-3p	hsa-miR-450a-2-3p	hsa-miR-553	hsa-miR-889-3p
hsa-miR-203a-5p	hsa-miR-4647	hsa-miR-556-3p	hsa-miR-891a-5p
hsa-miR-211-5p	hsa-miR-4787-3p	hsa-miR-572	hsa-miR-891b
hsa-miR-297	hsa-miR-487a-3p	hsa-miR-573	hsa-miR-922
hsa-miR-299-3p	hsa-miR-487b-5p	hsa-miR-579-5p	hsa-miR-924

Discussion

The lack of therapeutic options with a curative intent and the high side effects of the ones available are important issues associated with CRPC aggressiveness and patients' management [5]. Since CRPC is characterized by its resistance to ADT, there is an urgent need for new therapeutic strategies that explore other targets besides the androgen dependence of PCa cells [8]. Nanomedicine has been showing promising results in different tumor models, including PCa, where G-AgNPs have been shown cytotoxic against PC-3 and Du-145 cells, both CRPC cell models[14]. Nevertheless, when considering the development of a new therapeutic strategy, there are several aspects to be taken into consideration, such as their biocompatibility, the molecular pathways in which they intervene and if there are possible ways to evaluate cells' response to them, in order to select the patients that will benefit the most and monitor their response [9].

In the present study we assessed the cytotoxic capacity of both AgNPs and G-AgNPs against HepG2. Being initially classified as human hepatoma cells, they have later been classified as hepatoblastoma and are highly used in different studies on liver functions and hepatotoxicity[21]. In fact, they are highly capable of proliferation but, in spite of their cancer origin, they keep several features of differentiated hepatocytes such as albumin secretion, insulin-stimulated glycogen synthesis and glutathione-based detoxification [22]. Indeed, Giráldez-Pérez and collaborators have already used HepG2 to screen for NPs biocompatibility [23]. The liver is the first-step metabolic site, being particularly vulnerable to nanoparticles toxicity and it has been shown to accumulate administrated substances after cessation of exposure, making the evaluation of hepatocellular toxicity of uttermost importance [24]. With 73-85% viable cells after administration of AgNPs and G-AgNPs at IC50 doses of PC-3 and Du-145, HepG2 seem to be much less sensitive to the NPs than CRPC cells, which is a good first indication of their biocompatibility. Despite, our results be good indicators of AgNPs effect on CRPC, it would be important to study aspects related to

lung, dermal and neurotoxicity as well as perform *in vivo* studies that would help to ensure the NPs biocompatibility [24].

Regarding the PCa cells response to NPs treatment, we used a miRNA wide-screening approach (799 miRNAs) to define a miRNA profile that can assess cells' response to AgNPs and G-AgNPs. In this first screening, we showed that there was a consistent and common downregulation of two miRNAs among both cell lines and AgNPs and G-AgNPs. On the other hand, there were 156 and 241 upregulated miRNAs in Du-145 cells' response to AgNPs and G-AgNPs, respectively and 312 and 294 upregulated miRNAs in PC-3 cells' response to G-AgNPs, respectively. Interestingly, there was also a high overlap of upregulated miRNAs among cell lines and AgNPs and G-AgNPs treatments. This overlap indicates a common feature in the effect of AgNPs and G-AgNPs in both cell lines that deregulates miRNAs' expression pattern. ROS have been shown to interfere in miRNAs biogenesis and function through epigenetic mechanisms (such as deregulation of methylation and histones pattern), regulation of ROS-responsive transcription factors that can activate miRNA transcription and deregulation of miRNAs processing machinery [25]. In fact, we have previously observed an increase of ROS production after administration of AgNPs and G-AgNPs [14]. Therefore, we hypothesize that this increase of ROS production is one of the main responsible for this deregulation.

Moreover, when looking at the top deregulated genes (meaning the genes regulated by more deregulated miRNAs), we observed that they were mostly the same among cell lines and treatments. All this suggests that both AgNPs and G-AgNPs seem to act in the same way in both cell lines. Therefore, the difference of effect observed in the past may be due to a higher uptake due to the high glucose demands of the cells, and consequently, more efficacy of G-AgNPs [14, 26].

To further explore the pathways involved in cells response to AgNPs and G-AgNPs, we selected the top 10 upregulated and the downregulated miRNAs in each cell line for AgNPs and G-AgNPs to do an *in silico* analysis of all the deregulated genes of each miRNA. Interestingly, the molecular pathways and the more significative proteins and terms were consistent to the previous analysis and between cell lines and conditions. One of the main mechanisms of action of AgNPs is the induction of ROS, which will induce oxidative stress in the cell and trigger most of the cell damage observed [12]. The *in silico* analysis has revealed several terms regarding "oxidative stress" and "cytochrome c" which plays an important role in the response of cells to ROS [27, 28]. Moreover, ROS triggers the release of cytokines and interleukines to the microenvironment [29, 30]. The increase of ROS may indeed be responsible for the nuclear damage observed upon administration of AgNPs to cells [12]. The *in silico* analysis pointed out terms such as "chromosome" and "histones". Genotoxicity such as chromosomal instability has been widely reported in AgNPs studies

[31]. Moreover, “inhibition of replication initiation of damaged DNA” as well as the pathways involved in DNA repair “TC-NER” and “GG-NER” have also been pointed out in our analysis, further showing the occurrence of DNA damage through AgNPs and G-AgNPs action [32, 33]. Different pathways were highlighted in the *in silico* analysis that will disrupt the cell and lead to cell death. On one hand “cellular metabolism” was one of the most common terms. Indeed, AgNPs have been shown to be able to disrupt metabolism, by decreasing lactate production and downregulating different glycolytic enzymes [34, 35]. Proteoglycans seem to also be involved in AgNPs effect. Indeed, AgNPs have shown to alter proteoglycan expression in tendon injury and proteoglycans have been shown to have a pro- or anti-tumoral role in PCa depending on their structure [36, 37]. On the other hand, pathways such as AGE-RAGE, PI3K-Akt and mTOR, FOXO, JAK STAT, HIF1 and RUNX are associated with cell proliferation, migration, invasion and angiogenesis and seem to be targeted by AgNPs [38-44]. Moreover, most of these pathways are commonly disrupted in CRPC [6]. Thus, AgNPs will lead to a blockage of the cell cycle which is also highlighted in the *in silico* analysis and consequently apoptosis and senescence. Taken together, these results show that the main pathways affected by both AgNPs and G-AgNPs are the same, which is very promising because it demonstrates that this therapeutic approach has similar results in different cancer cell clones, which may help to surpass the treatment resistance caused by intratumor clonal heterogeneity in CRPC [45]. Furthermore, it also shows the role of these NPs as a potential multitarget approach, since they will simultaneously target multiple organelles and deregulated pathways of cancer cells, which can be a success approach to fight therapy resistance.

Additionally, this further proves the hypothesis that AgNPs and G-AgNPs act the same way in both cell lines, and that miRNAs are representative of the NPs’ response. Taking this into account, we selected the deregulated miRNAs common to AgNPs and G-AgNPs and to both cell lines, further narrowing them to the miRNAs present in the top10 most deregulated miRNAs. This allows us to propose this miRNA profile as a response biomarker for AgNPs and G-AgNPs administration in CRPC. The profile is composed by 2 downregulated miRNAs and 16 upregulated miRNAs. Interestingly, some of the upregulated miRNAs have already been reported as deregulated in PCa [46-52]. For example, miR-1-3p has been found to be downregulated in advanced PCa tissues samples and in metastatic PCa, targeting genes associated with invasion and migration promotion [46-49]. Moreover, miR-1272 has also been found downregulated in PCa, targeting HIP1 gene which promotes EGFR turnover and activation of AKT/ERK pathway [50]. miR-187-3p downregulation was associated with epithelial mesenchymal transition in PC-3 cells, regulating TNF expression [51]. Dong and collaborators have reported that overexpression of miR-590-3p led to abnormal mitosis, decreased cell proliferation and induction of

apoptosis in PC-3 cells [52]. Indeed, the upregulation of these tumor suppressor miRNAs after AgNPs and G-AgNPs administration will lead to tumor growth inhibition and apoptosis of PC-3 and Du-145 cells. These evidences strengthen and validate the applicability of this profile.

Conclusion

In conclusion, with this study we further proved the potential application of AgNPs and G-AgNPs in CRPC and we showed strong indications that their molecular mechanisms are similar and that their difference in efficacy is due to a higher uptake. We have also showed promising results regarding the biosafety of the AgNPs regarding liver toxicity. However, more studies need to be done regarding toxicity in other organs, using 3D *in vitro* models and *in vivo* models. Further, we explored the molecular pathways involved in the AgNPs and G-AgNPs damage on CRPC cells, showing that they target pathways commonly deregulated in CRPC and, that they seem to target multiple molecular pathways, which can pose as an advantage comparing to current therapies and may help surpass resistance to treatment that this form of PCa faces.

Finally, we propose a panel of 2 downregulated and 16 upregulated miRNAs that can be used as a biomarker of response to this possible future therapeutic approach. Future studies must include the replication of the study design in 3D spheroid models composed by different CRPC cell clones to mimic intratumoral heterogeneity and further explored in an *in vivo* study setting.

List of Abbreviations

ADT, androgen deprivation therapy; AGE, advanced glycation endproducts; AgNPs, Silver Nanoparticles; AHNK – Desmoyoki; AKT, protein kinase B; AR, Androgen Receptor; ASCL1, Achaete-Scute Family; BHLH, Transcription Factor 1; ATCC, American Type Culture Collection; BMP4, Bone Morphogenetic Protein-4; BRCA, Breast Cancer gene; BTG2, BTG Anti-Proliferation Factor 2; cAMP, Adenosine 3',5'-cyclic monophosphate; CCND1, Cyclin D1; CCND2, Cyclin D2; CDK6, Cell division protein kinase 6; CDKN1A, Cyclin Dependent Kinase Inhibitor 1a; CDKN1B, Cyclin Dependent Kinase Inhibitor 1B; CELF1, CUGBP Elav-Like Family Member 1; COP9, Constitutive photomorphogenesis 9; COPS6, COP9 Signalosome Subunit 6; COPS8, COP9 Signalosome Subunit 8; CRPC, Castration Resistant Prostate Cancer; DMEM, Dulbecco's Modified Eagle Medium; DNA, Deoxyribonucleic acid; EDTA, Ethylenediamine tetra acetic acid; EGFR, Epidermal growth factor receptor; ERG, ETS-related gene; ERK, Extracellular Signal-Regulated Kinase; ESR, Erythrocyte sedimentation rate; ETS, Erythroblast Transformation Specific; ETV1, ETS Variant Transcription Factor 1; ETV4, ETS Variant Transcription Factor 4; FBS, Fetal bovine

serum; FDR, False Discovery Rate; FGFR, fibroblast growth factor receptors; FLI1, Friend leukemia integration 1; FOXA1, Forkhead box protein A1; FOXO, Forkhead box transcription factors; G-AgNPs – Glucose functionalized Silver Nanoparticles; GG-NER, Global genome nucleotide excision repair; GIGYF1, GRB10 Interacting GYF Protein 1; GPS1, G Protein Pathway Suppressor 1; HIF1, Hypoxia Inducible Factor 1; HIP1, Huntingtin-interacting protein 1; HNRNPU, Heterogenous nuclear ribonucleoprotein U; IC50, Half maximal inhibitory concentration; IDH1, Isocitrate dehydrogenase 1; IGF1, Insulin-like growth factor 1; IGF1R, Type 1 insulin-like growth factor receptor; IL13, Interleukin 13; IL2, Interleukin 2; JAK, Janus kinase ; KEGG, Kyoto Encyclopedia of Genes and Genomes; KLHL15, Kelch Like Family Member 15; LRRC58, Leucine Rich Repeat Containing 58; LRRC58, Leucine Rich Repeat Containing 58; MAP1B, Microtubule-associated protein 1B; MAPK, Mitogen-activated protein kinase; MCL, Markov clustering; MCL1, Myeloid cell leukemia-1; mCRPC, metastatic Castration Resistant Prostate Cancer; MDM2, Mouse double minute 2 homolog; miRNA, microRNA; MKNK2, MAPK Interacting Serine/Threonine Kinase 2; mRNA, messenger RNA; mTOR, Mammalian target of rapamycin complex; NACC1, Nucleus Accumbens Associated 1; NEUROG1, Neurogenin 1; NOTCH, Neurogenic locus notch homolog protein; NPs, Nanoparticles; NTRK1, Neurotrophic tyrosine kinase receptor 1; NTRK3, Neurotrophic tyrosine kinase, receptor, type 3; NUFIP2, Nuclear FMR1 Interacting Protein 2; PAM, Partitioning Around Medoids; PARP, Poly ADP Ribose Polymerase; PCa, Prostate Cancer; PI3K, Phosphoinositide 3-kinase; PPI, protein-protein interaction; PTEN, Protein phosphatase and tensin homolog; PTK6, Protein tyrosine kinase 6; RAGE, Receptors for advanced glycation endproducts; RB1, Retinoblastoma protein; RISC, RNA-induced silencing complex; RNA, Ribonucleic acid; ROS, Reactive oxygen species ; RPMI, Roswell Pearl Memorial Institute; RUNX, Runt-related transcription factor; SOD2, Superoxide dismutase 2; SOX4, Sex-determining region Y-related high-mobility group box 4; SPOP, Speckle-type Poz protein; SRCAP, Snf2 Related CREBBP Activator Protein; SRRM2, Serine/Arginine Repetitive Matrix 2; STAT, Signal transducer and activator of transcription; STRINGapp, Search Tool for the Retrieval of Interacting Genes; TAOK1, Thousand and one amino-acid kinase 1; TC-NER, Transcription-coupled nucleotide excision repair; TLR4, Toll-like receptor 4; TNF, Tumor necrosis factor; TNPO1, Transportin 1; TNRC6A, Trinucleotide Repeat Containing Adaptor 6A; TP53, tumor protein p53; TXNIP, Thioredoxin Interacting Protein; VEGF, Vascular endothelial growth factor; WEE1, WEE1 G2 Checkpoint Kinase; XIAP, X-linked inhibitor of apoptosis protein; ZNF264, Zinc Finger Protein 264; ZNF460, Zinc Finger Protein 460; 223Ra, Radium-223

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing Interests

The authors declare that they have no competing interests.

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Authors Contribution

Mariana Morais has contributed to the design of the work, acquisition, analysis, and interpretation of the data and drafted the manuscript. Francisca Dias has contributed to the design of the work, analysis and interpretation of the data and revision of the manuscript. Patrícia Figueiredo contributed to the acquisition of the data and to the revision of the manuscript. Mariana Ferreira contributed to the acquisition and analysis of the data. Kirsi S Mikkonen contributed to the design of the work, funding and revision of the manuscript. Ana L Teixeira¹ contributed to the design of the work, analysis and interpretation of the data and to the revision of the manuscript. Rui Medeiros contributed to the design of the work, funding and revision of the manuscript.

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Supplementary Table 1 - Downregulated miRNAs in response to AgNPs or G-AgNPs administration to PC-3 and Du-145

PC-3+AgNPs			PC-3+G-AgNPs			Du-145+AgNPs			Du-145+G-AgNPs		
	log2FoldChange	-log10(padj)		log2FoldChange	-log10(padj)		log2FoldChange	-log10(padj)		log2FoldChange	-log10(padj)
hsa-let-7i-5p	-6.023905965	2.504101207	hsa-let-7i-5p	-6.50205209	2.321173135	hsa-let-7i-5p	-6.806248075	2.171754109	hsa-let-7i-5p	-6.983501168	2.197851516
hsa-miR-1283	-3.384699632	2.284170088	hsa-miR-125b-5p	-3.870247418	2.287513934	hsa-miR-1283	-4.238247532	2.020327188	hsa-miR-1283	-4.415500624	2.686612756
			hsa-miR-1283	-3.862845758	2.532061399						

Supplementary Table 2 - Upregulated miRNAs in response to AgNPs or G-AgNPs administration to PC-3 and Du-145

PC-3+AgNPs			PC-3+G-AgNPs			Du-145+AgNPs			Du-145+G-AgNPs		
	log2FoldChange	-log10(padj)		log2FoldChange	-log10(padj)		log2FoldChange	-log10(padj)		log2FoldChange	-log10(padj)
hsa-miR-597-5p	5.965319382	2.184318525	hsa-miR-597-5p	5.962093008	2.2539842	hsa-miR-572	5.459899343	2.15906612	hsa-miR-572	5.728602797	2.27673768
hsa-miR-525-3p	5.912553136	2.101946562	hsa-miR-1228-3p	5.67196465	2.00277596	hsa-miR-203a-5p	4.74442427	2.25417511	hsa-miR-597-5p	5.564159366	2.13431105
hsa-miR-572	5.838378787	2.319319204	hsa-miR-675-5p	5.642695856	2.41863072	hsa-miR-1272	4.644193519	2.27603513	hsa-miR-203a-5p	4.990028707	2.23410171
hsa-miR-1228-3p	5.721528856	2.222168377	hsa-miR-572	5.420101847	2.5320614	hsa-miR-1200	4.56057852	2.44099339	hsa-miR-675-5p	4.854019014	2.1533765
hsa-miR-675-5p	5.436998549	2.387693353	hsa-miR-203a-5p	5.231936344	2.49496586	hsa-miR-548g-3p	4.460708255	2.46287262	hsa-miR-1272	4.829834376	2.95341575
hsa-miR-637	5.202862424	2.188214886	hsa-miR-133b	5.103172225	2.49395985	hsa-miR-513a-5p	4.442288878	2.54508331	hsa-miR-495-5p	4.638773928	2.59845552
hsa-miR-517c-3p+hsa-miR-519a-3p	5.118304254	2.086751046	hsa-miR-3605-5p	5.07049937	2.60700105	hsa-miR-5001-3p	4.372221208	2.28289396	hsa-miR-548g-3p	4.580060857	2.40763963
hsa-miR-203a-5p	5.109276713	2.420823108	hsa-miR-518e-3p	5.042586638	2.73407775	hsa-miR-648	4.354360861	2.19623028	hsa-miR-187-3p	4.580024906	2.62833223
hsa-miR-518e-3p	4.981440389	2.504101207	hsa-miR-1248	5.007065658	2.6786006	hsa-miR-1228-3p	4.33635155	2.44099339	hsa-miR-1200	4.574135956	2.19625091
hsa-miR-494-5p	4.972892741	2.182341497	hsa-miR-208b-5p	4.950028352	2.45458696	hsa-miR-590-3p	4.2980904	2.17384807	hsa-miR-1-3p	4.57299908	2.66453028
hsa-miR-4787-3p	4.965319382	2.179368467	hsa-miR-3150b-3p	4.883726996	2.2406883	hsa-miR-564	4.27236778	2.39681735	hsa-miR-1228-3p	4.408302737	2.24317154
hsa-miR-33b-5p	4.931003948	2.204443999	hsa-miR-517c-3p+hsa-miR-519a-3p	4.78104169	2.08319765	hsa-miR-450a-2-3p	4.21360404	2.44573328	hsa-miR-1281	4.402409125	2.55284624
hsa-miR-1271-3p	4.929007925	2.504101207	hsa-miR-433-5p	4.707086617	2.56756134	hsa-miR-187-3p	4.195382969	2.44099339	hsa-miR-650	4.380627695	2.42775632
hsa-miR-3605-5p	4.925337665	2.086751046	hsa-miR-5010-3p	4.694248605	2.98568856	hsa-miR-548ad-3p	4.169660348	2.21983088	hsa-miR-450a-2-3p	4.333263346	2.15435273
hsa-miR-1248	4.887678352	2.504101207	hsa-miR-34c-5p	4.690825826	2.23152289	hsa-miR-650	4.139277719	2.11213699	hsa-miR-548ad-3p	4.325740589	2.31803682
hsa-miR-3168	4.88434369	2.44067641	hsa-miR-4647	4.668101992	2.65812028	hsa-miR-381-5p	4.106732339	2.44573328	hsa-miR-1185-5p	4.318450678	2.95341575
hsa-miR-5001-5p	4.846540007	2.100829187	hsa-miR-1910-3p	4.665757045	2.64296568	hsa-miR-512-3p	4.104353612	2.4978233	hsa-miR-648	4.280973559	2.62200032
hsa-miR-328-5p	4.838875327	2.001434926	hsa-miR-512-3p	4.655187425	2.19709009	hsa-miR-147b	4.095529541	2.20168633	hsa-miR-522-3p	4.278328369	2.51658382
hsa-miR-433-5p	4.835638619	2.504101207	hsa-miR-124-3p	4.618028996	2.41863072	hsa-miR-876-5p	4.089555572	2.23868755	hsa-miR-585-3p	4.275463149	2.56798037
hsa-miR-495-5p	4.827197692	2.504101207	hsa-miR-924	4.607138571	2.37121416	hsa-miR-517c-3p+hsa-miR-519a-3p	4.064888712	2.36146783	hsa-miR-937-3p	4.27441277	2.74134601
hsa-miR-184	4.818149479	2.180611538	hsa-miR-328-5p	4.606979571	2.17959551	hsa-miR-1-3p	4.052232761	2.46287262	hsa-miR-3168	4.271186847	2.74078409
hsa-miR-208b-5p	4.78657031	2.332612162	hsa-miR-708-5p	4.547364005	2.6038763	hsa-miR-494-5p	4.038887874	2.46287262	hsa-miR-876-5p	4.263174022	2.11642646
hsa-miR-345-3p	4.719664184	2.208968374	hsa-miR-885-5p	4.527411212	2.98568856	hsa-miR-128-2-5p	4.038887874	2.00741789	hsa-miR-499a-5p	4.235365071	2.6461702
hsa-miR-885-5p	4.714389195	2.284170088	hsa-miR-539-3p	4.521839163	2.3062405	hsa-miR-522-3p	4.033391833	2.46528898	hsa-miR-888-5p	4.224122396	2.91026002
hsa-miR-539-3p	4.705144361	2.184318525	hsa-miR-492	4.510867622	2.15436201	hsa-miR-924	4.025340546	2.15796642	hsa-miR-4516	4.199320166	2.46238611
hsa-miR-924	4.704866144	2.182341497	hsa-miR-3168	4.504263787	2.29038661	hsa-miR-182-3p	4.010542506	2.44573328	hsa-miR-564	4.177757192	2.52671381
hsa-miR-624-3p	4.696201075	2.182341497	hsa-miR-513a-3p	4.45234624	2.09852102	hsa-miR-345-3p	3.996509618	2.46528898	hsa-miR-1910-3p	4.175098285	2.33275795
hsa-miR-4707-3p	4.695206455	2.304142102	hsa-miR-302a-3p	4.441338749	2.42362522	hsa-miR-3168	3.995507829	2.46287262	hsa-miR-512-3p	4.163931647	2.57849081
hsa-miR-512-3p	4.631986049	2.407898513	hsa-miR-5001-5p	4.433700618	2.20537694	hsa-miR-874-3p	3.992103912	2.07035331	hsa-miR-134-3p	4.163931647	2.52437267

hsa-miR-513a-3p	4.630617916	2.37921641	hsa-miR-548m	4.414550342	2.51299538	hsa-miR-4516	3.982760262	2.30502996	hsa-miR-1271-3p	4.157821039	2.48157656
hsa-miR-525-5p	4.601463636	2.122892571	hsa-miR-4707-3p	4.414365417	2.42098292	hsa-miR-579-5p	3.966719694	2.36758071	hsa-miR-197-5p	4.14049755	2.6461702
hsa-miR-372-3p	4.599698869	2.097748185	hsa-miR-511-5p	4.405885273	2.06416787	hsa-miR-339-5p	3.96073279	2.27168551	hsa-miR-518e-3p	4.135635348	2.65017184
hsa-miR-34c-5p	4.597556779	2.222168377	hsa-miR-495-5p	4.404897085	2.70340881	hsa-miR-585-3p	3.940321252	2.44021586	hsa-miR-33b-5p	4.118890348	2.8396943
hsa-miR-5010-3p	4.592779349	2.284170088	hsa-miR-637	4.403327001	2.17172859	hsa-miR-197-5p	3.929260922	2.22964319	hsa-miR-630	4.118011638	2.55362364
hsa-miR-888-5p	4.589147802	2.504101207	hsa-miR-1271-3p	4.371039821	2.49540055	hsa-miR-495-5p	3.928240903	2.44573328	hsa-miR-5001-5p	4.117722961	2.83627232
hsa-miR-626	4.578379119	2.400986831	hsa-miR-494-5p	4.358581731	2.32245766	hsa-miR-1247-5p	3.909366112	2.46287262	hsa-miR-1262	4.095373305	2.5568746
hsa-miR-650	4.557855242	2.504101207	hsa-miR-522-3p	4.342974779	2.35030812	hsa-miR-612	3.890284794	2.66952732	hsa-miR-3182	4.091474259	2.78147812
hsa-miR-152-5p	4.515852613	2.300618986	hsa-miR-1-3p	4.320482055	2.57192733	hsa-miR-1323	3.889993499	2.0880876	hsa-miR-378d	4.089910772	2.54498596
hsa-miR-182-3p	4.508822884	2.169285759	hsa-miR-381-5p	4.315567749	2.2779431	hsa-miR-891b	3.878170643	2.19477473	hsa-miR-5001-3p	4.076253512	2.31777885
hsa-miR-1203	4.500383337	2.142583576	hsa-miR-187-3p	4.311325068	2.47461728	hsa-miR-638	3.860138802	2.28847676	hsa-miR-147b	4.069798403	2.29505362
hsa-miR-548a-3p	4.497770332	2.331163773	hsa-miR-345-3p	4.306125815	2.25009958	hsa-miR-637	3.849874343	2.13764374	hsa-miR-1323	4.062592348	2.24331791
hsa-miR-522-3p	4.488557624	2.214093741	hsa-miR-339-5p	4.302381863	2.41863072	hsa-miR-433-3p	3.841440531	2.44573328	hsa-miR-345-3p	4.052807501	2.67845589
hsa-miR-589-5p	4.482729815	2.198462732	hsa-miR-1272	4.297018946	2.12343344	hsa-miR-127-5p	3.834031553	2.0880876	hsa-miR-924	4.047065834	2.16293746
hsa-miR-133b	4.447805708	2.495062237	hsa-miR-650	4.288895675	2.74197649	hsa-miR-4787-3p	3.829102334	2.08617137	hsa-miR-299-3p	4.038275161	2.46731852
hsa-miR-302a-3p	4.444571907	2.331163773	hsa-miR-452-5p	4.287352566	2.05591631	hsa-miR-499a-5p	3.824800598	2.28895949	hsa-miR-3614-5p	4.032683977	2.83627232
hsa-miR-1185-5p	4.443973828	2.260149744	hsa-miR-556-5p	4.282087782	3.53938734	hsa-miR-5001-5p	3.813955617	2.40086162	hsa-miR-338-5p	4.026771754	2.23410171
hsa-miR-297	4.442972518	2.188861929	hsa-miR-4488	4.277848097	2.0910827	hsa-miR-1281	3.807981647	2.46287262	hsa-miR-182-3p	4.023674981	2.74134601
hsa-miR-124-3p	4.428174478	2.17577122	hsa-miR-1200	4.240267347	2.71030475	hsa-miR-604	3.796862115	2.51168895	hsa-miR-887-5p	4.007299914	2.18470032
hsa-miR-1272	4.419905047	2.305170252	hsa-miR-1281	4.236377983	2.14869576	hsa-miR-1910-3p	3.790937214	2.0069596	hsa-miR-3140-5p	3.998776864	2.55362364
hsa-miR-197-5p	4.415716388	2.400986831	hsa-miR-3614-3p	4.221684388	2.49421588	hsa-miR-1193	3.784983789	2.46287262	hsa-miR-596	3.997721104	2.78147812
hsa-miR-4647	4.40487575	2.504101207	hsa-miR-328-3p	4.21491824	2.44925046	hsa-miR-1469	3.783081057	2.44573328	hsa-miR-590-3p	3.99631702	2.37609189
hsa-miR-548m	4.392255751	2.419880874	hsa-miR-888-5p	4.204507621	2.70340881	hsa-miR-3185	3.777920581	2.21983088	hsa-miR-4488	3.995261261	2.61854971
hsa-miR-190a-3p	4.37561831	2.504101207	hsa-miR-190a-3p	4.193541461	2.5320614	hsa-miR-873-3p	3.773570571	2.46287262	hsa-miR-1247-5p	3.983742187	2.59845552
hsa-miR-187-3p	4.373495356	2.387693353	hsa-miR-4421	4.190720935	2.25127083	hsa-miR-33b-5p	3.769769567	2.3822821	hsa-miR-331-5p	3.964919869	2.78477594
hsa-miR-4421	4.357985483	2.182341497	hsa-miR-1302	4.187989013	2.44648216	hsa-miR-297	3.766418345	2.66952732	hsa-miR-492	3.962738921	2.09123207
hsa-miR-639	4.351417979	2.120460125	hsa-miR-5001-3p	4.17101669	2.01057852	hsa-miR-761	3.762569721	2.44573328	hsa-miR-494-5p	3.960317936	2.38057678
hsa-miR-876-5p	4.345810191	2.198462732	hsa-miR-182-3p	4.167277162	2.02849724	hsa-miR-878-5p	3.762196208	2.31010348	hsa-miR-369-5p	3.952640947	2.67845589
hsa-miR-4488	4.345219601	2.22066485	hsa-miR-181a-2-3p	4.162628696	2.77896629	hsa-miR-3182	3.750531068	2.46287262	hsa-miR-433-5p	3.947906725	2.38372563
hsa-miR-1-3p	4.33102316	2.036756086	hsa-miR-375	4.157008952	2.12493211	hsa-miR-627-5p	3.740857377	2.11213699	hsa-miR-337-3p	3.943780747	2.67845589
hsa-miR-548a-5p	4.32607533	2.436739762	hsa-miR-3179	4.155443216	2.57192733	hsa-miR-216a-5p	3.739397907	2.46287262	hsa-miR-515-3p	3.931383797	2.78147812
hsa-miR-1249-3p	4.315477047	2.179285006	hsa-miR-6721-5p	4.147203001	2.60664476	hsa-miR-328-5p	3.73575539	2.46287262	hsa-miR-513a-5p	3.924766686	2.41865085
hsa-miR-1910-3p	4.305569856	2.436739762	hsa-miR-519b-5p+hsa-miR-519c-5p+hsa-miR-523-5p+hsa-miR-518e-5p+hsa-miR-522-5p+hsa-miR-519a-5p	4.141222832	2.5320614	hsa-miR-134-3p	3.722429899	2.31540884	hsa-miR-891a-5p	3.920141121	2.36077992
hsa-miR-651-3p	4.302715852	2.419880874	hsa-miR-297	4.139041883	2.22968847	hsa-miR-95-3p	3.711811142	2.2905976	hsa-miR-199a-3p+hsa-miR-199b-3p	3.916943272	2.95341575
hsa-miR-590-3p	4.298652715	2.436739762	hsa-miR-576-3p	4.136286011	2.1362071	hsa-miR-1305	3.709682449	2.17175411	hsa-miR-133a-5p	3.912816535	2.27673768
hsa-miR-873-3p	4.297762687	2.436739762	hsa-miR-23c	4.117318879	2.54379704	hsa-miR-204-5p	3.705554541	2.46528898	hsa-miR-382-3p	3.882802429	2.56798037
hsa-miR-573	4.295142493	2.173693819	hsa-miR-1257	4.116829486	2.97646032	hsa-miR-891a-5p	3.702700536	2.44099339	hsa-miR-532-3p	3.879298323	2.51872511
hsa-miR-769-3p	4.292366373	2.051604065	hsa-miR-502-5p	4.110825941	2.73407775	hsa-miR-518e-3p	3.693379249	2.25417511	hsa-miR-300	3.870276659	2.3764683
hsa-miR-302f	4.288342096	2.24430651	hsa-miR-578	4.110745584	2.07334546	hsa-miR-433-5p	3.683183142	2.0880876	hsa-miR-663a	3.868511893	2.36713492
hsa-miR-450a-2-3p	4.286553847	2.436739762	hsa-miR-548v	4.10977309	2.71030475	hsa-miR-1203	3.681200053	2.39681735	hsa-miR-1295a	3.861033283	2.78147812
hsa-miR-339-5p	4.277370555	2.204443999	hsa-miR-512-5p	4.102884284	2.74197649	hsa-miR-1290	3.675131855	2.28289396	hsa-miR-548o-3p+hsa-miR-	3.855943611	2.67389493

									548ah-3p+hsa-miR-548av-3p		
hsa-miR-1302	4.267551475	2.204443999	hsa-miR-1185-5p	4.093534661	2.19962324	hsa-miR-2682-5p	3.671284272	2.08598752	hsa-miR-4647	3.855627334	2.87871455
hsa-miR-3150b-3p	4.261483277	2.171662356	hsa-miR-152-5p	4.088374186	2.42098292	hsa-miR-888-5p	3.668396112	2.08093203	hsa-miR-517c-3p+hsa-miR-519a-3p	3.84198036	2.86650523
hsa-miR-520e	4.254585882	2.504101207	hsa-miR-490-5p	4.088374186	2.23908216	hsa-miR-299-3p	3.659720033	2.44099339	hsa-miR-381-5p	3.834881591	2.59307784
hsa-miR-381-5p	4.247543218	2.369688436	hsa-miR-767-3p	4.079526923	2.57192733	hsa-miR-1302	3.654886843	2.3822821	hsa-miR-3140-3p	3.828456224	2.77718239
hsa-miR-492	4.238500826	2.188214886	hsa-miR-211-3p	4.072135757	2.11637196	hsa-miR-1262	3.654406185	2.27536427	hsa-miR-641	3.824624344	2.19625091
hsa-miR-2278	4.231700428	2.22066485	hsa-miR-887-5p	4.069513009	2.70674568	hsa-miR-492	3.651909135	2.00704531	hsa-miR-607	3.822771169	2.37609189
hsa-miR-6721-5p	4.230094373	2.173693819	hsa-miR-648	4.067907337	2.31096531	hsa-miR-5010-3p	3.647059699	2.46528898	hsa-miR-1976	3.818933173	2.80724761
						hsa-miR-199a-3p+hsa-miR-199b-3p					
hsa-miR-3614-3p	4.225844876	2.37192957	hsa-miR-211-5p	4.063707325	2.5320614	hsa-miR-1976	3.639717597	2.13764374	hsa-miR-935	3.817704824	2.52671381
hsa-miR-511-5p	4.217179806	2.100829187	hsa-miR-510-3p	4.052272224	2.28645969	hsa-miR-1185-5p	3.637884663	2.51168895	hsa-miR-5010-3p	3.816822637	2.50203291
hsa-miR-532-3p	4.208434889	2.37192957	hsa-miR-626	4.051566189	2.55569842	hsa-miR-382-3p	3.633566623	2.44099339	hsa-miR-2110	3.814516165	2.42387447
hsa-miR-1281	4.202546148	2.250213975	hsa-miR-518c-3p	4.049995615	2.31009969	hsa-miR-566	3.630532356	2.46287262	hsa-miR-483-5p	3.813821088	2.68661276
hsa-miR-337-3p	4.199884333	2.387693353	hsa-miR-548a-3p	4.046530558	2.49395985	hsa-miR-380-3p	3.62259576	2.92150737	hsa-miR-487a-3p	3.807135204	2.31010897
hsa-miR-922	4.196416929	2.436739762	hsa-miR-606	4.036219938	2.5320614		3.6191079	2.46528898	hsa-miR-922	3.804195555	2.59845552
hsa-miR-181a-2-3p	4.18599376	2.44067641	hsa-miR-2110	4.022744668	2.51854705	hsa-miR-4421	3.618554842	2.44573328	hsa-miR-127-5p	3.803110927	2.41865085
hsa-miR-576-3p	4.172996229	2.078986544	hsa-miR-515-3p	4.020168279	2.71030475	hsa-miR-184	3.614089785	2.23882554	hsa-miR-487b-5p	3.80213911	2.89539765
hsa-miR-1296-3p	4.168033613	2.504101207	hsa-miR-532-3p	4.017403877	2.17959551	hsa-miR-556-5p	3.59444104	2.44573328	hsa-miR-513b-5p	3.797237976	2.74134601
hsa-miR-1200	4.166447337	2.420823108	hsa-miR-890	4.009919662	2.5412181	hsa-miR-766-5p	3.590903074	2.28895949	hsa-miR-4787-3p	3.791133413	2.22035192
hsa-miR-548g-3p	4.158914273	2.400986831	hsa-miR-619-3p	4.00667002	2.41863072	hsa-miR-151b	3.589580106	2.24029036	hsa-miR-1245a	3.790583199	2.59845552
hsa-miR-337-5p	4.151577041	2.37192957	hsa-miR-519b-3p	4.000978848	2.18393415	hsa-miR-607	3.585452198	2.46528898	hsa-miR-216a-5p	3.789195248	2.78147812
hsa-miR-4461	4.143586894	2.161094541	hsa-miR-3614-5p	4.000797946	2.03184459	hsa-miR-3140-3p	3.584697848	2.46528898	hsa-miR-766-3p	3.789170868	2.56341499
hsa-miR-3179	4.141567031	2.436739762	hsa-miR-802	3.985119612	2.54782139	hsa-miR-3614-5p	3.582466297	2.47660768	hsa-miR-556-5p	3.78808163	2.74134601
hsa-miR-802	4.135276577	2.436739762	hsa-miR-571	3.983778283	2.55569842	hsa-miR-508-5p	3.576546833	2.46528898	hsa-miR-133b	3.779930616	2.43594487
hsa-miR-767-5p	4.12633409	2.369688436	hsa-miR-337-5p	3.983715528	2.35027526	hsa-miR-301a-5p	3.575060284	2.77474177	hsa-miR-891b	3.772350974	2.25664236
hsa-miR-1301-3p	4.125991448	2.204443999	hsa-miR-4536-3p	3.983058873	2.13731177	hsa-miR-1286	3.574030075	2.51168895	hsa-miR-512-5p	3.767501538	2.89539765
hsa-miR-887-5p	4.122623389	2.37921641	hsa-miR-363-5p	3.981457551	2.47976337	hsa-miR-934	3.555029512	2.05020799	hsa-miR-4524a-5p	3.764241198	2.24160647
hsa-miR-2110	4.122130753	2.380248297	hsa-miR-519e-3p	3.977040543	2.57353062	hsa-miR-515-3p	3.551597174	2.5325238	hsa-miR-1224-3p	3.763663542	2.53451986
hsa-miR-619-3p	4.120841982	2.37192957	hsa-miR-1193	3.974859595	2.08033129	hsa-miR-300	3.551365918	2.19827802	hsa-miR-626	3.760766068	2.67845589
hsa-miR-521	4.107016437	2.332612162	hsa-miR-105-5p	3.964384593	2.74197649	hsa-miR-552-3p	3.550905508	2.13764374	hsa-miR-639	3.759600406	2.89539765
hsa-miR-516b-5p	4.10328269	2.407898513	hsa-miR-1249-3p	3.962775802	2.64315364	hsa-miR-520g-3p	3.546582765	2.28289396	hsa-miR-638	3.759158607	2.08869631
hsa-miR-648	4.094399346	2.188214886	hsa-miR-133a-3p	3.96096465	2.84781678	hsa-miR-766-3p	3.543745599	2.40335634	hsa-miR-1197	3.75778609	2.605175
hsa-miR-211-3p	4.091489923	2.184318525	hsa-miR-338-5p	3.960483076	2.74197649	hsa-miR-639	3.54225444	2.16461163	hsa-miR-802	3.753121666	2.26430061
hsa-miR-4536-3p	4.089793649	2.142752551	hsa-miR-1224-5p	3.95256608	2.84735056	hsa-miR-212-3p	3.541541018	2.46287262	hsa-miR-767-5p	3.741764635	2.59845552
hsa-miR-137	4.077623556	2.400986831	hsa-miR-504-5p	3.95256608	2.5320614	hsa-miR-802	3.539721321	2.34192783	hsa-miR-1302	3.736762058	2.74134601
hsa-miR-146b-5p	4.076018828	2.182341497	hsa-miR-596	3.950028352	2.70340881	hsa-miR-378d	3.533658943	2.24419457	hsa-miR-18b-5p	3.734053699	2.78147812
hsa-miR-627-5p	4.072469746	2.081881824	hsa-miR-300	3.940030106	2.11168904	hsa-miR-626	3.529623347	2.46528898	hsa-miR-133a-3p	3.733435987	2.56341499
hsa-miR-1295a	4.071949409	2.369688436	hsa-miR-450a-2-3p	3.939593646	2.31382785	hsa-miR-889-3p	3.528716302	2.18113497	hsa-miR-3150b-3p	3.728602797	2.68310026
hsa-miR-105-5p	4.070327656	2.504101207	hsa-miR-1271-5p	3.939221193	2.57836198	hsa-miR-643	3.52327875	2.44573328	hsa-miR-297	3.718172961	2.44713054
hsa-miR-369-5p	4.061379789	2.016685993	hsa-miR-516a-3p+hsa-miR-516b-3p	3.936088294	2.17959551	hsa-miR-338-5p	3.522211637	2.2905976	hsa-miR-6720-3p	3.717002606	2.33275795
						hsa-miR-548o-3p+hsa-miR-548ah-3p+hsa-miR-548av-3p					
hsa-miR-130b-3p	4.057830707	2.284170088	hsa-miR-192-5p	3.926269215	2.49395985		3.515683029	2.24828713	hsa-miR-642a-5p	3.712618817	2.41865085

hsa-miR-582-3p	4.057038529	2.081796741	hsa-miR-370-5p	3.921451728	2.0050644	hsa-miR-211-5p	3.510472422	2.43560251	hsa-miR-637	3.712481789	2.24170039
hsa-miR-5196-5p	4.051268702	2.504101207	hsa-miR-516b-5p	3.920640343	2.54782139	hsa-miR-449b-5p	3.50457446	2.40113871	hsa-miR-873-3p	3.70651282	2.80724761
hsa-miR-507	4.047914926	2.504101207	hsa-miR-1301-3p	3.91801939	2.2779431	hsa-miR-516b-5p	3.498922444	2.44573328	hsa-miR-2682-5p	3.703374921	2.55943586
hsa-miR-339-3p	4.039866371	2.241480322	hsa-miR-487b-5p	3.917421952	2.14986856	hsa-miR-1295a	3.49495429	2.32431592	hsa-miR-449b-5p	3.700050768	2.23410171
hsa-miR-3147	4.035620928	2.419880874	hsa-miR-553	3.909240485	2.31009969	hsa-miR-3140-5p	3.490935811	2.34693382	hsa-miR-508-5p	3.69644114	2.74134601
hsa-miR-1197	4.034607987	2.036713353	hsa-miR-130b-3p	3.908993638	2.11559518	hsa-miR-573	3.490535592	2.46287262	hsa-miR-563	3.694884148	2.42387447
hsa-miR-199a-3p+hsa-miR-199b-3p	4.034485958	2.489593305	hsa-miR-651-3p	3.899077856	2.47647783	hsa-miR-133a-5p	3.489479751	2.46287262	hsa-miR-302a-3p	3.688584983	2.63196203
hsa-miR-98-3p	4.034208597	2.184318525	hsa-miR-767-5p	3.895846529	2.2779431	hsa-miR-192-5p	3.483241273	2.44099339	hsa-miR-5196-5p	3.684994403	2.78477594
hsa-miR-585-3p	4.032623079	2.436739762	hsa-miR-521	3.892297447	2.58963901	hsa-miR-337-3p	3.46843759	2.34693382	hsa-miR-301a-5p	3.67889413	2.31196666
hsa-miR-566	4.030145461	2.331163773	hsa-miR-548a-5p	3.890549519	2.28645969	hsa-miR-1258	3.463728047	2.34700536	hsa-miR-874-3p	3.676504987	2.89539765
hsa-miR-544a	4.024259419	2.259094513	hsa-miR-585-3p	3.889415578	2.5666323	hsa-miR-548k	3.459737723	2.36574187	hsa-miR-615-3p	3.667714759	2.6461702
hsa-miR-365b-5p	4.020039101	2.142583576	hsa-miR-876-5p	3.884326777	2.15436201	hsa-miR-4647	3.453213459	2.08598752	hsa-miR-4792	3.666647282	2.33267051
hsa-miR-513a-5p	4.019355817	2.436739762	hsa-miR-507	3.883749121	2.42098292	hsa-miR-553	3.447441253	2.11268698	hsa-miR-210-5p	3.664348912	2.95341575
hsa-miR-1271-5p	4.017359116	2.504101207	hsa-miR-1278	3.883726996	2.74197649	hsa-miR-548b-3p	3.446352015	2.0849971	hsa-miR-1290	3.661087701	2.20996127
hsa-miR-338-5p	4.016369784	2.44067641	hsa-miR-302f	3.883517865	2.20537694	hsa-miR-4707-5p	3.443380735	2.19477473	hsa-miR-328-5p	3.660978473	2.6461702
hsa-miR-487b-5p	4.015217026	2.198462732	hsa-miR-128-2-5p	3.881211393	2.4684252	hsa-miR-190a-3p	3.441038167	2.27168551	hsa-miR-514a-3p	3.657789814	2.05360504
hsa-miR-3185	4.01115389	2.258884508	hsa-miR-1469	3.864196278	2.35918805	hsa-miR-510-3p	3.438657477	2.3822821	hsa-miR-612	3.657481745	2.49341555
hsa-miR-370-3p	4.010170386	2.400986831	hsa-miR-337-3p	3.863506819	2.42098292	hsa-miR-767-5p	3.433824287	2.46287262	hsa-miR-184	3.65561907	2.95341575
hsa-miR-1193	4.009622853	2.190108252	hsa-miR-885-3p	3.856030478	2.71030475	hsa-miR-4458	3.42959143	2.18225828	hsa-miR-553	3.639159895	2.78147812
hsa-miR-1252-5p	3.998536799	2.504101207	hsa-miR-324-3p	3.84678824	2.11168904	hsa-miR-133a-3p	3.428862875	2.39681735	hsa-miR-380-3p	3.639159895	2.55362364
hsa-miR-556-5p	3.982496149	2.082142613	hsa-miR-127-5p	3.846268069	2.17959551	hsa-miR-412-3p	3.427722095	2.23868755	hsa-miR-509-3p	3.635610813	2.74936121
hsa-miR-328-3p	3.980189678	2.332612162	hsa-miR-4516	3.839626383	2.21373498	hsa-miR-532-3p	3.418965646	2.43869799	hsa-miR-4536-3p	3.630903634	2.30515718
hsa-miR-3614-5p	3.979204223	2.220716342	hsa-miR-524-3p	3.83799573	2.74197649	hsa-miR-548v	3.414377208	2.20168633	hsa-miR-6511a-5p	3.626786743	2.10750111
hsa-miR-301b-5p	3.971163925	2.332612162	hsa-miR-339-3p	3.833257158	2.32130769	hsa-miR-744-5p	3.410713613	2.40086162	hsa-miR-433-3p	3.625695032	2.05192793
hsa-miR-1275	3.970004581	2.35891815	hsa-miR-137	3.830440065	2.58963901	hsa-miR-487b-5p	3.409091103	2.34700536	hsa-miR-181b-2-3p	3.621670755	2.15670309
hsa-miR-891a-5p	3.952981186	2.191745248	hsa-miR-516a-5p	3.82050659	2.44662795	hsa-miR-146b-5p	3.408948847	2.44573328	hsa-miR-582-5p	3.617294206	2.56341499
hsa-miR-433-3p	3.951613053	2.400986831	hsa-miR-548g-3p	3.814815419	2.5320614	hsa-miR-1275	3.400364135	2.29434655	hsa-miR-582-3p	3.615979584	2.6607919
hsa-miR-4707-5p	3.947684176	2.263526855	hsa-miR-384	3.813343655	2.04569774	hsa-miR-373-3p	3.399233398	2.16870245	hsa-miR-1286	3.613362558	2.78147812
hsa-miR-563	3.938719564	2.284170088	hsa-miR-1306-3p	3.813050652	2.27112772	hsa-miR-601	3.389710348	2.51168895	hsa-miR-3196	3.610943953	2.55284624
hsa-miR-1293	3.936679157	2.436739762	hsa-miR-518d-3p	3.812078158	2.59801425	hsa-miR-1257	3.388551003	2.44099339	hsa-miR-5010-5p	3.596507355	2.6461702
hsa-miR-571	3.932372081	2.304142102	hsa-miR-197-5p	3.80785064	2.04409828	hsa-miR-939-5p	3.387850409	2.27748467	hsa-miR-516b-5p	3.594811936	2.55731015
hsa-miR-1297	3.931512175	2.311304206	hsa-miR-627-3p	3.805075372	2.55569842	hsa-miR-1245a	3.385768536	2.3822821	hsa-miR-6721-5p	3.594183368	2.89539765
hsa-miR-151b	3.926826976	2.05779406	hsa-miR-1224-3p	3.803390963	2.49395985	hsa-miR-5010-5p	3.385768536	2.17328518	hsa-miR-1268b	3.591215995	2.51872511
hsa-miR-578	3.924916143	2.369688436	hsa-miR-513b-5p	3.803170822	2.18993254	hsa-miR-512-5p	3.378274331	2.51168895	hsa-miR-548al	3.590374385	2.74936121
hsa-miR-516a-5p	3.923475755	2.37921641	hsa-miR-146b-5p	3.802858449	2.31009969	hsa-miR-514a-3p	3.378195177	2.27748467	hsa-miR-337-5p	3.587402959	2.67389493
hsa-miR-548v	3.913400973	2.400986831	hsa-miR-299-3p	3.801684273	2.4950762	hsa-miR-3144-3p	3.376670679	2.28289396	hsa-miR-211-5p	3.586925815	2.57849081
hsa-miR-376b-3p	3.913400973	2.000353028	hsa-miR-580-3p	3.800672919	2.12343344	hsa-miR-1185-2-3p	3.375770289	2.0849971	hsa-miR-301b-5p	3.586852902	2.78147812
hsa-miR-1323	3.910797442	2.241480322	hsa-miR-2117	3.79878903	2.70340881	hsa-miR-922	3.367055351	2.3637547	hsa-miR-520a-3p	3.584943116	2.41865085
hsa-miR-598-3p	3.903793841	2.181750558	hsa-miR-3180-5p	3.792428613	2.71030475	hsa-miR-369-5p	3.348758098	2.44573328	hsa-miR-152-5p	3.584943116	2.33771849
hsa-miR-1183	3.897001179	2.436739762	hsa-miR-188-3p	3.790679254	2.03184459	hsa-miR-1261	3.347730082	2.18225828	hsa-miR-526b-5p	3.583456566	2.82723925
hsa-miR-628-3p	3.892397707	2.097748185	hsa-miR-369-3p	3.784083309	2.5320614	hsa-miR-487a-3p	3.346875911	2.27956222	hsa-miR-3074-3p	3.580714696	2.56341499
hsa-miR-508-3p	3.886306316	2.180611538	hsa-miR-499a-5p	3.783759733	2.22963721	hsa-miR-516a-3p+hsa-miR-516b-3p	3.341184739	2.07157552	hsa-miR-3916	3.575836247	2.56341499
hsa-miR-1224-3p	3.883550444	2.44067641	hsa-miR-181d-3p	3.769397093	2.21247187	hsa-miR-363-3p	3.339673667	2.02286908	hsa-miR-4421	3.568444785	2.59307784
hsa-miR-607	3.878476865	2.387693353	hsa-miR-615-3p	3.759126351	2.59801425	hsa-miR-4488	3.338207031	2.40086162	hsa-miR-1537-3p	3.568024966	2.78477594

hsa-miR-643	3.876390535	2.250213975	hsa-miR-942-3p	3.755040852	2.11559518	hsa-miR-490-5p	3.33037758	2.19708715	hsa-miR-3180-3p	3.566793854	2.2954983
hsa-miR-512-5p	3.875186784	2.504101207	hsa-miR-671-3p	3.753535692	2.57836198	hsa-miR-486-3p	3.328693171	2.26118663	hsa-miR-129-5p	3.557455729	2.22484898
hsa-miR-596	3.874362281	2.311304206	hsa-miR-873-3p	3.746825424	2.17959551	hsa-miR-105-5p	3.326229518	2.44099339	hsa-miR-1245b-3p	3.554302286	2.62773117
hsa-miR-23c	3.871354904	2.350347492	hsa-miR-509-3p	3.745381163	2.74008728				hsa-miR-1185-2-3p	3.547281747	2.22022943
hsa-miR-133a-3p	3.871315079	2.421991029	hsa-miR-1288-3p	3.744489785	2.5320614				hsa-miR-205-5p	3.531733109	2.55362364
hsa-miR-432-5p	3.869872936	2.436739762	hsa-miR-2113	3.741787464	2.2779431				hsa-miR-6503-5p	3.524862783	2.83627232
hsa-miR-378d	3.868017298	2.173693819	hsa-miR-125a-3p	3.739033681	2.71030475				hsa-miR-1275	3.5078346	2.53857887
hsa-miR-449b-5p	3.86290901	2.436739762	hsa-miR-509-3-5p	3.731970473	2.74197649				hsa-miR-604	3.506583889	2.31676741
hsa-miR-1273c	3.859755567	2.016316553	hsa-miR-4435	3.730373992	3.2538809				hsa-miR-10b-5p	3.50625067	2.95341575
hsa-miR-766-5p	3.856759306	2.184318525	hsa-miR-5196-5p	3.727367323	2.84735056				hsa-miR-130b-3p	3.501174745	2.25664236
hsa-miR-514b-3p	3.848525825	2.436739762	hsa-miR-302e	3.725202792	2.74197649				hsa-miR-520b	3.49726498	2.89539765
hsa-miR-487a-3p	3.845196885	2.504101207	hsa-miR-922	3.724168766	2.71030475				hsa-miR-375	3.493240703	2.20161321
hsa-miR-519b-5p+hsa-miR-519c-5p+hsa-miR-523-5p+hsa-miR-518e-5p+hsa-miR-522-5p+hsa-miR-519a-5p	3.843211977	2.204443999	hsa-miR-642a-3p	3.708595292	3.33888096				hsa-miR-3185	3.485485327	2.19625091
hsa-miR-509-3-5p	3.841144864	2.436739762	hsa-miR-376c-5p	3.705741287	2.27612122				hsa-miR-767-3p	3.484852149	2.4922591
hsa-miR-516a-3p+hsa-miR-516b-3p	3.840079078	2.221575065	hsa-miR-1262	3.705318705	2.04053343				hsa-miR-744-5p	3.484371491	2.56341499
hsa-miR-410-3p	3.8391877	2.332612162	hsa-miR-766-5p	3.702390066	2.15434517				hsa-miR-363-3p	3.48290874	2.28609449
hsa-miR-1185-2-3p	3.827056814	2.32517759	hsa-miR-875-3p	3.696516997	2.49395985				hsa-miR-510-3p	3.476359847	2.36713492
hsa-miR-128-2-5p	3.824831459	2.181750558	hsa-miR-891a-5p	3.69271413	2.19485714				hsa-miR-934	3.473966253	2.37609189
hsa-miR-1245b-5p	3.82169856	2.024797372	hsa-miR-548l	3.689077898	2.13731177				hsa-miR-589-5p	3.470765674	2.15670309
hsa-miR-3144-3p	3.802259728	2.311304206	hsa-miR-329-3p	3.677278498	2.30546816				hsa-miR-513a-3p	3.469433093	2.83627232
hsa-miR-1305	3.801943243	2.436739762	hsa-miR-561-5p	3.675988928	3.40635441				hsa-miR-761	3.467154846	2.33475518
hsa-miR-323b-3p	3.796744292	2.109895989	hsa-miR-367-3p	3.670852366	2.58963901				hsa-miR-548v	3.456663963	2.77718239
hsa-miR-1205	3.7887124	2.159981087	hsa-miR-513a-5p	3.670035206	2.16501439				hsa-miR-3180-5p	3.453499404	2.23118853
hsa-miR-449a	3.787508649	2.504101207	hsa-miR-628-3p	3.664394199	2.0071485				hsa-miR-579-5p	3.449447384	2.08874819
hsa-miR-764	3.785202177	2.419880874	hsa-miR-514a-5p	3.661671546	2.71030475				hsa-miR-365b-5p	3.441561652	2.01901456
hsa-miR-548ai+hsa-miR-570-5p	3.78387921	2.421991029	hsa-miR-103a-3p	3.659034797	2.57192733				hsa-miR-519b-5p+hsa-miR-519c-5p+hsa-miR-523-5p+hsa-miR-518e-5p+hsa-miR-522-5p+hsa-miR-519a-5p	3.439372602	2.11830659
hsa-miR-299-5p	3.781925523	2.304120758	hsa-miR-548ai+hsa-miR-570-5p	3.657446936	2.5320614				hsa-miR-370-3p	3.439215118	2.59737943

hsa-miR-298	3.772791725	2.480596659	hsa-miR-3144-3p	3.655941775	2.49395985				hsa-miR-4707-3p	3.436879361	2.03233503
hsa-miR-3613-5p	3.770415147	2.263526855	hsa-miR-142-5p	3.655187425	2.50089776				hsa-miR-889-3p	3.430907865	2.52444276
hsa-miR-208a-3p	3.768462547	2.436739762	hsa-miR-370-3p	3.652564677	2.49395985				hsa-miR-151b	3.426683255	2.33275795
hsa-miR-3916	3.764198912	2.387693353	hsa-miR-891b	3.650700005	2.57949057				hsa-miR-573	3.422242795	2.16001789
hsa-miR-642a-5p	3.757639568	2.169285871	hsa-miR-1305	3.647430078	2.32245766				hsa-miR-3613-3p	3.414321067	2.67071997
hsa-miR-3605-3p	3.757516931	2.436739762	hsa-miR-887-3p	3.641164819	2.70674568				hsa-miR-339-3p	3.414306199	2.66453028
hsa-miR-513c-5p	3.752663197	2.128244261	hsa-miR-764	3.640389385	2.13331714				hsa-miR-23c	3.413135926	2.48560671
hsa-miR-761	3.747960483	2.067983384	hsa-miR-449b-5p	3.638749933	2.6038763				hsa-miR-3202	3.411653958	2.59845552
hsa-miR-548y	3.746248331	2.403674085	hsa-miR-98-3p	3.637256486	2.35123766				hsa-miR-455-3p	3.408500515	2.0808356
hsa-miR-490-5p	3.743127293	2.495062237	hsa-miR-526b-5p	3.634376076	2.0926752				hsa-miR-643	3.398467313	2.25280768
hsa-miR-4792	3.734020425	2.260149744	hsa-miR-1205	3.63295581	2.17783242				hsa-miR-2113	3.39066753	2.89539765
hsa-miR-548h-5p	3.72586941	2.400986831	hsa-miR-620	3.632922332	2.49395985				hsa-miR-521	3.388745199	2.46238611
hsa-miR-612	3.717792383	2.284170088	hsa-miR-3182	3.626601788	2.49395985				hsa-miR-431-5p	3.387813526	2.22035192
hsa-miR-1469	3.717041436	2.292984134	hsa-miR-1296-3p	3.625058679	2.71030475				hsa-miR-212-3p	3.384889687	2.95341575
hsa-miR-553	3.715837685	2.097748185	hsa-miR-299-5p	3.62219106	2.38612959				hsa-miR-1257	3.378950596	2.63106359
hsa-miR-211-5p	3.71420427	2.400986831	hsa-miR-1910-5p	3.615370089	2.6669779				hsa-miR-4755-5p	3.37589147	2.78147812
hsa-miR-4787-5p	3.713229805	2.204443999	hsa-miR-3147	3.612005158	2.13277674				hsa-miR-190b	3.375157576	2.25664236
hsa-miR-4755-5p	3.708857025	2.37921641	hsa-miR-642a-5p	3.61147207	2.14400977				hsa-miR-601	3.374266197	2.78147812
hsa-miR-487b-3p	3.704866144	2.399649024	hsa-miR-326	3.60758815	2.2703361				hsa-miR-323b-3p	3.366492506	2.60649358
hsa-miR-664b-5p	3.704866144	2.198462732	hsa-miR-301b-5p	3.603581389	2.03724957				hsa-miR-4787-5p	3.363514798	2.65971601
hsa-miR-518b	3.700875264	2.161094541	hsa-miR-200c-3p	3.602613062	2.54379704				hsa-miR-514b-3p	3.36028728	2.30642748
hsa-miR-520a-5p	3.699830513	2.37921641	hsa-miR-4792	3.583334681	2.35123766				hsa-miR-455-5p	3.347139494	2.38800248
hsa-miR-323b-5p	3.694950363	2.304120758	hsa-miR-3074-3p	3.575397673	2.60993329				hsa-miR-940	3.346861276	2.68310026
hsa-miR-376c-3p	3.694555524	2.204443999	hsa-miR-1323	3.573993411	2.46399816				hsa-miR-933	3.338627796	2.41174212
hsa-miR-324-3p	3.694431438	2.358227195	hsa-miR-526a+hsa-miR-518c-5p+hsa-miR-518d-5p	3.57095474	2.54379704				hsa-miR-1249-3p	3.338470491	2.83627232
hsa-miR-579-5p	3.693003225	2.284170088	hsa-miR-487a-3p	3.570161531	2.28737692				hsa-miR-346	3.338147138	2.46731852
hsa-miR-652-5p	3.692690852	2.436739762	hsa-miR-1185-2-3p	3.567931872	2.49395985				hsa-miR-708-5p	3.337304829	2.42775632
hsa-miR-502-5p	3.686565984	2.436739762	hsa-miR-566	3.565071467	2.42328481				hsa-miR-324-3p	3.331641257	2.60315931
hsa-miR-708-5p	3.673829676	2.400986831	hsa-miR-1275	3.564655285	2.40323039				hsa-miR-1296-3p	3.327800359	2.68661276
hsa-miR-767-3p	3.673829676	2.182341497	hsa-miR-1295a	3.563602433	2.37173639				hsa-miR-329-3p	3.323043207	2.20996127
hsa-miR-548o-3p+hsa-miR-548ah-3p+hsa-miR-548av-3p	3.66762445	2.2943528	hsa-miR-520c-3p	3.560823406	2.62685817				hsa-miR-506-3p	3.320624601	2.87871455
hsa-miR-548k	3.666135138	2.173693819	hsa-miR-576-5p	3.560726408	2.35125322						
hsa-miR-499a-5p	3.662349461	2.155149466	hsa-miR-520e	3.560053352	2.16845696						
hsa-miR-1224-5p	3.648224477	2.284170088	hsa-miR-1204	3.55880264	2.42098292						
hsa-miR-513b-5p	3.646223167	2.204443999	hsa-miR-889-3p	3.556682913	2.60993329						
hsa-miR-892b	3.645964966	2.387693353	hsa-miR-513c-5p	3.543118562	2.49395985						
hsa-miR-744-5p	3.6442002	2.436739762	hsa-miR-1183	3.540846115	2.31009969						
hsa-miR-369-3p	3.640971923	2.387693353	hsa-miR-563	3.540422122	2.04409828						
hsa-miR-125a-3p	3.638191275	2.188135863	hsa-miR-548al	3.537681953	2.71030475						
hsa-miR-889-3p	3.633753723	2.44067641	hsa-miR-1908-3p	3.536059547	2.58309269						
hsa-miR-301a-5p	3.627265409	2.284170088	hsa-miR-544a	3.535386491	3.53938734						
hsa-miR-299-3p	3.62710379	2.165397593	hsa-miR-1255a	3.533041161	2.03184459						
hsa-miR-135a-5p	3.621019889	2.400986831	hsa-miR-1233-3p	3.523042914	2.55630398						
hsa-miR-146b-3p	3.621019889	2.332612162	hsa-miR-1827	3.520287042	2.74197649						
hsa-miR-615-3p	3.617188009	2.369688436	hsa-miR-561-3p	3.519101103	2.35477758						
hsa-miR-1264	3.610980404	2.250850796	hsa-miR-573	3.519067706	2.6669779						

hsa-miR-584-3p	3.608007189	2.173693819	hsa-miR-1269b	3.515076825	2.24063956						
hsa-miR-660-3p	3.607272228	2.182341497	hsa-miR-3196	3.513607791	2.05513571						
hsa-miR-592	3.605672838	2.006267048	hsa-miR-34c-3p	3.512574313	2.28737692						
hsa-miR-3182	3.60429884	2.436739762	hsa-miR-3185	3.509385654	2.65812028						
hsa-miR-3131	3.60324795	2.400986831	hsa-miR-3613-5p	3.509385654	2.17959551						
hsa-miR-376a-2-5p	3.597556779	2.220716342	hsa-miR-548y	3.508963072	2.34837942						
hsa-miR-638	3.59481661	2.504101207	hsa-miR-582-5p	3.505158672	2.32117313						
hsa-miR-302e	3.584867432	2.504101207	hsa-miR-1202	3.501812295	2.49569063						
hsa-miR-363-3p	3.580617552	2.44067641	hsa-miR-1293	3.501744791	2.57192733						
hsa-miR-542-5p	3.566084912	2.182341497	hsa-miR-3192-5p	3.500278785	2.41863072						
hsa-miR-514b-5p	3.560427879	2.44067641	hsa-miR-520d-5p+hsa-miR-527+hsa-miR-518a-5p	3.498787627	2.05513571						
hsa-miR-375	3.557696241	2.32416138	hsa-miR-2053	3.498578495	2.6786006						
hsa-miR-765	3.557192391	2.32517759	hsa-miR-363-3p	3.488227019	2.71030475						
hsa-miR-937-3p	3.556324205	2.221575065	hsa-miR-483-3p	3.487245512	2.22871666						
hsa-miR-526a+hsa-miR-518c-5p+hsa-miR-518d-5p	3.551881272	2.284170088	hsa-miR-1245b-5p	3.485031167	2.00827918						
hsa-miR-502-3p	3.55097696	2.172072468	hsa-miR-382-5p	3.478902006	2.60993329						
hsa-miR-223-3p	3.549462761	2.27832244	hsa-miR-383-5p	3.477689065	2.57836198						
hsa-miR-606	3.549031171	2.179368467	hsa-miR-598-3p	3.475682236	2.23912932						
hsa-miR-378c	3.546889081	2.167745396	hsa-miR-590-3p	3.472375217	2.31087879						
hsa-miR-181b-2-3p	3.543479371	2.480596659	hsa-miR-409-3p	3.468864994	2.58309269						
hsa-miR-203a-3p	3.536851976	2.369688436	hsa-miR-548o-3p+hsa-miR-548ah-3p+hsa-miR-548av-3p	3.468078861	2.1641394						
hsa-miR-873-5p	3.533341753	2.332612162	hsa-miR-579-5p	3.467191549	2.64273826						
hsa-miR-188-3p	3.528027905	2.155149466	hsa-miR-199a-3p+hsa-miR-199b-3p	3.465921529	2.6669779						
hsa-miR-663a	3.524003628	2.155149466	hsa-miR-873-5p	3.460199925	2.04384509						
hsa-miR-548l	3.515852613	2.260149744	hsa-miR-301a-5p	3.454494074	2.20350863						
hsa-miR-4485-3p	3.511129356	2.198462732	hsa-miR-1268b	3.451832331	2.74197649						
hsa-miR-483-5p	3.505045454	2.305170252	hsa-miR-761	3.450014828	2.11559518						
hsa-miR-627-3p	3.503509036	2.504101207	hsa-miR-449a	3.449392765	2.31087879						
hsa-miR-520b	3.501366947	2.222281551	hsa-miR-410-3p	3.44871971	2.05790264						
hsa-miR-1268b	3.500218955	2.141151103	hsa-miR-514b-3p	3.446770018	2.54782139						
hsa-miR-758-3p+hsa-miR-411-3p	3.495759943	2.173693819	hsa-miR-3916	3.445401885	2.23912932						
hsa-miR-3130-3p	3.4895355	2.289651911	hsa-miR-210-5p	3.445049449	2.78698127						
hsa-miR-1296-5p	3.487507245	2.246487414	hsa-miR-514a-3p	3.443914536	2.04053343						
hsa-miR-2053	3.484677155	2.37192957	hsa-miR-376b-3p	3.440568695	2.90340416						
hsa-miR-1262	3.484477587	2.169285871	hsa-miR-1296-5p	3.438884286	2.38612959						
hsa-miR-34b-3p	3.482391257	2.489593305	hsa-miR-25-5p	3.436268019	2.21247187						
hsa-miR-1285-5p	3.47309733	2.24430651	hsa-miR-6503-3p	3.435647577	2.29969279						
hsa-miR-890	3.470215965	2.033739594	hsa-miR-933	3.43366267	2.291779						
hsa-miR-103a-3p	3.4643657	2.441790469	hsa-miR-577	3.427915124	2.5320614						
hsa-miR-891b	3.460414697	2.419880874	hsa-miR-1297	3.427365212	2.21211458						

hsa-miR-1249-5p	3.458040673	2.256045546	hsa-miR-202-3p	3.42002311	2.6786006						
hsa-miR-520d-3p	3.456708434	2.304120758	hsa-miR-591	3.40990574	2.31096531						
hsa-miR-212-3p	3.455576634	2.436739762	hsa-miR-320b	3.40921595	2.71030475						
hsa-miR-510-3p	3.455186669	2.142583576	hsa-miR-381-3p	3.40228724	2.7993099						
hsa-miR-199b-5p	3.453897098	2.305745508	hsa-miR-584-3p	3.399633209	2.29969279						
hsa-miR-608	3.449331116	2.159981087	hsa-miR-1908-5p	3.399430349	2.0277808						
hsa-miR-382-5p	3.44726929	2.260149744	hsa-miR-1264	3.396674477	2.6786006						
hsa-miR-519b-3p	3.444854706	2.006040639	hsa-miR-302b-3p	3.391858075	2.14986856						
hsa-miR-580-3p	3.442312396	2.214093741	hsa-miR-758-3p+hsa-miR-411-3p	3.390880547	2.28645969						
hsa-miR-196a-3p	3.441061685	2.504101207	hsa-miR-652-5p	3.388528961	2.2539842						
hsa-miR-6511a-3p	3.428174478	2.250850796	hsa-miR-509-5p	3.387555667	2.62685817						
hsa-miR-556-3p	3.425021035	2.349535509	hsa-miR-4707-5p	3.384697081	2.19148361						
hsa-miR-577	3.423658189	2.142583576	hsa-miR-892b	3.379863892	2.29969279						
hsa-miR-576-5p	3.416509338	2.020282779	hsa-miR-4787-5p	3.377420393	2.41467226						
hsa-miR-1910-5p	3.416116607	2.37192957	hsa-miR-548h-5p	3.375873011	2.54782139						
hsa-miR-548ar-3p	3.414744571	2.24993301	hsa-miR-661	3.374391043	2.10922923						
hsa-miR-582-5p	3.414371058	2.184318525	hsa-miR-1304-5p	3.374365118	2.60664476						
hsa-miR-654-5p	3.413376438	2.284170088	hsa-miR-514b-5p	3.370480565	2.29969279						
hsa-miR-545-3p	3.413376438	2.086479653	hsa-miR-607	3.364692338	2.58963901						
hsa-miR-4431	3.412862383	2.32416138	hsa-miR-617	3.360030221	2.07242145						
hsa-miR-642a-3p	3.41248506	2.064158428	hsa-miR-548j-3p	3.351408591	2.38295569						
hsa-miR-25-5p	3.412075959	2.202779139	hsa-miR-1279	3.349341478	2.37121416						
hsa-miR-367-3p	3.411080976	2.289651911	hsa-miR-1197	3.348261265	2.01115622						
hsa-miR-1306-3p	3.404711369	2.258884508	hsa-miR-298	3.338393777	2.10922923						
hsa-miR-518c-3p	3.401201146	2.37192957	hsa-miR-567	3.336652131	2.40775957						
hsa-miR-383-5p	3.39982911	2.250155279	hsa-miR-147a	3.327008845	2.2703361						
hsa-miR-373-3p	3.393591836	2.420823108	hsa-miR-142-3p	3.326391253	2.56756134						
hsa-miR-4741	3.383429208	2.086751046	hsa-miR-520a-5p	3.322227605	2.16905756						
hsa-miR-526b-5p	3.382716998	2.137964264	hsa-miR-597-5p	5.962093008	2.2539842						
hsa-miR-302d-3p	3.369455367	2.182341497	hsa-miR-1228-3p	5.67196465	2.00277596						
hsa-miR-513c-3p	3.368712285	2.208768217	hsa-miR-675-5p	5.642695856	2.41863072						
hsa-miR-219a-2-3p	3.366218963	2.02082792	hsa-miR-572	5.420101847	2.5320614						
hsa-miR-370-5p	3.365327585	2.001434926	hsa-miR-203a-5p	5.231936344	2.49496586						
hsa-miR-1972	3.364856651	2.169285871	hsa-miR-133b	5.103172225	2.49395985						
hsa-miR-18b-5p	3.357592752	2.489593305	hsa-miR-3605-5p	5.07049937	2.60700105						
hsa-miR-217	3.346915188	2.250213975	hsa-miR-518e-3p	5.042586638	2.73407775						
hsa-miR-548z+hsa-miR-548h-3p	3.345531974	2.311304206	hsa-miR-1248	5.007065658	2.6786006						
hsa-miR-450b-3p	3.34452062	2.086751046	hsa-miR-208b-5p	4.950028352	2.45458696						
hsa-miR-548e-5p	3.344438155	2.407898513	hsa-miR-3150b-3p	4.883726996	2.2406883						
hsa-miR-302a-5p	3.343847564	2.084251205	hsa-miR-517c-3p+hsa-miR-519a-3p	4.78104169	2.08319765						
hsa-miR-942-5p	3.337179277	2.182341497	hsa-miR-433-5p	4.707086617	2.56756134						
hsa-miR-3615	3.336703322	2.284170088	hsa-miR-5010-3p	4.694248605	2.98568856						
hsa-miR-210-5p	3.335380355	2.258884508	hsa-miR-34c-5p	4.690825826	2.23152289						
hsa-miR-4425	3.327784633	2.181750558	hsa-miR-4647	4.668101992	2.65812028						

hsa-miR-1289	3.324528708	2.400986831	hsa-miR-1910-3p	4.665757045	2.64296568						
hsa-miR-1-5p	3.322347081	2.37192957	hsa-miR-512-3p	4.655187425	2.19709009						
			hsa-miR-124-3p	4.618028996	2.41863072						
			hsa-miR-924	4.607138571	2.37121416						
			hsa-miR-328-5p	4.606979571	2.17959551						
			hsa-miR-708-5p	4.547364005	2.6038763						
			hsa-miR-885-5p	4.527411212	2.98568856						
			hsa-miR-539-3p	4.521839163	2.3062405						
			hsa-miR-492	4.510867622	2.15436201						
			hsa-miR-3168	4.504263787	2.29038661						
			hsa-miR-513a-3p	4.45234624	2.09852102						
			hsa-miR-302a-3p	4.441338749	2.42362522						
			hsa-miR-5001-5p	4.433700618	2.20537694						
			hsa-miR-548m	4.414550342	2.51299538						
			hsa-miR-4707-3p	4.414365417	2.42098292						
			hsa-miR-511-5p	4.405885273	2.06416787						
			hsa-miR-495-5p	4.404897085	2.70340881						
			hsa-miR-637	4.403327001	2.17172859						
			hsa-miR-1271-3p	4.371039821	2.49540055						
			hsa-miR-494-5p	4.358581731	2.32245766						
			hsa-miR-522-3p	4.342974779	2.35030812						
			hsa-miR-1-3p	4.320482055	2.57192733						
			hsa-miR-381-5p	4.315567749	2.2779431						
			hsa-miR-187-3p	4.311325068	2.47461728						
			hsa-miR-345-3p	4.306125815	2.25009958						
			hsa-miR-339-5p	4.302381863	2.41863072						
			hsa-miR-1272	4.297018946	2.12343344						
			hsa-miR-650	4.288895675	2.74197649						
			hsa-miR-452-5p	4.287352566	2.05591631						
			hsa-miR-556-5p	4.282087782	3.53938734						
			hsa-miR-4488	4.277848097	2.0910827						
			hsa-miR-1200	4.240267347	2.71030475						
			hsa-miR-1281	4.236377983	2.14869576						
			hsa-miR-3614-3p	4.221684388	2.49421588						
			hsa-miR-328-3p	4.21491824	2.44925046						
			hsa-miR-888-5p	4.204507621	2.70340881						
			hsa-miR-190a-3p	4.193541461	2.5320614						
			hsa-miR-4421	4.190720935	2.25127083						
			hsa-miR-1302	4.187989013	2.44648216						
			hsa-miR-5001-3p	4.17101669	2.01057852						
			hsa-miR-182-3p	4.167277162	2.02849724						
			hsa-miR-181a-2-3p	4.162628696	2.77896629						
			hsa-miR-375	4.157008952	2.12493211						
			hsa-miR-3179	4.155443216	2.57192733						
			hsa-miR-6721-5p	4.147203001	2.60664476						
			hsa-miR-519b-5p+hsa-miR-519c-5p+hsa-miR-523-5p+hsa-miR-518c-5p+hsa-miR-522-5p+hsa-miR-519a-5p	4.141222832	2.5320614						

		hsa-miR-297	4.139041883	2.22968847						
		hsa-miR-576-3p	4.136286011	2.1362071						
		hsa-miR-23c	4.117318879	2.54379704						
		hsa-miR-1257	4.116829486	2.97646032						
		hsa-miR-502-5p	4.110825941	2.73407775						
		hsa-miR-578	4.110745584	2.07334546						
		hsa-miR-548v	4.10977309	2.71030475						
		hsa-miR-512-5p	4.102884284	2.74197649						
		hsa-miR-1185-5p	4.093534661	2.19962324						
		hsa-miR-152-5p	4.088374186	2.42098292						
		hsa-miR-490-5p	4.088374186	2.23908216						
		hsa-miR-767-3p	4.079526923	2.57192733						
		hsa-miR-211-3p	4.072135757	2.11637196						
		hsa-miR-887-5p	4.069513009	2.70674568						
		hsa-miR-648	4.067907337	2.31096531						
		hsa-miR-211-5p	4.063707325	2.5320614						
		hsa-miR-510-3p	4.052272224	2.28645969						
		hsa-miR-626	4.051566189	2.55569842						
		hsa-miR-518c-3p	4.049995615	2.31009969						
		hsa-miR-548a-3p	4.046530558	2.49395985						
		hsa-miR-606	4.036219938	2.5320614						
		hsa-miR-2110	4.022744668	2.51854705						
		hsa-miR-515-3p	4.020168279	2.71030475						
		hsa-miR-532-3p	4.017403877	2.17959551						
		hsa-miR-890	4.009919662	2.5412181						
		hsa-miR-619-3p	4.00667002	2.41863072						
		hsa-miR-519b-3p	4.000978848	2.18393415						
		hsa-miR-3614-5p	4.000797946	2.03184459						
		hsa-miR-802	3.985119612	2.54782139						
		hsa-miR-571	3.983778283	2.55569842						
		hsa-miR-337-5p	3.983715528	2.35027526						
		hsa-miR-4536-3p	3.983058873	2.13731177						
		hsa-miR-363-5p	3.981457551	2.47976337						
		hsa-miR-519e-3p	3.977040543	2.57353062						
		hsa-miR-1193	3.974859595	2.08033129						
		hsa-miR-105-5p	3.964384593	2.74197649						
		hsa-miR-1249-3p	3.962775802	2.64315364						
		hsa-miR-133a-3p	3.96096465	2.84781678						
		hsa-miR-338-5p	3.960483076	2.74197649						
		hsa-miR-1224-5p	3.95256608	2.84735056						
		hsa-miR-504-5p	3.95256608	2.5320614						
		hsa-miR-596	3.950028352	2.70340881						
		hsa-miR-300	3.940030106	2.11168904						
		hsa-miR-450a-2-3p	3.939593646	2.31382785						
		hsa-miR-1271-5p	3.939221193	2.57836198						
		hsa-miR-516a-3p+hsa-miR-516b-3p	3.936088294	2.17959551						
		hsa-miR-192-5p	3.926269215	2.49395985						
		hsa-miR-370-5p	3.921451728	2.0050644						
		hsa-miR-516b-5p	3.920640343	2.54782139						
		hsa-miR-1301-3p	3.91801939	2.2779431						

		hsa-miR-487b-5p	3.917421952	2.14986856						
		hsa-miR-553	3.909240485	2.31009969						
		hsa-miR-130b-3p	3.908993638	2.11559518						
		hsa-miR-651-3p	3.899077856	2.47647783						
		hsa-miR-767-5p	3.895846529	2.2779431						
		hsa-miR-521	3.892297447	2.58963901						
		hsa-miR-548a-5p	3.890549519	2.28645969						
		hsa-miR-585-3p	3.889415578	2.5666323						
		hsa-miR-876-5p	3.884326777	2.15436201						
		hsa-miR-507	3.883749121	2.42098292						
		hsa-miR-1278	3.883726996	2.74197649						
		hsa-miR-302f	3.883517865	2.20537694						
		hsa-miR-128-2-5p	3.881211393	2.4684252						
		hsa-miR-1469	3.864196278	2.35918805						
		hsa-miR-337-3p	3.863506819	2.42098292						
		hsa-miR-885-3p	3.856030478	2.71030475						
		hsa-miR-324-3p	3.84678824	2.11168904						
		hsa-miR-127-5p	3.846268069	2.17959551						
		hsa-miR-4516	3.839626383	2.21373498						
		hsa-miR-524-3p	3.83799573	2.74197649						
		hsa-miR-339-3p	3.833257158	2.32130769						
		hsa-miR-137	3.830440065	2.58963901						
		hsa-miR-516a-5p	3.82050659	2.44662795						
		hsa-miR-548g-3p	3.814815419	2.5320614						
		hsa-miR-384	3.813343655	2.04569774						
		hsa-miR-1306-3p	3.813050652	2.27112772						
		hsa-miR-518d-3p	3.812078158	2.59801425						
		hsa-miR-197-5p	3.80785064	2.04409828						
		hsa-miR-627-3p	3.805075372	2.55569842						
		hsa-miR-1224-3p	3.803390963	2.49395985						
		hsa-miR-513b-5p	3.803170822	2.18993254						
		hsa-miR-146b-5p	3.802858449	2.31009969						
		hsa-miR-299-3p	3.801684273	2.4950762						
		hsa-miR-580-3p	3.800672919	2.12343344						
		hsa-miR-2117	3.79878903	2.70340881						
		hsa-miR-3180-5p	3.792428613	2.71030475						
		hsa-miR-188-3p	3.790679254	2.03184459						
		hsa-miR-369-3p	3.784083309	2.5320614						
		hsa-miR-499a-5p	3.783759733	2.22963721						
		hsa-miR-181d-3p	3.769397093	2.21247187						
		hsa-miR-615-3p	3.759126351	2.59801425						
		hsa-miR-942-3p	3.755040852	2.11559518						
		hsa-miR-671-3p	3.753535692	2.57836198						
		hsa-miR-873-3p	3.746825424	2.17959551						
		hsa-miR-509-3p	3.745381163	2.74008728						
		hsa-miR-1288-3p	3.744489785	2.5320614						
		hsa-miR-2113	3.741787464	2.2779431						
		hsa-miR-125a-3p	3.739033681	2.71030475						
		hsa-miR-509-3-5p	3.731970473	2.74197649						
		hsa-miR-4435	3.730373992	3.2538809						
		hsa-miR-5196-5p	3.727367323	2.84735056						

		hsa-miR-302e	3.725202792	2.74197649					
		hsa-miR-922	3.724168766	2.71030475					
		hsa-miR-642a-3p	3.708595292	3.33888096					
		hsa-miR-376c-5p	3.705741287	2.27612122					
		hsa-miR-1262	3.705318705	2.04053343					
		hsa-miR-766-5p	3.702390066	2.15434517					
		hsa-miR-875-3p	3.696516997	2.49395985					
		hsa-miR-891a-5p	3.69271413	2.19485714					
		hsa-miR-548l	3.689077898	2.13731177					
		hsa-miR-329-3p	3.677278498	2.30546816					
		hsa-miR-561-5p	3.675988928	3.40635441					
		hsa-miR-367-3p	3.670852366	2.58963901					
		hsa-miR-513a-5p	3.670035206	2.16501439					
		hsa-miR-628-3p	3.664394199	2.0071485					
		hsa-miR-514a-5p	3.661671546	2.71030475					
		hsa-miR-103a-3p	3.659034797	2.57192733					
		hsa-miR-548ai+hsa-miR-570-5p	3.657446936	2.5320614					
		hsa-miR-3144-3p	3.655941775	2.49395985					
		hsa-miR-142-5p	3.655187425	2.50089776					
		hsa-miR-370-3p	3.652564677	2.49395985					
		hsa-miR-891b	3.650700005	2.57949057					
		hsa-miR-1305	3.647430078	2.32245766					
		hsa-miR-887-3p	3.641164819	2.70674568					
		hsa-miR-764	3.640389385	2.13331714					
		hsa-miR-449b-5p	3.638749933	2.6038763					
		hsa-miR-98-3p	3.637256486	2.35123766					
		hsa-miR-526b-5p	3.634376076	2.0926752					
		hsa-miR-1205	3.63295581	2.17783242					
		hsa-miR-620	3.632922332	2.49395985					
		hsa-miR-3182	3.626601788	2.49395985					
		hsa-miR-1296-3p	3.625058679	2.71030475					
		hsa-miR-299-5p	3.62219106	2.38612959					
		hsa-miR-1910-5p	3.615370089	2.6669779					
		hsa-miR-3147	3.612005158	2.13277674					
		hsa-miR-642a-5p	3.61147207	2.14400977					
		hsa-miR-326	3.60758815	2.2703361					
		hsa-miR-301b-5p	3.603581389	2.03724957					
		hsa-miR-200c-3p	3.602613062	2.54379704					
		hsa-miR-4792	3.583334681	2.35123766					
		hsa-miR-3074-3p	3.575397673	2.60993329					
		hsa-miR-1323	3.573993411	2.46399816					
		hsa-miR-526a+hsa-miR-518c-5p+hsa-miR-518d-5p	3.57095474	2.54379704					
		hsa-miR-487a-3p	3.570161531	2.28737692					
		hsa-miR-1185-2-3p	3.567931872	2.49395985					
		hsa-miR-566	3.565071467	2.42328481					
		hsa-miR-1275	3.564655285	2.40323039					
		hsa-miR-1295a	3.563602433	2.37173639					
		hsa-miR-520c-3p	3.560823406	2.62685817					

			hsa-miR-576-5p	3.560726408	2.35125322						
			hsa-miR-520e	3.560053352	2.16845696						
			hsa-miR-1204	3.55880264	2.42098292						
			hsa-miR-889-3p	3.556682913	2.60993329						
			hsa-miR-513c-5p	3.543118562	2.49395985						

Supplementary Table 3 – GO, KEGG pathways and Reactome pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the top 20 targets deregulated genes.

GO Biological Processes				GO Cellular Component			
# genes	term name	description	FDR value	# genes	term name	description	FDR value
31	GO:0050794	Regulation of cellular process	3.40E-04	31	GO:0043229	Intracellular organelle	0.0112
27	GO:0031323	Regulation of cellular metabolic process	3.39E-06	30	GO:0043227	Membrane-bounded organelle	0.0366
26	GO:0051171	Regulation of nitrogen compound metabolic process	5.31E-06	29	GO:0043231	Intracellular membrane-bounded organelle	0.0112
26	GO:0080090	Regulation of primary metabolic process	1.04E-05	27	GO:0005634	Nucleus	4.90E-04
26	GO:0060255	Regulation of macromolecule metabolic process	2.86E-05	21	GO:0070013	Intracellular organelle lumen	0.0112
24	GO:0048519	Negative regulation of biological process	2.86E-05	19	GO:0005654	Nucleoplasm	0.0013
23	GO:0048523	Negative regulation of cellular process	2.86E-05	19	GO:0032991	Protein-containing complex	0.0144
22	GO:0010468	Regulation of gene expression	9.61E-05	11	GO:1902494	Catalytic complex	0.0024
22	GO:0051716	Cellular response to stimulus	0.0094	7	GO:1990234	Transferase complex	0.0255
21	GO:0019219	Regulation of nucleobase-containing compound metabolic process	2.86E-05	5	GO:1902911	Protein kinase complex	5.60E-04
21	GO:0048522	Positive regulation of cellular process	0.0037	4	GO:0000307	Cyclin-dependent protein kinase holoenzyme complex	5.60E-04
20	GO:0051252	Regulation of RNA metabolic process	4.89E-05	2	GO:0016442	RISC complex	0.0345
19	GO:0009892	Negative regulation of metabolic process	2.67E-05	GO Molecular Component			
19	GO:2000112	Regulation of cellular macromolecule biosynthetic process	3.90E-04	# genes	term name	description	FDR value
19	GO:0007165	Signal transduction	0.0077	11	GO:0003723	RNA binding	0.0282
18	GO:0031324	Negative regulation of cellular metabolic process	1.46E-05	7	GO:0004672	Protein kinase activity	0.0271
18	GO:0010605	Negative regulation of macromolecule metabolic process	3.49E-05	5	GO:0019887	Protein kinase regulator activity	0.027
17	GO:0051172	Negative regulation of nitrogen compound metabolic process	2.73E-05	4	GO:0016538	Cyclin-dependent protein serine/threonine kinase regulator activity	0.0055
15	GO:0042127	Regulation of cell population proliferation	1.01E-05	3	GO:0030332	Cyclin binding	0.0271
15	GO:0010629	Negative regulation of gene expression	6.94E-05	Reactome Pathways			
15	GO:0006355	Regulation of transcription, DNA-templated	0.0159	# genes	term name	description	FDR value
15	GO:0010604	Positive regulation of macromolecule metabolic process	0.0272	9	hsa05206	MicroRNAs in cancer	2.31E-09
14	GO:0032268	Regulation of cellular protein metabolic process	0.0064	8	hsa04151	PI3K-Akt signaling pathway	8.97E-06
14	GO:0051173	Positive regulation of nitrogen compound metabolic process	0.0319	8	hsa05200	Pathways in cancer	4.64E-05
14	GO:0031325	Positive regulation of cellular metabolic process	0.0498	7	hsa04068	FoxO signaling pathway	2.53E-07
13	GO:2000113	Negative regulation of cellular macromolecule biosynthetic process	8.36E-05	7	hsa04110	Cell cycle	2.53E-07
13	GO:0033554	Cellular response to stress	4.00E-04	6	hsa05203	Viral carcinogenesis	2.16E-05
13	GO:0007166	Cell surface receptor signaling pathway	0.0064	6	hsa05169	Epstein-Barr virus infection	2.31E-05
12	GO:0007049	Cell cycle	1.80E-04	6	hsa05165	Human papillomavirus infection	3.30E-04
12	GO:0006357	Regulation of transcription by RNA polymerase II	0.0131	5	hsa04115	p53 signaling pathway	1.20E-05
11	GO:0007346	Regulation of mitotic cell cycle	5.31E-06	5	hsa05214	Glioma	1.20E-05
11	GO:0010564	Regulation of cell cycle process	1.46E-05	5	hsa05218	Melanoma	1.20E-05
11	GO:0045934	Negative regulation of nucleobase-containing compound metabolic process	0.0033	5	hsa05220	Chronic myeloid leukemia	1.20E-05
11	GO:0042981	Regulation of apoptotic process	0.0037	5	hsa05222	Small cell lung cancer	2.12E-05
11	GO:0045595	Regulation of cell differentiation	0.0158	5	hsa01522	Endocrine resistance	2.16E-05
10	GO:1901990	Regulation of mitotic cell cycle phase transition	1.61E-06	5	hsa05215	Prostate cancer	2.16E-05
10	GO:1903047	Mitotic cell cycle process	2.17E-05	5	hsa04218	Cellular senescence	1.30E-04
10	GO:0008284	Positive regulation of cell population proliferation	3.70E-04	5	hsa05202	Transcriptional misregulation in cancer	2.20E-04

10	GO:0060548	Negative regulation of cell death	6.60E-04	4	hsa04066	HIF-1 signaling pathway	6.30E-04
10	GO:0032269	Negative regulation of cellular protein metabolic process	9.10E-04	4	hsa05162	Measles	0.0015
10	GO:1901700	Response to oxygen-containing compound	0.0172	4	hsa05224	Breast cancer	0.0018
10	GO:0035556	Intracellular signal transduction	0.0299	4	hsa04934	Cushing syndrome	0.0021
9	GO:2000045	Regulation of G1/S transition of mitotic cell cycle	7.11E-08	4	hsa04630	JAK-STAT signaling pathway	0.0023
9	GO:1901991	Negative regulation of mitotic cell cycle phase transition	2.59E-07	4	hsa05225	Hepatocellular carcinoma	0.0023
9	GO:0010608	Posttranscriptional regulation of gene expression	8.36E-05	4	hsa04510	Focal adhesion	0.0041
9	GO:0006974	Cellular response to DNA damage stimulus	8.20E-04	4	hsa05205	Proteoglycans in cancer	0.0041
9	GO:0009725	Response to hormone	0.0012	4	hsa05166	Human T-cell leukemia virus 1 infection	0.005
9	GO:0043066	Negative regulation of apoptotic process	0.0018	4	hsa05163	Human cytomegalovirus infection	0.0055
9	GO:0045892	Negative regulation of transcription, DNA-templated	0.0181	3	hsa05219	Bladder cancer	9.70E-04
8	GO:2000134	Negative regulation of G1/S transition of mitotic cell cycle	8.66E-08	3	hsa05223	Non-small cell lung cancer	0.0031
8	GO:0007093	Mitotic cell cycle checkpoint	4.32E-07	3	hsa01524	Platinum drug resistance	0.0032
8	GO:0090068	Positive regulation of cell cycle process	1.46E-05	3	hsa05212	Pancreatic cancer	0.0035
8	GO:0000122	Negative regulation of transcription by RNA polymerase II	0.0101	3	hsa05226	Gastric cancer	0.0195
8	GO:0014070	Response to organic cyclic compound	0.0111	3	hsa05160	Hepatitis C	0.0236
8	GO:0009894	Regulation of catabolic process	0.0231	3	hsa05167	Kaposi sarcoma-associated herpesvirus infection	0.0369
8	GO:0012501	Programmed cell death	0.0247	2	hsa05216	Thyroid cancer	0.0195
7	GO:0044773	Mitotic DNA damage checkpoint	7.73E-07	2	hsa04340	Hedgehog signaling pathway	0.0295
7	GO:0008285	Negative regulation of cell population proliferation	0.0135	2	hsa05213	Endometrial cancer	0.0402
7	GO:0080135	Regulation of cellular response to stress	0.0186	2	hsa04213	Longevity regulating pathway - multiple species	0.0445
7	GO:0043086	Negative regulation of catalytic activity	0.0277	KEGG Pathways			
7	GO:0033993	Response to lipid	0.0373	# genes	term name	description	FDR value
7	GO:0031329	Regulation of cellular catabolic process	0.0412	14	HSA-74160	Gene expression (Transcription)	8.77E-06
6	GO:0031571	Mitotic G1 DNA damage checkpoint	2.19E-06	13	HSA-212436	Generic Transcription Pathway	8.77E-06
6	GO:0071156	Regulation of cell cycle arrest	1.68E-05	9	HSA-1640170	Cell Cycle	1.50E-04
6	GO:0044772	Mitotic cell cycle phase transition	0.0011	8	HSA-69278	Cell Cycle, Mitotic	2.80E-04
6	GO:1903311	Regulation of mRNA metabolic process	0.0028	7	HSA-2262752	Cellular responses to stress	0.0025
6	GO:0006417	Regulation of translation	0.0061	6	HSA-453279	Mitotic G1 phase and G1/S transition	3.77E-05
6	GO:0071900	Regulation of protein serine/threonine kinase activity	0.0197	6	HSA-2559583	Cellular Senescence	6.44E-05
6	GO:0010035	Response to inorganic substance	0.0227	6	HSA-1257604	PIP3 activates AKT signaling	6.70E-04
6	GO:0033674	Positive regulation of kinase activity	0.0415	6	HSA-3700989	Transcriptional Regulation by TP53	0.0025
5	GO:0006977	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	2.73E-05	5	HSA-8934593	Regulation of RUNX1 Expression and Activity	5.52E-07
5	GO:0016458	Gene silencing	8.60E-04	5	HSA-9661069	Defective binding of RB1 mutants to E2F1,(E2F2, E2F3)	5.52E-07
5	GO:0034504	Protein localization to nucleus	0.0013	5	HSA-69231	Cyclin D associated events in G1	8.77E-06
5	GO:0009743	Response to carbohydrate	0.0016	5	HSA-8939211	ESR-mediated signaling	0.0016
5	GO:0061013	Regulation of mRNA catabolic process	0.0032	5	HSA-69620	Cell Cycle Checkpoints	0.0058
5	GO:0048545	Response to steroid hormone	0.0197	4	HSA-2559585	Oncogene Induced Senescence	7.07E-05
5	GO:0043434	Response to peptide hormone	0.0378	4	HSA-9614085	FOXO-mediated transcription	6.70E-04
4	GO:1904031	Positive regulation of cyclin-dependent protein kinase activity	1.10E-04	4	HSA-69202	Cyclin E associated events during G1/S transition	0.0013
4	GO:1904705	Regulation of vascular associated smooth muscle cell proliferation	4.30E-04	4	HSA-69656	Cyclin A:Cdk2-associated events at S phase entry	0.0014
4	GO:0035194	Post-transcriptional gene silencing by RNA	5.70E-04	4	HSA-2559580	Oxidative Stress Induced Senescence	0.0019
4	GO:0000082	G1/S transition of mitotic cell cycle	0.0079	3	HSA-198323	AKT phosphorylates targets in the cytosol	4.00E-04
4	GO:0017148	Negative regulation of translation	0.0094	3	HSA-5674400	Constitutive Signaling by AKT1 E17K in Cancer	0.0015
4	GO:0009749	Response to glucose	0.0097	3	HSA-8853884	Transcriptional Regulation by VENTX	0.0033
4	GO:0032355	Response to estradiol	0.0105	3	HSA-6791312	TP53 Regulates Transcription of Cell Cycle Genes	0.0058
4	GO:0045931	Positive regulation of mitotic cell cycle	0.014	3	HSA-187577	SCF(Skp2)-mediated degradation of p27/p21	0.0086
4	GO:0070507	Regulation of microtubule cytoskeleton organization	0.0261	3	HSA-1912408	Pre-NOTCH Transcription and Translation	0.0096
3	GO:0060213	Positive regulation of nuclear-transcribed mRNA poly(A) tail shortening	3.90E-04	3	HSA-69563	p53-Dependent G1 DNA Damage Response	0.0098
3	GO:1904706	Negative regulation of vascular associated smooth muscle cell proliferation	9.10E-04	3	HSA-9009391	Extra-nuclear estrogen signaling	0.0144

3	GO:0045737	Positive regulation of cyclin-dependent protein serine/threonine kinase activity	0.003	3	HSA-2559582	Senescence-Associated Secretory Phenotype (SASP)	0.0168
3	GO:1900087	Positive regulation of G1/S transition of mitotic cell cycle	0.0046	3	HSA-8878159	Transcriptional regulation by RUNX3	0.0248
3	GO:0035195	Gene silencing by miRNA	0.0089	3	HSA-211000	Gene Silencing by RNA	0.0344
3	GO:1903317	Regulation of protein maturation	0.0211	3	HSA-6785807	Interleukin-4 and Interleukin-13 signaling	0.0347
3	GO:0033273	Response to vitamin	0.032	3	HSA-9018519	Estrogen-dependent gene expression	0.0451
3	GO:0031110	Regulation of microtubule polymerization or depolymerization	0.0329	2	HSA-8849470	PTK6 Regulates Cell Cycle	0.0054
3	GO:0006606	Protein import into nucleus	0.0454	2	HSA-426496	Post-transcriptional silencing by small RNAs	0.0061
3	GO:2001243	Negative regulation of intrinsic apoptotic signaling pathway	0.0478	2	HSA-8948700	Competing endogenous RNAs (ceRNAs) regulate PTEN translation	0.0074
3	GO:0002065	Columnar/cuboidal epithelial cell differentiation	0.0498	2	HSA-6804116	TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest	0.0157
2	GO:1905179	Negative regulation of cardiac muscle tissue regeneration	0.0022	2	HSA-9617828	FOXO-mediated transcription of cell cycle genes	0.0209
2	GO:0035278	miRNA mediated inhibition of translation	0.0242	2	HSA-9634638	Estrogen-dependent nuclear events downstream of ESR-membrane signaling	0.0357
2	GO:0048009	Insulin-like growth factor receptor signaling pathway	0.0242				
2	GO:0016246	RNA interference	0.0266				
2	GO:0071236	Cellular response to antibiotic	0.0266				
2	GO:0021542	Dentate gyrus development	0.032				
2	GO:0071850	Mitotic cell cycle arrest	0.032				
2	GO:0032026	Response to magnesium ion	0.0415				
2	GO:0051412	Response to corticosterone	0.0451				
2	GO:0007095	Mitotic G2 DNA damage checkpoint	0.0483				

Supplementary Table 4 – GO, KEGG pathways and Reactome pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the downregulated miRNAs in Du-145's response to AgNPs or G-AgNPs and PC-3's response to AgNPs .

GO Biological Processes				GO Cellular Component			
# genes	term name	description	FDR value	# genes	term name	description	FDR value
15	GO:0050896	Response to stimulus	3.60E-04	3	GO:0008180	COP9 signalosome	5.00E-04
14	GO:0048519	Negative regulation of biological process	9.85E-05	KEGG Pathways			
14	GO:0051171	Regulation of nitrogen compound metabolic process	1.70E-04	# genes	term name	description	FDR value
14	GO:0080090	Regulation of primary metabolic process	2.20E-04	4	hsa04151	PI3K-Akt signaling pathway	0.0167
14	GO:0031323	Regulation of cellular metabolic process	2.90E-04	4	hsa05200	Pathways in cancer	0.03
14	GO:0060255	Regulation of macromolecule metabolic process	3.60E-04	3	hsa05321	Inflammatory bowel disease	0.0047
14	GO:0051716	Cellular response to stimulus	4.00E-04	3	hsa04550	Signaling pathways regulating pluripotency of stem cells	0.0167
13	GO:0010646	Regulation of cell communication	4.08E-05	3	hsa04630	JAK-STAT signaling pathway	0.0167
13	GO:0023051	Regulation of signaling	4.08E-05	3	hsa04935	Growth hormone synthesis, secretion and action	0.0167
13	GO:0048583	Regulation of response to stimulus	8.61E-05	Reactome Pathways			
13	GO:0048523	Negative regulation of cellular process	2.50E-04	# genes	term name	description	FDR value
13	GO:0065009	Regulation of molecular function	2.60E-04	3	HSA-5696394	DNA Damage Recognition in GG-NER	0.0083
13	GO:0048522	Positive regulation of cellular process	6.70E-04	3	HSA-6781823	Formation of TC-NER Pre-Incision Complex	0.0107
12	GO:0010605	Negative regulation of macromolecule metabolic process	4.08E-05	3	HSA-8856825	Cargo recognition for clathrin-mediated endocytosis	0.0307
12	GO:0009966	Regulation of signal transduction	8.61E-05				
12	GO:0051173	Positive regulation of nitrogen compound metabolic process	8.61E-05				
12	GO:0031325	Positive regulation of cellular metabolic process	1.20E-04				
12	GO:0010604	Positive regulation of macromolecule metabolic process	1.60E-04				
12	GO:0010468	Regulation of gene expression	0.0012				
12	GO:0007165	Signal transduction	0.0013				
12	GO:0044260	Cellular macromolecule metabolic process	0.0015				

12	GO:0006807	Nitrogen compound metabolic process	0.0226			
12	GO:0044238	Primary metabolic process	0.0394			
11	GO:0051241	Negative regulation of multicellular organismal process	8.87E-07			
11	GO:0045595	Regulation of cell differentiation	3.89E-05			
11	GO:2000026	Regulation of multicellular organismal development	4.08E-05			
11	GO:0051172	Negative regulation of nitrogen compound metabolic process	8.61E-05			
11	GO:0031324	Negative regulation of cellular metabolic process	1.20E-04			
11	GO:0032268	Regulation of cellular protein metabolic process	1.20E-04			
11	GO:1901564	Organonitrogen compound metabolic process	0.0125			
10	GO:0051240	Positive regulation of multicellular organismal process	8.61E-05			
10	GO:0010628	Positive regulation of gene expression	2.60E-04			
10	GO:0050790	Regulation of catalytic activity	2.90E-04			
10	GO:0032879	Regulation of localization	6.50E-04			
10	GO:0006464	Cellular protein modification process	0.0013			
10	GO:2001141	Regulation of RNA biosynthetic process	0.0031			
9	GO:0051093	Negative regulation of developmental process	3.89E-05			
9	GO:0051248	Negative regulation of protein metabolic process	4.08E-05			
9	GO:0051338	Regulation of transferase activity	4.08E-05			
9	GO:0016310	Phosphorylation	8.61E-05			
9	GO:0051094	Positive regulation of developmental process	9.85E-05			
9	GO:0001932	Regulation of protein phosphorylation	1.20E-04			
9	GO:0009967	Positive regulation of signal transduction	2.10E-04			
9	GO:0010941	Regulation of cell death	2.50E-04			
9	GO:0010557	Positive regulation of macromolecule biosynthetic process	4.30E-04			
9	GO:0045935	Positive regulation of nucleobase-containing compound metabolic process	4.50E-04			
9	GO:0031328	Positive regulation of cellular biosynthetic process	5.60E-04			
9	GO:0006357	Regulation of transcription by RNA polymerase II	8.70E-04			
9	GO:0007166	Cell surface receptor signaling pathway	0.0013			
9	GO:0010033	Response to organic substance	0.0068			
9	GO:0030154	Cell differentiation	0.0238			
9	GO:0065008	Regulation of biological quality	0.0401			
8	GO:0045596	Negative regulation of cell differentiation	4.08E-05			
8	GO:0043549	Regulation of kinase activity	8.61E-05			
8	GO:0006468	Protein phosphorylation	9.85E-05			
8	GO:0045597	Positive regulation of cell differentiation	1.20E-04			
8	GO:0060548	Negative regulation of cell death	1.20E-04			
8	GO:0032269	Negative regulation of cellular protein metabolic process	1.30E-04			
8	GO:0045944	Positive regulation of transcription by RNA polymerase II	2.90E-04			
8	GO:0042981	Regulation of apoptotic process	8.10E-04			
8	GO:1901700	Response to oxygen-containing compound	8.50E-04			
8	GO:0042127	Regulation of cell population proliferation	0.0011			
8	GO:0048585	Negative regulation of response to stimulus	0.0011			
8	GO:0051049	Regulation of transport	0.0017			
8	GO:1902531	Regulation of intracellular signal transduction	0.0018			
8	GO:0044093	Positive regulation of molecular function	0.0021			
8	GO:0010629	Negative regulation of gene expression	0.0034			
8	GO:0071310	Cellular response to organic substance	0.0085			
7	GO:0051347	Positive regulation of transferase activity	1.60E-04			
7	GO:0045859	Regulation of protein kinase activity	2.60E-04			
7	GO:0008284	Positive regulation of cell population proliferation	4.30E-04			
7	GO:0001934	Positive regulation of protein phosphorylation	6.70E-04			

7	GO:0009628	Response to abiotic stimulus	0.0012				
7	GO:0009719	Response to endogenous stimulus	0.0037				
7	GO:0002682	Regulation of immune system process	0.0047				
7	GO:0022008	Neurogenesis	0.0072				
7	GO:0035556	Intracellular signal transduction	0.0085				
7	GO:0051128	Regulation of cellular component organization	0.0425				
6	GO:0001933	Negative regulation of protein phosphorylation	1.60E-04				
6	GO:0051249	Regulation of lymphocyte activation	2.00E-04				
6	GO:0010720	Positive regulation of cell development	4.10E-04				
6	GO:0033674	Positive regulation of kinase activity	6.40E-04				
6	GO:0051046	Regulation of secretion	8.90E-04				
6	GO:0001817	Regulation of cytokine production	0.0012				
6	GO:0043066	Negative regulation of apoptotic process	0.0028				
6	GO:1902533	Positive regulation of intracellular signal transduction	0.0055				
6	GO:0071495	Cellular response to endogenous stimulus	0.0091				
6	GO:0051130	Positive regulation of cellular component organization	0.0102				
6	GO:0080134	Regulation of response to stress	0.0215				
6	GO:0048699	Generation of neurons	0.0287				
6	GO:0048468	Cell development	0.0356				
5	GO:0050670	Regulation of lymphocyte proliferation	1.70E-04				
5	GO:0051251	Positive regulation of lymphocyte activation	3.70E-04				
5	GO:0071214	Cellular response to abiotic stimulus	4.90E-04				
5	GO:0043405	Regulation of MAP kinase activity	6.50E-04				
5	GO:0032147	Activation of protein kinase activity	8.70E-04				
5	GO:0045666	Positive regulation of neuron differentiation	8.70E-04				
5	GO:0008283	Cell population proliferation	0.0023				
5	GO:1903706	Regulation of hemopoiesis	0.0023				
5	GO:0007423	Sensory organ development	0.0037				
5	GO:0051223	Regulation of protein transport	0.0054				
5	GO:1903530	Regulation of secretion by cell	0.0058				
5	GO:0007346	Regulation of mitotic cell cycle	0.0073				
5	GO:0040008	Regulation of growth	0.0073				
5	GO:0010942	Positive regulation of cell death	0.0091				
5	GO:0033993	Response to lipid	0.0172				
5	GO:0051050	Positive regulation of transport	0.0224				
5	GO:0010243	Response to organonitrogen compound	0.0284				
5	GO:0032101	Regulation of response to external stimulus	0.0308				
5	GO:0030182	Neuron differentiation	0.0312				
5	GO:1901701	Cellular response to oxygen-containing compound	0.0359				
5	GO:0044092	Negative regulation of molecular function	0.0497				
4	GO:0042509	Regulation of tyrosine phosphorylation of STAT protein	1.60E-04				
4	GO:1902106	Negative regulation of leukocyte differentiation	2.80E-04				
4	GO:0050671	Positive regulation of lymphocyte proliferation	5.00E-04				
4	GO:0007088	Regulation of mitotic nuclear division	8.20E-04				
4	GO:0045165	Cell fate commitment	0.0028				
4	GO:0043406	Positive regulation of MAP kinase activity	0.0034				
4	GO:1903532	Positive regulation of secretion by cell	0.0057				
4	GO:1903037	Regulation of leukocyte cell-cell adhesion	0.0058				
4	GO:1901214	Regulation of neuron death	0.0059				
4	GO:0050863	Regulation of T cell activation	0.0066				
4	GO:0050708	Regulation of protein secretion	0.0068				

4	GO:0043687	Post-translational protein modification	0.0085			
4	GO:0045787	Positive regulation of cell cycle	0.0105			
4	GO:0001819	Positive regulation of cytokine production	0.0174			
4	GO:0071417	Cellular response to organonitrogen compound	0.0359			
4	GO:0010638	Positive regulation of organelle organization	0.0434			
4	GO:0043065	Positive regulation of apoptotic process	0.0487			
3	GO:0000338	Protein deneddylation	8.61E-05			
3	GO:0000715	Nucleotide-excision repair, DNA damage recognition	2.60E-04			
3	GO:0070229	Negative regulation of lymphocyte apoptotic process	4.10E-04			
3	GO:0030890	Positive regulation of B cell proliferation	7.40E-04			
3	GO:0045581	Negative regulation of T cell differentiation	8.10E-04			
3	GO:0045840	Positive regulation of mitotic nuclear division	0.0013			
3	GO:0048663	Neuron fate commitment	0.0018			
3	GO:0042531	Positive regulation of tyrosine phosphorylation of STAT protein	0.0019			
3	GO:0002088	Lens development in camera-type eye	0.0024			
3	GO:0006283	Transcription-coupled nucleotide-excision repair	0.0024			
3	GO:1901224	Positive regulation of NIK/NF-kappaB signaling	0.003			
3	GO:0048661	Positive regulation of smooth muscle cell proliferation	0.0034			
3	GO:1901216	Positive regulation of neuron death	0.0044			
3	GO:0002702	Positive regulation of production of molecular mediator of immune response	0.0053			
3	GO:0021782	Glial cell development	0.006			
3	GO:0045667	Regulation of osteoblast differentiation	0.0066			
3	GO:0045471	Response to ethanol	0.0073			
3	GO:0048634	Regulation of muscle organ development	0.0091			
3	GO:0120161	Regulation of cold-induced thermogenesis	0.0103			
3	GO:0030856	Regulation of epithelial cell differentiation	0.0115			
3	GO:0034249	Negative regulation of cellular amide metabolic process	0.0119			
3	GO:0000187	Activation of MAPK activity	0.0124			
3	GO:0042129	Regulation of T cell proliferation	0.0139			
3	GO:0050714	Positive regulation of protein secretion	0.0142			
3	GO:0043409	Negative regulation of MAPK cascade	0.0144			
3	GO:0030307	Positive regulation of cell growth	0.0151			
3	GO:0050679	Positive regulation of epithelial cell proliferation	0.0199			
3	GO:0048839	Inner ear development	0.0215			
3	GO:0060348	Bone development	0.0215			
3	GO:0002703	Regulation of leukocyte mediated immunity	0.0238			
3	GO:0050870	Positive regulation of T cell activation	0.0238			
3	GO:0009612	Response to mechanical stimulus	0.0245			
3	GO:0006469	Negative regulation of protein kinase activity	0.0309			
3	GO:0001503	Ossification	0.0401			
3	GO:0090596	Sensory organ morphogenesis	0.0401			
3	GO:0001818	Negative regulation of cytokine production	0.0454			
3	GO:0097190	Apoptotic signaling pathway	0.0475			
2	GO:1905459	Regulation of vascular associated smooth muscle cell apoptotic process	0.0027			
2	GO:0002903	Negative regulation of B cell apoptotic process	0.0038			
2	GO:0045591	Positive regulation of regulatory T cell differentiation	0.0049			
2	GO:0097094	Craniofacial suture morphogenesis	0.0065			
2	GO:2000727	Positive regulation of cardiac muscle cell differentiation	0.0073			
2	GO:0002089	Lens morphogenesis in camera-type eye	0.0091			
2	GO:0042104	Positive regulation of activated T cell proliferation	0.0103			
2	GO:0043032	Positive regulation of macrophage activation	0.0109			

2	GO:0046639	Negative regulation of alpha-beta T cell differentiation	0.0109			
2	GO:0070306	Lens fiber cell differentiation	0.0115			
2	GO:1903203	Regulation of oxidative stress-induced neuron death	0.0128			
2	GO:0032660	Regulation of interleukin-17 production	0.0135			
2	GO:0060428	Lung epithelium development	0.0142			
2	GO:0032148	Activation of protein kinase B activity	0.0156			
2	GO:0048665	Neuron fate specification	0.0164			
2	GO:0032733	Positive regulation of interleukin-10 production	0.0215			
2	GO:1904646	Cellular response to amyloid-beta	0.0215			
2	GO:0002067	Glandular epithelial cell differentiation	0.022			
2	GO:0045839	Negative regulation of mitotic nuclear division	0.022			
2	GO:0001709	Cell fate determination	0.0236			
2	GO:0002639	Positive regulation of immunoglobulin production	0.0271			
2	GO:0043370	Regulation of CD4-positive, alpha-beta T cell differentiation	0.028			
2	GO:0120163	Negative regulation of cold-induced thermogenesis	0.028			
2	GO:0070169	Positive regulation of biomineral tissue development	0.0287			
2	GO:0030282	Bone mineralization	0.0301			
2	GO:0090102	Cochlea development	0.031			
2	GO:0009948	Anterior/posterior axis specification	0.0319			
2	GO:0048146	Positive regulation of fibroblast proliferation	0.0319			
2	GO:0042116	Macrophage activation	0.0328			
2	GO:0043525	Positive regulation of neuron apoptotic process	0.0381			
2	GO:2000242	Negative regulation of reproductive process	0.0381			
2	GO:0032720	Negative regulation of tumor necrosis factor production	0.0401			
2	GO:0045669	Positive regulation of osteoblast differentiation	0.0401			
2	GO:0030858	Positive regulation of epithelial cell differentiation	0.0417			
2	GO:0042490	Mechanoreceptor differentiation	0.0425			
2	GO:1903036	Positive regulation of response to wounding	0.0487			
2	GO:0032729	Positive regulation of interferon-gamma production	0.0497			

Supplementary Table 5 – GO, KEGG pathways and Reactome pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs in Du-145's response to AgNPs

GO Biological Process				KEGG pathways			
# genes	term name	description	FDR value	# genes	term name	description	FDR value
22	GO:0009893	Positive regulation of metabolic process	2.40E-04	10	hsa05200	Pathways in cancer	3.06E-06
15	GO:0007166	Cell surface receptor signaling pathway	0.0024	9	hsa05206	MicroRNAs in cancer	4.16E-09
10	GO:0000122	Negative regulation of transcription by RNA polymerase II	0.0019	5	hsa04066	HIF-1 signaling pathway	1.70E-04
10	GO:0008285	Negative regulation of cell population proliferation	5.40E-04	4	hsa04010	MAPK signaling pathway	0.022
9	GO:0051726	Regulation of cell cycle	0.0267	4	hsa05167	Kaposi sarcoma-associated herpesvirus infection	0.006
8	GO:0051270	Regulation of cellular component movement	0.0334	3	hsa04630	JAK-STAT signaling pathway	0.0309
8	GO:0001558	Regulation of cell growth	7.10E-04	3	hsa05211	Renal cell carcinoma	0.0053
7	GO:0010942	Positive regulation of cell death	0.027	2	hsa05213	Endometrial cancer	0.0477
6	GO:0048598	Embryonic morphogenesis	0.0432	Reactome Pathways			
6	GO:0010720	Positive regulation of cell development	0.0391	# genes	term name	description	FDR value
6	GO:0071902	Positive regulation of protein serine/threonine kinase activity	0.0075	5	HSA-6785807	Interleukin-4 and Interleukin-13 signaling	6.80E-04
5	GO:0030856	Regulation of epithelial cell differentiation	0.0033	4	HSA-9009391	Extra-nuclear estrogen signaling	0.003
5	GO:0001101	Response to acid chemical	0.0024	4	HSA-9661069	Defective binding of RB1 mutants to E2F1,(E2F2, E2F3)	1.10E-04
4	GO:0045807	Positive regulation of endocytosis	0.0077				

3	GO:0031110	Regulation of microtubule polymerization or depolymerization	0.0436			
3	GO:0010827	Regulation of glucose transmembrane transport	0.0337			
3	GO:0048146	Positive regulation of fibroblast proliferation	0.0181			
2	GO:1902746	Regulation of lens fiber cell differentiation	0.0212			

Supplementary Table 6 - GO, KEGG pathways and Reactome pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs in Du-145's response to G-AgNPs

GO Biological Processes				GO Cellular Processes			
# genes	term name	description	FDR value	# genes	term name	description	FDR value
75	GO:0010033	Response to organic substance	6.18E-24	59	GO:0043232	Intracellular non-membrane-bounded organelle	6.95E-05
71	GO:0044238	Primary metabolic process	0.0013	46	GO:0012505	Endomembrane system	0.0375
67	GO:0016043	Cellular component organization	6.63E-07	26	GO:0005694	Chromosome	0.0042
58	GO:0045595	Regulation of cell differentiation	1.79E-21	15	GO:0009986	Cell surface	0.0172
50	GO:0042325	Regulation of phosphorylation	4.63E-18	15	GO:0070161	Anchoring junction	0.0172
50	GO:0051128	Regulation of cellular component organization	6.96E-12	14	GO:0048471	Perinuclear region of cytoplasm	0.0171
48	GO:0007166	Cell surface receptor signaling pathway	3.39E-11	10	GO:0045121	Membrane raft	0.005
44	GO:0009891	Positive regulation of biosynthetic process	1.12E-10	9	GO:0030016	Myofibril	0.0021
43	GO:0009888	Tissue development	4.47E-12	6	GO:0042470	Melanosome	0.0055
43	GO:0048584	Positive regulation of response to stimulus	8.58E-09	2	GO:0044354	Macropinosome	0.0458
41	GO:0009605	Response to external stimulus	1.63E-07	GO Molecular Processes			
39	GO:0051641	Cellular localization	4.10E-04	# genes	term name	description	FDR value
38	GO:0043069	Negative regulation of programmed cell death	3.38E-17	66	GO:0043167	Ion binding	6.80E-04
37	GO:0051270	Regulation of cellular component movement	3.62E-15	42	GO:0019899	Enzyme binding	2.15E-07
35	GO:0071495	Cellular response to endogenous stimulus	9.79E-12	34	GO:0036094	Small molecule binding	0.0032
35	GO:0006796	Phosphate-containing compound metabolic process	1.16E-05	30	GO:0042802	Identical protein binding	7.90E-04
34	GO:0034097	Response to cytokine	7.89E-12	26	GO:0005102	Signaling receptor binding	0.0015
34	GO:0044092	Negative regulation of molecular function	3.24E-11	25	GO:0008134	Transcription factor binding	4.32E-09
33	GO:0072359	Circulatory system development	1.09E-13	23	GO:0044877	Protein-containing complex binding	7.60E-04
33	GO:0008284	Positive regulation of cell population proliferation	4.31E-13	20	GO:0019900	Kinase binding	2.41E-05
33	GO:0009628	Response to abiotic stimulus	1.12E-10	20	GO:0008092	Cytoskeletal protein binding	9.20E-04
33	GO:2000113	Negative regulation of cellular macromolecule biosynthetic process	3.93E-08	17	GO:0030234	Enzyme regulator activity	0.0281
33	GO:1901576	Organic substance biosynthetic process	0.0068	16	GO:0019904	Protein domain specific binding	0.0024
32	GO:0010243	Response to organonitrogen compound	1.36E-11	14	GO:0050839	Cell adhesion molecule binding	0.0017
32	GO:0010648	Negative regulation of cell communication	3.69E-08	13	GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	0.0012
31	GO:0014070	Response to organic cyclic compound	1.06E-11	9	GO:0042826	Histone deacetylase binding	2.39E-05
31	GO:0080134	Regulation of response to stress	3.09E-07	9	GO:0031625	Ubiquitin protein ligase binding	0.0119
31	GO:0043085	Positive regulation of catalytic activity	6.46E-07	8	GO:0061629	RNA polymerase II-specific DNA-binding transcription factor binding	0.0318
31	GO:0002682	Regulation of immune system process	9.03E-07	6	GO:0001085	RNA polymerase II transcription factor binding	5.20E-04
30	GO:0042592	Homeostatic process	2.13E-05	6	GO:0033613	Activating transcription factor binding	0.0036
30	GO:0003008	System process	2.80E-04	6	GO:1902936	Phosphatidylinositol bisphosphate binding	0.0088
29	GO:0009725	Response to hormone	6.39E-11	6	GO:0002020	Protease binding	0.0194
28	GO:0051050	Positive regulation of transport	1.98E-09	5	GO:0035035	Histone acetyltransferase binding	4.50E-04
27	GO:0040008	Regulation of growth	1.49E-11	5	GO:0097718	Disordered domain specific binding	7.50E-04
27	GO:0030155	Regulation of cell adhesion	4.59E-11	5	GO:0051087	Chaperone binding	0.0352
26	GO:0009887	Animal organ morphogenesis	9.70E-08	4	GO:0070888	E-box binding	0.0197

26	GO:0035556	Intracellular signal transduction	0.0014	3	GO:0019211	Phosphatase activator activity	0.0141
26	GO:0044419	Interspecies interaction between organisms	0.0053	2	GO:0140327	Flippase activity	0.0477
25	GO:0060284	Regulation of cell development	3.22E-07	KEGG Pathways			
25	GO:0030182	Neuron differentiation	9.73E-07	# genes	term name	description	FDR value
25	GO:0043933	Protein-containing complex subunit organization	7.20E-04	30	hsa05200	Pathways in cancer	6.15E-17
25	GO:0033554	Cellular response to stress	0.0034	18	hsa04151	PI3K-Akt signaling pathway	8.69E-10
24	GO:0002520	Immune system development	1.53E-09	17	hsa05206	MicroRNAs in cancer	4.24E-13
24	GO:0008285	Negative regulation of cell population proliferation	5.04E-09	16	hsa05205	Proteoglycans in cancer	2.83E-11
23	GO:0071900	Regulation of protein serine/threonine kinase activity	1.53E-10	15	hsa05226	Gastric cancer	1.05E-11
23	GO:0060322	Head development	2.08E-07	15	hsa05203	Viral carcinogenesis	1.22E-10
23	GO:0001775	Cell activation	3.04E-05	14	hsa04933	AGE-RAGE signaling pathway in diabetic complications	2.27E-12
23	GO:0006928	Movement of cell or subcellular component	0.003	13	hsa04218	Cellular senescence	1.26E-09
22	GO:0001501	Skeletal system development	4.67E-10	12	hsa04934	Cushing syndrome	1.57E-08
22	GO:0009894	Regulation of catabolic process	5.92E-05	12	hsa05202	Transcriptional misregulation in cancer	4.30E-08
21	GO:0034762	Regulation of transmembrane transport	1.99E-08	11	hsa05222	Small cell lung cancer	1.50E-09
21	GO:0051336	Regulation of hydrolase activity	0.0025	10	hsa04010	MAPK signaling pathway	1.80E-04
20	GO:0070848	Response to growth factor	3.71E-08	9	hsa05162	Measles	6.03E-06
20	GO:0043408	Regulation of MAPK cascade	4.48E-06	9	hsa05161	Hepatitis B	1.64E-05
20	GO:0032880	Regulation of protein localization	1.40E-04	8	hsa05219	Bladder cancer	1.59E-08
19	GO:0006915	Apoptotic process	3.50E-04	8	hsa05211	Renal cell carcinoma	3.92E-07
19	GO:0007010	Cytoskeleton organization	0.0035	8	hsa04115	p53 signaling pathway	6.77E-07
18	GO:0050678	Regulation of epithelial cell proliferation	2.44E-09	8	hsa04068	FoxO signaling pathway	2.74E-05
18	GO:0062012	Regulation of small molecule metabolic process	1.25E-07	8	hsa04150	mTOR signaling pathway	7.97E-05
18	GO:0044089	Positive regulation of cellular component biogenesis	1.57E-06	8	hsa04530	Tight junction	9.56E-05
18	GO:0010638	Positive regulation of organelle organization	1.23E-05	8	hsa04630	JAK-STAT signaling pathway	1.10E-04
18	GO:0140352	Export from cell	0.0034	7	hsa05213	Endometrial cancer	2.28E-06
17	GO:0051098	Regulation of binding	6.01E-08	7	hsa04917	Prolactin signaling pathway	7.07E-06
17	GO:0048732	Gland development	2.13E-07	7	hsa05323	Rheumatoid arthritis	2.26E-05
17	GO:0010035	Response to inorganic substance	7.01E-06	7	hsa04066	HIF-1 signaling pathway	7.66E-05
17	GO:0007423	Sensory organ development	1.21E-05	7	hsa04722	Neurotrophin signaling pathway	1.10E-04
17	GO:0051129	Negative regulation of cellular component organization	2.00E-04	7	hsa04071	Sphingolipid signaling pathway	1.20E-04
17	GO:0022402	Cell cycle process	0.005	7	hsa04152	AMPK signaling pathway	1.40E-04
16	GO:0010632	Regulation of epithelial cell migration	7.22E-10	7	hsa04926	Relaxin signaling pathway	2.00E-04
16	GO:0001666	Response to hypoxia	1.30E-07	7	hsa05418	Fluid shear stress and atherosclerosis	2.10E-04
16	GO:0061024	Membrane organization	0.0019	7	hsa04371	Apelin signaling pathway	2.20E-04
15	GO:0051147	Regulation of muscle cell differentiation	5.64E-11	7	hsa04024	cAMP signaling pathway	0.0028
15	GO:0051348	Negative regulation of transferase activity	1.40E-07	6	hsa05217	Basal cell carcinoma	4.84E-05
15	GO:0001525	Angiogenesis	3.13E-07	6	hsa05221	Acute myeloid leukemia	6.44E-05
15	GO:0051090	Regulation of DNA-binding transcription factor activity	1.33E-05	6	hsa04916	Melanogenesis	3.30E-04
15	GO:0006351	Transcription, DNA-templated	2.10E-04	6	hsa04668	TNF signaling pathway	7.40E-04
14	GO:0090257	Regulation of muscle system process	6.43E-08	6	hsa05034	Alcoholism	0.0025
14	GO:0042493	Response to drug	5.74E-07	6	hsa05164	Influenza A	0.0045
14	GO:1901342	Regulation of vasculature development	4.02E-06	6	hsa04360	Axon guidance	0.0062
14	GO:0006979	Response to oxidative stress	2.10E-05	6	hsa05131	Shigellosis	0.0154
14	GO:0043434	Response to peptide hormone	2.15E-05	6	hsa05016	Huntington disease	0.0486
14	GO:0008283	Cell population proliferation	2.00E-04	5	hsa05230	Central carbon metabolism in cancer	7.40E-04
14	GO:0050878	Regulation of body fluid levels	2.70E-04	5	hsa05410	Hypertrophic cardiomyopathy	0.0021
14	GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	3.20E-04	5	hsa04810	Regulation of actin cytoskeleton	0.0435
14	GO:0071396	Cellular response to lipid	3.90E-04	4	hsa05144	Malaria	0.0018
14	GO:0045786	Negative regulation of cell cycle	8.20E-04	4	hsa04213	Longevity regulating pathway - multiple species	0.0043
14	GO:1903530	Regulation of secretion by cell	0.002	4	hsa04137	Mitophagy - animal	0.0046

14	GO:0010942	Positive regulation of cell death	0.0058	4	hsa04350	TGF-beta signaling pathway	0.0151
14	GO:0030162	Regulation of proteolysis	0.0079	4	hsa04657	IL-17 signaling pathway	0.0154
14	GO:0050801	Ion homeostasis	0.0128	4	hsa05146	Amoebiasis	0.0194
13	GO:0061448	Connective tissue development	3.51E-07	4	hsa04928	Parathyroid hormone synthesis, secretion and action	0.0211
13	GO:0051271	Negative regulation of cellular component movement	1.05E-05	4	hsa04670	Leukocyte transendothelial migration	0.0248
13	GO:0002696	Positive regulation of leukocyte activation	2.00E-05	4	hsa04114	Oocyte meiosis	0.0327
13	GO:0032535	Regulation of cellular component size	7.98E-05	3	hsa05134	Legionellosis	0.0248
13	GO:0048589	Developmental growth	9.22E-05	3	hsa04730	Long-term depression	0.0289
13	GO:0048608	Reproductive structure development	1.90E-04	3	hsa04929	GnRH secretion	0.0332
13	GO:0009991	Response to extracellular stimulus	6.40E-04	Reactome Pathways			
12	GO:0009612	Response to mechanical stimulus	1.61E-06	# genes	term name	description	FDR value
12	GO:2000027	Regulation of animal organ morphogenesis	1.03E-05	35	HSA-212436	Generic Transcription Pathway	1.87E-10
12	GO:0001503	Ossification	1.34E-05	21	HSA-109582	Hemostasis	1.70E-07
12	GO:0043270	Positive regulation of ion transport	2.62E-05	21	HSA-1266738	Developmental Biology	6.60E-04
12	GO:0090068	Positive regulation of cell cycle process	3.49E-05	20	HSA-5663202	Diseases of signal transduction by growth factor receptors and second messengers	1.80E-09
12	GO:0060562	Epithelial tube morphogenesis	5.18E-05	18	HSA-449147	Signaling by Interleukins	2.21E-07
12	GO:0071496	Cellular response to external stimulus	5.48E-05	18	HSA-9006934	Signaling by Receptor Tyrosine Kinases	1.10E-06
12	GO:0032496	Response to lipopolysaccharide	6.15E-05	15	HSA-9006931	Signaling by Nuclear Receptors	1.15E-07
12	GO:1902903	Regulation of supramolecular fiber organization	2.50E-04	15	HSA-9006925	Intracellular signaling by second messengers	4.28E-07
12	GO:0000165	MAPK cascade	2.60E-04	15	HSA-2262752	Cellular responses to stress	3.00E-04
12	GO:0042060	Wound healing	0.0011	12	HSA-8878166	Transcriptional regulation by RUNX2	2.20E-08
12	GO:0001817	Regulation of cytokine production	0.0423	12	HSA-195721	Signaling by WNT	7.35E-05
11	GO:0048660	Regulation of smooth muscle cell proliferation	2.42E-07	11	HSA-5683057	MAPK family signaling cascades	6.60E-04
11	GO:0048762	Mesenchymal cell differentiation	6.46E-07	9	HSA-157118	Signaling by NOTCH	6.60E-04
11	GO:0048863	Stem cell differentiation	8.10E-07	8	HSA-69231	Cyclin D associated events in G1	4.22E-07
11	GO:0048639	Positive regulation of developmental growth	2.33E-06	8	HSA-69620	Cell Cycle Checkpoints	0.0103
11	GO:0043523	Regulation of neuron apoptotic process	1.20E-05	7	HSA-2559585	Oncogene Induced Senescence	8.70E-07
11	GO:0003007	Heart morphogenesis	5.06E-05	7	HSA-187037	Signaling by NTRK1 (TRKA)	8.60E-04
11	GO:0010721	Negative regulation of cell development	4.50E-04	7	HSA-397014	Muscle contraction	0.008
11	GO:0045666	Positive regulation of neuron differentiation	0.0013	6	HSA-190236	Signaling by FGFR	0.0015
11	GO:0010959	Regulation of metal ion transport	0.0016	6	HSA-2219528	PI3K/AKT Signaling in Cancer	0.0034
11	GO:0046649	Lymphocyte activation	0.0016	6	HSA-9018519	Estrogen-dependent gene expression	0.0055
11	GO:0022411	Cellular component disassembly	0.0018	5	HSA-8951936	RUNX3 regulates p14-ARF	2.18E-06
11	GO:0006091	Generation of precursor metabolites and energy	0.0021	5	HSA-8934593	Regulation of RUNX1 Expression and Activity	2.33E-05
11	GO:0051051	Negative regulation of transport	0.0039	5	HSA-9616222	Transcriptional regulation of granulopoiesis	0.0025
11	GO:0048667	Cell morphogenesis involved in neuron differentiation	0.0041	5	HSA-5628897	TP53 Regulates Metabolic Genes	0.0079
11	GO:0050804	Modulation of chemical synaptic transmission	0.0041	5	HSA-6811558	PI3P, PP2A and IER3 Regulate PI3K/AKT Signaling	0.0145
11	GO:0022604	Regulation of cell morphogenesis	0.0085	5	HSA-6807070	PTEN Regulation	0.0361
11	GO:0032103	Positive regulation of response to external stimulus	0.01	5	HSA-3858494	Beta-catenin independent WNT signaling	0.0398
11	GO:0007610	Behavior	0.0207	4	HSA-9703648	Signaling by FLT3 ITD and TKD mutants	4.90E-04
10	GO:0055024	Regulation of cardiac muscle tissue development	3.61E-08	4	HSA-350054	Notch-HLH transcription pathway	0.0023
10	GO:0035051	Cardiocyte differentiation	3.88E-07	4	HSA-4791275	Signaling by WNT in cancer	0.0038
10	GO:0045667	Regulation of osteoblast differentiation	6.87E-07	4	HSA-5673000	RAF activation	0.0038
10	GO:1901888	Regulation of cell junction assembly	4.86E-05	4	HSA-5655302	Signaling by FGFR1 in disease	0.0053
10	GO:0048872	Homeostasis of number of cells	5.85E-05	4	HSA-9656223	Signaling by RAF1 mutants	0.0057
10	GO:0006936	Muscle contraction	2.50E-04	4	HSA-8848021	Signaling by PTK6	0.0108
10	GO:0048511	Rhythmic process	4.70E-04	4	HSA-8986944	Transcriptional Regulation by MECP2	0.0143
10	GO:0022409	Positive regulation of cell-cell adhesion	4.80E-04	4	HSA-381340	Transcriptional regulation of white adipocyte differentiation	0.0385
10	GO:0048871	Multicellular organismal homeostasis	0.0029	4	HSA-3371556	Cellular response to heat stress	0.0437
10	GO:0032102	Negative regulation of response to external stimulus	0.0038	3	HSA-9028731	Activated NTRK2 signals through FRS2 and FRS3	0.0035
10	GO:0001701	In utero embryonic development	0.0049	3	HSA-5218921	VEGFR2 mediated cell proliferation	0.0085

10	GO:0009314	Response to radiation	0.0101	3	HSA-8941326	RUNX2 regulates bone development	0.0228
10	GO:0002683	Negative regulation of immune system process	0.0131	3	HSA-8864260	Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors	0.03
10	GO:1902532	Negative regulation of intracellular signal transduction	0.0264	3	HSA-8853884	Transcriptional Regulation by VENTX	0.0361
10	GO:0060627	Regulation of vesicle-mediated transport	0.0407	3	HSA-76009	Platelet Aggregation (Plug Formation)	0.0379
9	GO:0000079	Regulation of cyclin-dependent protein serine/threonine kinase activity	1.78E-06	2	HSA-1296061	HCN channels	0.0119
9	GO:0002761	Regulation of myeloid leukocyte differentiation	9.27E-06	2	HSA-9682706	Replication of the SARS-CoV-1 genome	0.0194
9	GO:0030856	Regulation of epithelial cell differentiation	4.48E-05	2	HSA-170968	Frs2-mediated activation	0.0474
9	GO:0009743	Response to carbohydrate	1.20E-04	2	HSA-9634600	Regulation of glycolysis by fructose 2,6-bisphosphate metabolism	0.0474
9	GO:2000377	Regulation of reactive oxygen species metabolic process	2.00E-04				
9	GO:0051302	Regulation of cell division	2.70E-04				
9	GO:0005996	Monosaccharide metabolic process	2.80E-04				
9	GO:1905475	Regulation of protein localization to membrane	2.80E-04				
9	GO:0010810	Regulation of cell-substrate adhesion	4.40E-04				
9	GO:2001234	Negative regulation of apoptotic signaling pathway	8.00E-04				
9	GO:0097305	Response to alcohol	8.30E-04				
9	GO:0045165	Cell fate commitment	0.0013				
9	GO:0050730	Regulation of peptidyl-tyrosine phosphorylation	0.0016				
9	GO:0007568	Aging	0.0023				
9	GO:0007162	Negative regulation of cell adhesion	0.0026				
9	GO:0031647	Regulation of protein stability	0.0034				
9	GO:0001655	Urogenital system development	0.0052				
9	GO:0016055	Wnt signaling pathway	0.0095				
9	GO:0033044	Regulation of chromosome organization	0.0108				
9	GO:0030111	Regulation of Wnt signaling pathway	0.0128				
9	GO:0031331	Positive regulation of cellular catabolic process	0.0191				
9	GO:0051346	Negative regulation of hydrolase activity	0.0361				
9	GO:0034248	Regulation of cellular amide metabolic process	0.0384				
9	GO:0097435	Supramolecular fiber organization	0.0494				
8	GO:1902893	Regulation of pri-miRNA transcription by RNA polymerase II	3.45E-07				
8	GO:0033138	Positive regulation of peptidyl-serine phosphorylation	4.94E-05				
8	GO:0002573	Myeloid leukocyte differentiation	5.20E-05				
8	GO:0001889	Liver development	1.20E-04				
8	GO:0046683	Response to organophosphorus	2.00E-04				
8	GO:0062013	Positive regulation of small molecule metabolic process	2.30E-04				
8	GO:0031099	Regeneration	3.90E-04				
8	GO:0030324	Lung development	6.20E-04				
8	GO:0003205	Cardiac chamber development	6.40E-04				
8	GO:1905330	Regulation of morphogenesis of an epithelium	8.80E-04				
8	GO:0030308	Negative regulation of cell growth	0.0011				
8	GO:0002064	Epithelial cell development	0.0014				
8	GO:0071695	Anatomical structure maturation	0.0016				
8	GO:0035303	Regulation of dephosphorylation	0.0019				
8	GO:0070374	Positive regulation of ERK1 and ERK2 cascade	0.0021				
8	GO:0009636	Response to toxic substance	0.0027				
8	GO:0043583	Ear development	0.0031				
8	GO:0051896	Regulation of protein kinase B signaling	0.0032				
8	GO:0071356	Cellular response to tumor necrosis factor	0.0049				
8	GO:0021537	Telencephalon development	0.0062				
8	GO:0032412	Regulation of ion transmembrane transporter activity	0.0066				
8	GO:0007517	Muscle organ development	0.0118				
8	GO:0010506	Regulation of autophagy	0.0256				

8	GO:1903829	Positive regulation of cellular protein localization	0.0264			
8	GO:0032386	Regulation of intracellular transport	0.0333			
8	GO:0032147	Activation of protein kinase activity	0.0405			
7	GO:0051385	Response to mineralocorticoid	3.66E-07			
7	GO:0048146	Positive regulation of fibroblast proliferation	5.70E-06			
7	GO:0043388	Positive regulation of DNA binding	1.15E-05			
7	GO:0045639	Positive regulation of myeloid cell differentiation	2.00E-04			
7	GO:0043279	Response to alkaloid	2.80E-04			
7	GO:0035270	Endocrine system development	5.60E-04			
7	GO:0002576	Platelet degranulation	8.30E-04			
7	GO:0045598	Regulation of fat cell differentiation	9.80E-04			
7	GO:0001936	Regulation of endothelial cell proliferation	0.001			
7	GO:0061041	Regulation of wound healing	0.0013			
7	GO:0071560	Cellular response to transforming growth factor beta stimulus	0.0017			
7	GO:0048592	Eye morphogenesis	0.0019			
7	GO:0007369	Gastrulation	0.0024			
7	GO:2000241	Regulation of reproductive process	0.003			
7	GO:0001667	Ameboidal-type cell migration	0.0036			
7	GO:0071456	Cellular response to hypoxia	0.0055			
7	GO:0071248	Cellular response to metal ion	0.0061			
7	GO:0044706	Multi-multicellular organism process	0.0095			
7	GO:0042063	Gliogenesis	0.0131			
7	GO:0045732	Positive regulation of protein catabolic process	0.0165			
7	GO:0090287	Regulation of cellular response to growth factor stimulus	0.0324			
6	GO:0003180	Aortic valve morphogenesis	4.14E-06			
6	GO:0043491	Protein kinase B signaling	1.77E-05			
6	GO:2000144	Positive regulation of DNA-templated transcription, initiation	2.87E-05			
6	GO:0086001	Cardiac muscle cell action potential	6.58E-05			
6	GO:0043536	Positive regulation of blood vessel endothelial cell migration	1.10E-04			
6	GO:0072091	Regulation of stem cell proliferation	2.70E-04			
6	GO:0043502	Regulation of muscle adaptation	6.50E-04			
6	GO:0050810	Regulation of steroid biosynthetic process	9.70E-04			
6	GO:0046620	Regulation of organ growth	0.0014			
6	GO:0048771	Tissue remodeling	0.0014			
6	GO:0002065	Columnar/cuboidal epithelial cell differentiation	0.0017			
6	GO:0062014	Negative regulation of small molecule metabolic process	0.0019			
6	GO:0060349	Bone morphogenesis	0.0024			
6	GO:2000736	Regulation of stem cell differentiation	0.0034			
6	GO:0007219	Notch signaling pathway	0.0035			
6	GO:0043244	Regulation of protein-containing complex disassembly	0.0037			
6	GO:0048565	Digestive tract development	0.0047			
6	GO:0001101	Response to acid chemical	0.0051			
6	GO:0055002	Striated muscle cell development	0.0053			
6	GO:0008584	Male gonad development	0.0055			
6	GO:0007006	Mitochondrial membrane organization	0.0062			
6	GO:0032355	Response to estradiol	0.0071			
6	GO:1902107	Positive regulation of leukocyte differentiation	0.0104			
6	GO:0007584	Response to nutrient	0.0116			
6	GO:0043467	Regulation of generation of precursor metabolites and energy	0.0119			
6	GO:0006367	Transcription initiation from RNA polymerase II promoter	0.0122			
6	GO:0050714	Positive regulation of protein secretion	0.0138			

6	GO:0051100	Negative regulation of binding	0.0157				
6	GO:0050954	Sensory perception of mechanical stimulus	0.0166				
6	GO:0003018	Vascular process in circulatory system	0.0208				
6	GO:0035966	Response to topologically incorrect protein	0.0222				
6	GO:0032872	Regulation of stress-activated MAPK cascade	0.0453				
6	GO:0007265	Ras protein signal transduction	0.0468				
5	GO:1905209	Positive regulation of cardiocyte differentiation	8.35E-05				
5	GO:0051145	Smooth muscle cell differentiation	1.40E-04				
5	GO:0010762	Regulation of fibroblast migration	1.60E-04				
5	GO:1904707	Positive regulation of vascular associated smooth muscle cell proliferation	2.00E-04				
5	GO:0045773	Positive regulation of axon extension	5.10E-04				
5	GO:0050850	Positive regulation of calcium-mediated signaling	5.10E-04				
5	GO:1904645	Response to amyloid-beta	7.20E-04				
5	GO:0033077	T cell differentiation in thymus	8.40E-04				
5	GO:0045912	Negative regulation of carbohydrate metabolic process	0.0016				
5	GO:0061515	Myeloid cell development	0.0016				
5	GO:0048844	Artery morphogenesis	0.0017				
5	GO:0040014	Regulation of multicellular organism growth	0.0026				
5	GO:0010827	Regulation of glucose transmembrane transport	0.0034				
5	GO:1901983	Regulation of protein acetylation	0.0034				
5	GO:0030500	Regulation of bone mineralization	0.0035				
5	GO:0071260	Cellular response to mechanical stimulus	0.0043				
5	GO:0002062	Chondrocyte differentiation	0.0052				
5	GO:0045778	Positive regulation of ossification	0.0062				
5	GO:0010717	Regulation of epithelial to mesenchymal transition	0.0067				
5	GO:0070542	Response to fatty acid	0.007				
5	GO:0045833	Negative regulation of lipid metabolic process	0.0081				
5	GO:0044070	Regulation of anion transport	0.0107				
5	GO:0045444	Fat cell differentiation	0.0118				
5	GO:0044344	Cellular response to fibroblast growth factor stimulus	0.0126				
5	GO:2000278	Regulation of DNA biosynthetic process	0.0154				
5	GO:1903828	Negative regulation of cellular protein localization	0.0172				
5	GO:0042542	Response to hydrogen peroxide	0.0178				
5	GO:0051928	Positive regulation of calcium ion transport	0.0209				
5	GO:0030879	Mammary gland development	0.0215				
5	GO:0001649	Osteoblast differentiation	0.0222				
5	GO:0050671	Positive regulation of lymphocyte proliferation	0.0264				
5	GO:0030168	Platelet activation	0.0269				
5	GO:0098727	Maintenance of cell number	0.0283				
5	GO:0017038	Protein import	0.0334				
5	GO:0051592	Response to calcium ion	0.0381				
5	GO:1905952	Regulation of lipid localization	0.0386				
5	GO:0042113	B cell activation	0.0473				
5	GO:0007088	Regulation of mitotic nuclear division	0.0483				
4	GO:0006098	Pentose-phosphate shunt	1.30E-04				
4	GO:0010649	Regulation of cell communication by electrical coupling	2.00E-04				
4	GO:0010832	Negative regulation of myotube differentiation	2.80E-04				
4	GO:0060055	Angiogenesis involved in wound healing	2.80E-04				
4	GO:2000726	Negative regulation of cardiac muscle cell differentiation	2.80E-04				
4	GO:0060149	Negative regulation of posttranscriptional gene silencing	4.70E-04				
4	GO:2000810	Regulation of bicellular tight junction assembly	7.20E-04				

4	GO:0045736	Negative regulation of cyclin-dependent protein serine/threonine kinase activity	0.0015				
4	GO:0043516	Regulation of DNA damage response, signal transduction by p53 class mediator	0.0025				
4	GO:0043537	Negative regulation of blood vessel endothelial cell migration	0.0025				
4	GO:0044275	Cellular carbohydrate catabolic process	0.0025				
4	GO:0010259	Multicellular organism aging	0.003				
4	GO:0045445	Myoblast differentiation	0.003				
4	GO:0090049	Regulation of cell migration involved in sprouting angiogenesis	0.0032				
4	GO:0040019	Positive regulation of embryonic development	0.0035				
4	GO:1903053	Regulation of extracellular matrix organization	0.004				
4	GO:2000826	Regulation of heart morphogenesis	0.0043				
4	GO:2000772	Regulation of cellular senescence	0.0046				
4	GO:0007173	Epidermal growth factor receptor signaling pathway	0.007				
4	GO:0032330	Regulation of chondrocyte differentiation	0.0074				
4	GO:0002762	Negative regulation of myeloid leukocyte differentiation	0.0079				
4	GO:0048260	Positive regulation of receptor-mediated endocytosis	0.0083				
4	GO:0140115	Export across plasma membrane	0.0083				
4	GO:0001954	Positive regulation of cell-matrix adhesion	0.0093				
4	GO:0045661	Regulation of myoblast differentiation	0.0093				
4	GO:0051353	Positive regulation of oxidoreductase activity	0.0102				
4	GO:2000378	Negative regulation of reactive oxygen species metabolic process	0.0102				
4	GO:0050994	Regulation of lipid catabolic process	0.0118				
4	GO:0051893	Regulation of focal adhesion assembly	0.0136				
4	GO:0043550	Regulation of lipid kinase activity	0.0156				
4	GO:0001570	Vasculogenesis	0.0199				
4	GO:0003151	Outflow tract morphogenesis	0.0208				
4	GO:0007589	Body fluid secretion	0.0208				
4	GO:0031016	Pancreas development	0.0208				
4	GO:0043627	Response to estrogen	0.0215				
4	GO:0032410	Negative regulation of transporter activity	0.0284				
4	GO:0032642	Regulation of chemokine production	0.0303				
4	GO:0046889	Positive regulation of lipid biosynthetic process	0.0313				
4	GO:0035264	Multicellular organism growth	0.0324				
4	GO:0050673	Epithelial cell proliferation	0.0343				
4	GO:0003158	Endothelium development	0.0354				
4	GO:0032465	Regulation of cytokinesis	0.0354				
4	GO:0051702	Interaction with symbiont	0.0398				
4	GO:1903426	Regulation of reactive oxygen species biosynthetic process	0.0421				
4	GO:0035265	Organ growth	0.0431				
4	GO:0110020	Regulation of actomyosin structure organization	0.0455				
4	GO:0009408	Response to heat	0.0494				
3	GO:1900222	Negative regulation of amyloid-beta clearance	0.0011				
3	GO:0042989	Sequestering of actin monomers	0.0017				
3	GO:0048642	Negative regulation of skeletal muscle tissue development	0.0021				
3	GO:0071236	Cellular response to antibiotic	0.0034				
3	GO:2001028	Positive regulation of endothelial cell chemotaxis	0.0039				
3	GO:0060977	Coronary vasculature morphogenesis	0.0045				
3	GO:2000269	Regulation of fibroblast apoptotic process	0.0051				
3	GO:0055012	Ventricular cardiac muscle cell differentiation	0.0057				
3	GO:0009299	mRNA transcription	0.0071				
3	GO:0030851	Granulocyte differentiation	0.0071				
3	GO:2000178	Negative regulation of neural precursor cell proliferation	0.0079				

3	GO:0045649	Regulation of macrophage differentiation	0.0088				
3	GO:0009651	Response to salt stress	0.0095				
3	GO:0010288	Response to lead ion	0.0095				
3	GO:0044321	Response to leptin	0.0095				
3	GO:0048596	Embryonic camera-type eye morphogenesis	0.0147				
3	GO:0070371	ERK1 and ERK2 cascade	0.0157				
3	GO:0048873	Homeostasis of number of cells within a tissue	0.017				
3	GO:0014896	Muscle hypertrophy	0.0196				
3	GO:0046320	Regulation of fatty acid oxidation	0.0196				
3	GO:1901889	Negative regulation of cell junction assembly	0.0196				
3	GO:1902003	Regulation of amyloid-beta formation	0.0196				
3	GO:1902745	Positive regulation of lamellipodium organization	0.0209				
3	GO:1901031	Regulation of response to reactive oxygen species	0.0235				
3	GO:0033198	Response to ATP	0.0253				
3	GO:1900087	Positive regulation of G1/S transition of mitotic cell cycle	0.0253				
3	GO:0014002	Astrocyte development	0.0265				
3	GO:0032965	Regulation of collagen biosynthetic process	0.0265				
3	GO:0086004	Regulation of cardiac muscle cell contraction	0.0265				
3	GO:0003156	Regulation of animal organ formation	0.0281				
3	GO:0006984	ER-nucleus signaling pathway	0.0281				
3	GO:0010470	Regulation of gastrulation	0.0281				
3	GO:0021983	Pituitary gland development	0.0345				
3	GO:0045746	Negative regulation of Notch signaling pathway	0.0363				
3	GO:0071364	Cellular response to epidermal growth factor stimulus	0.0363				
3	GO:0010799	Regulation of peptidyl-threonine phosphorylation	0.0398				
3	GO:0001754	Eye photoreceptor cell differentiation	0.0417				
3	GO:1900544	Positive regulation of purine nucleotide metabolic process	0.0433				
3	GO:0060324	Face development	0.0454				
3	GO:0005977	Glycogen metabolic process	0.0473				
2	GO:0018963	Phthalate metabolic process	0.0046				
2	GO:0060532	Bronchus cartilage development	0.0046				
2	GO:0014834	Skeletal muscle satellite cell maintenance involved in skeletal muscle regeneration	0.0094				
2	GO:0046016	Positive regulation of transcription by glucose	0.0094				
2	GO:0035900	Response to isolation stress	0.0156				
2	GO:0038030	Non-canonical Wnt signaling pathway via MAPK cascade	0.0156				
2	GO:0042421	Norepinephrine biosynthetic process	0.0156				
2	GO:0043615	Astrocyte cell migration	0.0156				
2	GO:0034392	Negative regulation of smooth muscle cell apoptotic process	0.0191				
2	GO:0035280	miRNA loading onto RISC involved in gene silencing by miRNA	0.0191				
2	GO:0036035	Osteoclast development	0.0191				
2	GO:0044387	Negative regulation of protein kinase activity by regulation of protein phosphorylation	0.0225				
2	GO:2000586	Regulation of platelet-derived growth factor receptor-beta signaling pathway	0.0225				
2	GO:0033622	Integrin activation	0.0264				
2	GO:0048668	Collateral sprouting	0.0264				
2	GO:0071374	Cellular response to parathyroid hormone stimulus	0.0264				
2	GO:0045899	Positive regulation of RNA polymerase II transcription preinitiation complex assembly	0.0302				
2	GO:1900122	Positive regulation of receptor binding	0.0302				
2	GO:0045793	Positive regulation of cell size	0.0343				
2	GO:0038180	Nerve growth factor signaling pathway	0.0384				
2	GO:0048935	Peripheral nervous system neuron development	0.0427				
2	GO:0055119	Relaxation of cardiac muscle	0.0427				

2	GO:0061029	Eyelid development in camera-type eye	0.0427				
2	GO:1901163	Regulation of trophoblast cell migration	0.0427				
2	GO:0010870	Positive regulation of receptor biosynthetic process	0.0473				
2	GO:0050872	White fat cell differentiation	0.0473				
2	GO:0051645	Golgi localization	0.0473				

Supplementary Table 7 - GO, KEGG pathways and Reactome pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs in PC-3's response to AgNPs

GO Biological Processes				GO Cellular Component			
# genes	term name	description	FDR value	# genes	term name	description	FDR value
29	GO:0043170	Macromolecule metabolic process	8.68E-05	14	GO:0030054	Cell junction	0.0104
23	GO:0048583	Regulation of response to stimulus	1.70E-04	13	GO:1902494	Catalytic complex	0.002
21	GO:0042127	Regulation of cell population proliferation	4.32E-09	9	GO:0016604	Nuclear body	0.0067
17	GO:0051338	Regulation of transferase activity	1.25E-08	6	GO:0098978	Glutamatergic synapse	0.0132
15	GO:0010243	Response to organonitrogen compound	3.78E-07	GO Molecular Component			
15	GO:0051270	Regulation of cellular component movement	4.81E-07	# genes	term name	description	FDR value
15	GO:0051130	Positive regulation of cellular component organization	3.08E-06	12	GO:0044877	Protein-containing complex binding	0.0015
15	GO:0009056	Catabolic process	7.00E-04	11	GO:0005102	Signaling receptor binding	0.0354
15	GO:0006139	Nucleobase-containing compound metabolic process	0.0093	8	GO:0031625	Ubiquitin protein ligase binding	1.70E-04
14	GO:0045597	Positive regulation of cell differentiation	2.32E-06	7	GO:0016773	Phosphotransferase activity, alcohol group as acceptor	0.0417
14	GO:0009894	Regulation of catabolic process	3.57E-06	3	GO:0016538	Cyclin-dependent protein serine/threonine kinase regulator activity	0.0181
13	GO:0009628	Response to abiotic stimulus	5.31E-05	KEGG Pathways			
12	GO:0030097	Hemopoiesis	6.06E-07	# genes	term name	description	FDR value
12	GO:0030155	Regulation of cell adhesion	3.92E-06	16	hsa05200	Pathways in cancer	3.22E-12
11	GO:0043086	Negative regulation of catalytic activity	7.16E-05	10	hsa05206	MicroRNAs in cancer	3.08E-10
11	GO:0030182	Neuron differentiation	4.40E-04	9	hsa04110	Cell cycle	5.82E-10
10	GO:0001501	Skeletal system development	1.18E-05	8	hsa05161	Hepatitis B	4.42E-08
10	GO:0043933	Protein-containing complex subunit organization	0.0307	7	hsa04068	FoxO signaling pathway	2.38E-07
9	GO:0022411	Cellular component disassembly	1.74E-05	7	hsa05202	Transcriptional misregulation in cancer	1.28E-06
9	GO:0061024	Membrane organization	0.0018	6	hsa01522	Endocrine resistance	1.01E-06
9	GO:0032880	Regulation of protein localization	0.005	6	hsa04066	HIF-1 signaling pathway	1.74E-06
9	GO:0001775	Cell activation	0.012	5	hsa04926	Relaxin signaling pathway	9.21E-05
8	GO:0045862	Positive regulation of proteolysis	1.00E-04	4	hsa05217	Basal cell carcinoma	1.10E-04
8	GO:0051090	Regulation of DNA-binding transcription factor activity	2.50E-04	4	hsa05211	Renal cell carcinoma	1.40E-04
8	GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	6.80E-04	4	hsa04928	Parathyroid hormone synthesis, secretion and action	6.30E-04
8	GO:0060322	Head development	0.008	4	hsa04670	Leukocyte transendothelial migration	7.40E-04
8	GO:0018193	Peptidyl-amino acid modification	0.0133	4	hsa04728	Dopaminergic synapse	0.0012
7	GO:0048771	Tissue remodeling	5.58E-07	3	hsa04340	Hedgehog signaling pathway	0.0011
7	GO:0002761	Regulation of myeloid leukocyte differentiation	1.85E-06	3	hsa04371	Apelin signaling pathway	0.0147
7	GO:0010632	Regulation of epithelial cell migration	5.31E-05	3	hsa04530	Tight junction	0.0219
7	GO:0030336	Negative regulation of cell migration	1.10E-04	Reactome Pathways			
7	GO:0007568	Aging	1.40E-04	# genes	term name	description	FDR value
7	GO:0042493	Response to drug	1.60E-04	21	HSA-74160	Gene expression (Transcription)	2.55E-10

7	GO:0002696	Positive regulation of leukocyte activation	3.90E-04	14	HSA-5663202	Diseases of signal transduction by growth factor receptors and second messengers	2.10E-10
7	GO:0008283	Cell population proliferation	0.0032	12	HSA-392499	Metabolism of proteins	0.0177
7	GO:0070848	Response to growth factor	0.0043	11	HSA-449147	Signaling by Interleukins	4.53E-07
6	GO:0043467	Regulation of generation of precursor metabolites and energy	1.00E-04	10	HSA-8878166	Transcriptional regulation by RUNX2	2.10E-10
6	GO:1904018	Positive regulation of vasculature development	2.20E-04	9	HSA-109582	Hemostasis	3.60E-04
6	GO:0070374	Positive regulation of ERK1 and ERK2 cascade	3.40E-04	7	HSA-157118	Signaling by NOTCH	3.11E-05
6	GO:0009612	Response to mechanical stimulus	3.60E-04	6	HSA-8939211	ESR-mediated signaling	2.60E-04
6	GO:0021700	Developmental maturation	6.20E-04	4	HSA-8940973	RUNX2 regulates osteoblast differentiation	3.43E-05
6	GO:0031334	Positive regulation of protein-containing complex assembly	7.60E-04	3	HSA-8941855	RUNX3 regulates CDKN1A transcription	9.05E-05
6	GO:0071375	Cellular response to peptide hormone stimulus	0.0011	3	HSA-8951936	RUNX3 regulates p14-ARF	1.40E-04
6	GO:0010975	Regulation of neuron projection development	0.0191	3	HSA-8934593	Regulation of RUNX1 Expression and Activity	6.20E-04
6	GO:0071396	Cellular response to lipid	0.0217	3	HSA-389513	CTLA4 inhibitory signaling	9.50E-04
6	GO:0006351	Transcription, DNA-templated	0.0291	2	HSA-9682706	Replication of the SARS-CoV-1 genome	0.0046
5	GO:2000736	Regulation of stem cell differentiation	3.60E-04	2	HSA-8941332	RUNX2 regulates genes involved in cell migration	0.0066
5	GO:0001952	Regulation of cell-matrix adhesion	3.70E-04	2	HSA-9634600	Regulation of glycolysis by fructose 2,6-bisphosphate metabolism	0.0109
5	GO:0050680	Negative regulation of epithelial cell proliferation	5.20E-04	2	HSA-113501	Inhibition of replication initiation of damaged DNA by RB1/E2F1	0.0118
5	GO:0010001	Glial cell differentiation	0.0017	2	HSA-3232118	SUMOylation of transcription factors	0.0177
5	GO:0043405	Regulation of MAP kinase activity	0.0203	2	HSA-9670439	Signaling by phosphorylated juxtamembrane, extracellular and kinase domain KIT mutants	0.0208
5	GO:0048871	Multicellular organismal homeostasis	0.0222	2	HSA-982772	Growth hormone receptor signaling	0.0258
5	GO:0033044	Regulation of chromosome organization	0.0239				
5	GO:0032147	Activation of protein kinase activity	0.0282				
5	GO:0048589	Developmental growth	0.0317				
4	GO:0060965	Negative regulation of gene silencing by miRNA	1.38E-05				
4	GO:0045737	Positive regulation of cyclin-dependent protein serine/threonine kinase activity	6.23E-05				
4	GO:0045746	Negative regulation of Notch signaling pathway	1.90E-04				
4	GO:1904705	Regulation of vascular associated smooth muscle cell proliferation	3.30E-04				
4	GO:0010660	Regulation of muscle cell apoptotic process	6.90E-04				
4	GO:0043550	Regulation of lipid kinase activity	6.90E-04				
4	GO:0060070	Canonical Wnt signaling pathway	0.0015				
4	GO:0051153	Regulation of striated muscle cell differentiation	0.0028				
4	GO:0030183	B cell differentiation	0.0035				
4	GO:0043271	Negative regulation of ion transport	0.0093				
4	GO:0030856	Regulation of epithelial cell differentiation	0.0094				
4	GO:0016241	Regulation of macroautophagy	0.0146				
4	GO:0002064	Epithelial cell development	0.0203				
4	GO:0051302	Regulation of cell division	0.0208				
4	GO:0060348	Bone development	0.0214				
3	GO:0060218	Hematopoietic stem cell differentiation	4.30E-04				
3	GO:0106027	Neuron projection organization	0.0034				

3	GO:1904645	Response to amyloid-beta	0.0058				
3	GO:0048146	Positive regulation of fibroblast proliferation	0.0066				
3	GO:0035690	Cellular response to drug	0.0093				
3	GO:0031646	Positive regulation of nervous system process	0.0121				
3	GO:0010633	Negative regulation of epithelial cell migration	0.0129				
3	GO:0030279	Negative regulation of ossification	0.0155				
3	GO:0050764	Regulation of phagocytosis	0.0242				
3	GO:0045807	Positive regulation of endocytosis	0.0314				
3	GO:0043112	Receptor metabolic process	0.0329				
3	GO:0032091	Negative regulation of protein binding	0.0334				
3	GO:0043279	Response to alkaloid	0.0334				
3	GO:0035270	Endocrine system development	0.0436				
2	GO:0010727	Negative regulation of hydrogen peroxide metabolic process	0.006				
2	GO:0034983	Peptidyl-lysine deacetylation	0.006				
2	GO:0045820	Negative regulation of glycolytic process	0.0148				
2	GO:0021542	Dentate gyrus development	0.0162				
2	GO:1905048	Regulation of metalloproteinase activity	0.0177				
2	GO:0051895	Negative regulation of focal adhesion assembly	0.0192				
2	GO:2000178	Negative regulation of neural precursor cell proliferation	0.0239				
2	GO:0010226	Response to lithium ion	0.0274				
2	GO:0045453	Bone resorption	0.0274				
2	GO:0038083	Peptidyl-tyrosine autophosphorylation	0.0443				
2	GO:0035025	Positive regulation of Rho protein signal transduction	0.0462				
2	GO:0008156	Negative regulation of DNA replication	0.0482				

Supplementary Table 8 – GO, KEGG pathways and Reactome pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the downregulated miRNAs in PC-3's response to G-AgNPs

GO Biological Processes				GO Cellular Component			
# genes	term name	description	FDR value	# genes	term name	description	FDR value
45	GO:0009987	Cellular process	0.0027	45	GO:0043226	Organelle	9.51E-05
44	GO:0050794	Regulation of cellular process	2.93E-07	44	GO:0043227	Membrane-bounded organelle	4.99E-05
40	GO:0048519	Negative regulation of biological process	4.05E-13	44	GO:0043229	Intracellular organelle	5.94E-05
40	GO:0050896	Response to stimulus	4.71E-08	41	GO:0043231	Intracellular membrane-bounded organelle	7.74E-05
39	GO:0048523	Negative regulation of cellular process	3.14E-13	36	GO:0005634	Nucleus	8.94E-06
39	GO:0048518	Positive regulation of biological process	8.81E-11	32	GO:0070013	Intracellular organelle lumen	1.02E-05
39	GO:0031323	Regulation of cellular metabolic process	1.41E-10	30	GO:0031981	Nuclear lumen	4.15E-06
38	GO:0048522	Positive regulation of cellular process	5.28E-11	28	GO:0005654	Nucleoplasm	4.15E-06
37	GO:0051171	Regulation of nitrogen compound metabolic process	1.10E-09	27	GO:0032991	Protein-containing complex	2.50E-04
37	GO:0080090	Regulation of primary metabolic process	2.90E-09	27	GO:0005829	Cytosol	3.80E-04
37	GO:0060255	Regulation of macromolecule metabolic process	1.71E-08	22	GO:0043232	Intracellular non-membrane-bounded organelle	0.0353
37	GO:0008152	Metabolic process	1.31E-05	16	GO:0005694	Chromosome	9.51E-05
35	GO:0032502	Developmental process	4.07E-08	14	GO:0000785	Chromatin	6.14E-05
35	GO:0051716	Cellular response to stimulus	4.92E-07	13	GO:0000228	Nuclear chromosome	3.80E-04
34	GO:0044237	Cellular metabolic process	6.97E-05	13	GO:0098805	Whole membrane	0.0074
34	GO:0071704	Organic substance metabolic process	1.50E-04	10	GO:0000790	Nuclear chromatin	0.0084
33	GO:0010604	Positive regulation of macromolecule metabolic process	7.74E-12	8	GO:0005741	Mitochondrial outer membrane	8.74E-06
33	GO:0043170	Macromolecule metabolic process	2.42E-06	8	GO:0005667	Transcription regulator complex	5.70E-04
32	GO:0051173	Positive regulation of nitrogen compound metabolic process	4.32E-12	6	GO:0090575	RNA polymerase II transcription regulator complex	2.50E-04

32	GO:0065009	Regulation of molecular function	7.53E-08	4	GO:0097136	Bcl-2 family protein complex	4.89E-06
32	GO:0006807	Nitrogen compound metabolic process	1.20E-04	4	GO:0000792	Heterochromatin	0.0025
32	GO:0032501	Multicellular organismal process	1.50E-04	3	GO:0035985	Senescence-associated heterochromatin focus	5.94E-05
32	GO:0044238	Primary metabolic process	4.90E-04	3	GO:0046930	Pore complex	0.0022
31	GO:0009892	Negative regulation of metabolic process	1.31E-11	2	GO:0097145	BAK complex	0.0023
31	GO:0031325	Positive regulation of cellular metabolic process	9.35E-11	2	GO:0071141	SMAD protein complex	0.0112
31	GO:0044260	Cellular macromolecule metabolic process	4.53E-07	GO Molecular Component			
30	GO:0031324	Negative regulation of cellular metabolic process	1.86E-12	# genes	term name	description	FDR value
30	GO:0042221	Response to chemical	1.11E-07	41	GO:0005488	Binding	0.0126
30	GO:0010468	Regulation of gene expression	9.17E-07	33	GO:0005515	Protein binding	1.70E-04
29	GO:0048869	Cellular developmental process	3.13E-08	25	GO:1901363	Heterocyclic compound binding	0.0403
29	GO:0007275	Multicellular organism development	9.66E-06	25	GO:0097159	Organic cyclic compound binding	0.045
29	GO:0016043	Cellular component organization	4.75E-05	23	GO:0098772	Molecular function regulator	1.20E-04
28	GO:0010605	Negative regulation of macromolecule metabolic process	6.42E-10	21	GO:0003676	Nucleic acid binding	0.0106
28	GO:2000112	Regulation of cellular macromolecule biosynthetic process	2.50E-07	19	GO:0003677	DNA binding	2.10E-04
28	GO:0019219	Regulation of nucleobase-containing compound metabolic process	4.14E-07	16	GO:0043565	Sequence-specific DNA binding	1.67E-05
28	GO:0048583	Regulation of response to stimulus	7.25E-07	16	GO:0140110	Transcription regulator activity	1.40E-04
27	GO:0051172	Negative regulation of nitrogen compound metabolic process	1.14E-10	15	GO:0000976	Transcription regulatory region sequence-specific DNA binding	1.04E-05
27	GO:0010033	Response to organic substance	1.08E-08	14	GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	2.13E-05
27	GO:0006950	Response to stress	1.47E-07	14	GO:0019899	Enzyme binding	0.0403
27	GO:0051252	Regulation of RNA metabolic process	4.92E-07	13	GO:0042802	Identical protein binding	0.0294
27	GO:0023052	Signaling	2.80E-04	12	GO:0003682	Chromatin binding	1.04E-05
27	GO:0007154	Cell communication	3.60E-04	12	GO:0008134	Transcription factor binding	1.67E-05
26	GO:0010941	Regulation of cell death	1.21E-12	12	GO:0000987	Cis-regulatory region sequence-specific DNA binding	2.13E-05
26	GO:0030154	Cell differentiation	1.84E-06	12	GO:0046983	Protein dimerization activity	6.30E-04
26	GO:0007165	Signal transduction	2.60E-04	12	GO:0044877	Protein-containing complex binding	0.0026
25	GO:0042981	Regulation of apoptotic process	1.48E-12	11	GO:0000977	RNA polymerase II transcription regulatory region sequence-specific DNA binding	7.50E-04
25	GO:0045935	Positive regulation of nucleobase-containing compound metabolic process	7.15E-11	10	GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	2.52E-05
25	GO:0006355	Regulation of transcription, DNA-templated	1.63E-06	10	GO:0046914	Transition metal ion binding	0.0173
25	GO:0048731	System development	1.70E-04	9	GO:0000978	RNA polymerase II cis-regulatory region sequence-specific DNA binding	0.0033
24	GO:1901564	Organonitrogen compound metabolic process	0.0064	9	GO:0019904	Protein domain specific binding	0.0051
23	GO:0042127	Regulation of cell population proliferation	1.73E-10	8	GO:0042803	Protein homodimerization activity	0.0172
23	GO:0051254	Positive regulation of RNA metabolic process	6.49E-10	8	GO:0008270	Zinc ion binding	0.045
23	GO:0010629	Negative regulation of gene expression	7.47E-09	7	GO:0046982	Protein heterodimerization activity	0.0021
23	GO:0006357	Regulation of transcription by RNA polymerase II	2.61E-08	5	GO:0051400	BH domain binding	1.11E-06
23	GO:0010628	Positive regulation of gene expression	7.53E-08	4	GO:0019903	Protein phosphatase binding	0.0403
23	GO:0051128	Regulation of cellular component organization	1.15E-07	3	GO:0051434	BH3 domain binding	1.90E-04
23	GO:0050793	Regulation of developmental process	5.35E-07	3	GO:0051721	Protein phosphatase 2A binding	0.0093
23	GO:0070887	Cellular response to chemical stimulus	2.58E-06	3	GO:0097718	Disordered domain specific binding	0.0106
23	GO:0009966	Regulation of signal transduction	7.65E-06	3	GO:0005518	Collagen binding	0.0465
23	GO:0048513	Animal organ development	1.18E-05	2	GO:0051575	5-deoxyribose-5-phosphate lyase activity	0.0125
22	GO:0010557	Positive regulation of macromolecule biosynthetic process	1.77E-08	2	GO:0003680	Minor groove of adenine-thymine-rich DNA binding	0.0235
22	GO:0031328	Positive regulation of cellular biosynthetic process	3.82E-08	2	GO:0097371	MDM2/MDM4 family protein binding	0.0278
22	GO:0032268	Regulation of cellular protein metabolic process	3.26E-06	2	GO:0004861	Cyclin-dependent protein serine/threonine kinase inhibitor activity	0.0403
22	GO:0065008	Regulation of biological quality	0.0014	2	GO:0003906	DNA-(apurinic or apyrimidinic site) endonuclease activity	0.0449
21	GO:0051726	Regulation of cell cycle	9.22E-11	KEGG Pathways			
21	GO:0033554	Cellular response to stress	2.16E-08	# genes	description	FDR value	
21	GO:0048584	Positive regulation of response to stimulus	1.02E-06	17	hsa05200	Pathways in cancer	2.74E-13

21	GO:1901360	Organic cyclic compound metabolic process	1.20E-04	14	hsa05206	MicroRNAs in cancer	6.94E-16
21	GO:0019538	Protein metabolic process	0.0072	9	hsa01522	Endocrine resistance	2.44E-10
20	GO:0045893	Positive regulation of transcription, DNA-templated	3.65E-08	9	hsa05226	Gastric cancer	4.07E-09
20	GO:0071310	Cellular response to organic substance	9.94E-06	9	hsa05225	Hepatocellular carcinoma	8.51E-09
20	GO:0050790	Regulation of catalytic activity	1.10E-05	8	hsa01524	Platinum drug resistance	8.40E-10
20	GO:0006725	Cellular aromatic compound metabolic process	1.50E-04	8	hsa05212	Pancreatic cancer	9.17E-10
20	GO:0051239	Regulation of multicellular organismal process	6.80E-04	8	hsa05224	Breast cancer	5.19E-08
20	GO:0034641	Cellular nitrogen compound metabolic process	8.60E-04	8	hsa05169	Epstein-Barr virus infection	4.16E-07
19	GO:2000113	Negative regulation of cellular macromolecule biosynthetic process	6.71E-08	8	hsa05205	Proteoglycans in cancer	4.39E-07
19	GO:1902531	Regulation of intracellular signal transduction	1.02E-06	8	hsa05163	Human cytomegalovirus infection	8.70E-07
19	GO:0006139	Nucleobase-containing compound metabolic process	1.90E-04	7	hsa05223	Non-small cell lung cancer	1.79E-08
19	GO:0006996	Organelle organization	0.0045	7	hsa04115	p53 signaling pathway	2.31E-08
19	GO:0044267	Cellular protein metabolic process	0.0095	7	hsa05214	Glioma	2.31E-08
18	GO:0009628	Response to abiotic stimulus	1.71E-08	7	hsa05218	Melanoma	2.31E-08
18	GO:0045944	Positive regulation of transcription by RNA polymerase II	4.78E-08	7	hsa05220	Chronic myeloid leukemia	2.31E-08
18	GO:0033043	Regulation of organelle organization	7.82E-08	7	hsa05210	Colorectal cancer	3.47E-08
18	GO:0051253	Negative regulation of RNA metabolic process	2.48E-07	7	hsa04210	Apoptosis	6.12E-07
18	GO:1901700	Response to oxygen-containing compound	7.94E-07	7	hsa05161	Hepatitis B	1.79E-06
18	GO:0031399	Regulation of protein modification process	8.62E-06	7	hsa05202	Transcriptional misregulation in cancer	2.62E-06
18	GO:0009653	Anatomical structure morphogenesis	5.48E-05	7	hsa05166	Human T-cell leukemia virus 1 infection	9.11E-06
18	GO:0090304	Nucleic acid metabolic process	5.95E-05	7	hsa04151	PI3K-Akt signaling pathway	1.80E-04
18	GO:0009605	Response to external stimulus	1.20E-04	6	hsa05219	Bladder cancer	3.06E-08
17	GO:0045892	Negative regulation of transcription, DNA-templated	3.42E-07	6	hsa05222	Small cell lung cancer	1.79E-06
17	GO:0007049	Cell cycle	4.92E-07	6	hsa05215	Prostate cancer	2.06E-06
17	GO:0009059	Macromolecule biosynthetic process	8.12E-06	6	hsa04110	Cell cycle	6.36E-06
17	GO:0009967	Positive regulation of signal transduction	8.82E-06	6	hsa05162	Measles	1.29E-05
17	GO:0051247	Positive regulation of protein metabolic process	1.35E-05	6	hsa04218	Cellular senescence	1.86E-05
17	GO:0019220	Regulation of phosphate metabolic process	2.88E-05	6	hsa05160	Hepatitis C	2.24E-05
17	GO:0045595	Regulation of cell differentiation	4.05E-05	6	hsa05167	Kaposi sarcoma-associated herpesvirus infection	5.96E-05
17	GO:2000026	Regulation of multicellular organismal development	1.50E-04	6	hsa04010	MAPK signaling pathway	5.90E-04
17	GO:0007166	Cell surface receptor signaling pathway	5.20E-04	6	hsa05165	Human papillomavirus infection	9.90E-04
16	GO:0043065	Positive regulation of apoptotic process	7.72E-10	6	hsa05168	Herpes simplex virus 1 infection	0.0066
16	GO:0008285	Negative regulation of cell population proliferation	1.48E-09	5	hsa05213	Endometrial cancer	4.65E-06
16	GO:0051248	Negative regulation of protein metabolic process	3.42E-07	5	hsa01521	EGFR tyrosine kinase inhibitor resistance	1.67E-05
16	GO:0051094	Positive regulation of developmental process	5.44E-06	4	hsa04215	Apoptosis - multiple species	1.40E-05
16	GO:0001932	Regulation of protein phosphorylation	9.66E-06	4	hsa05217	Basal cell carcinoma	1.70E-04
16	GO:0034645	Cellular macromolecule biosynthetic process	2.83E-05	4	hsa04211	Longevity regulating pathway	5.90E-04
16	GO:0032270	Positive regulation of cellular protein metabolic process	3.74E-05	4	hsa04933	AGE-RAGE signaling pathway in diabetic complications	8.60E-04
16	GO:0048585	Negative regulation of response to stimulus	3.75E-05	4	hsa04928	Parathyroid hormone synthesis, secretion and action	9.90E-04
16	GO:0035556	Intracellular signal transduction	6.14E-05	4	hsa04066	HIF-1 signaling pathway	0.001
16	GO:0032879	Regulation of localization	0.0085	4	hsa05418	Fluid shear stress and atherosclerosis	0.0022
15	GO:0007346	Regulation of mitotic cell cycle	1.09E-08	4	hsa04550	Signaling pathways regulating pluripotency of stem cells	0.0028
15	GO:0006974	Cellular response to DNA damage stimulus	5.75E-08	4	hsa04934	Cushing syndrome	0.0038
15	GO:0060548	Negative regulation of cell death	6.88E-07	4	hsa05203	Viral carcinogenesis	0.0067
15	GO:0032269	Negative regulation of cellular protein metabolic process	1.11E-06	4	hsa05170	Human immunodeficiency virus 1 infection	0.0099
15	GO:0022414	Reproductive process	3.17E-05	4	hsa05131	Shigellosis	0.0123
15	GO:0080134	Regulation of response to stress	4.10E-05	3	hsa05216	Thyroid cancer	9.20E-04
15	GO:0044093	Positive regulation of molecular function	5.70E-04	3	hsa05221	Acute myeloid leukemia	0.0042
15	GO:0010467	Gene expression	0.0017	3	hsa04722	Neurotrophin signaling pathway	0.0175
15	GO:0007399	Nervous system development	0.0064	3	hsa04071	Sphingolipid signaling pathway	0.018
14	GO:0043066	Negative regulation of apoptotic process	1.29E-06	3	hsa04068	FoxO signaling pathway	0.0227

14	GO:0000122	Negative regulation of transcription by RNA polymerase II	1.31E-06	3	hsa04217	Necroptosis	0.0338
14	GO:0022402	Cell cycle process	3.37E-06	3	hsa04932	Non-alcoholic fatty liver disease	0.0338
14	GO:0009968	Negative regulation of signal transduction	5.44E-05	3	hsa04310	Wnt signaling pathway	0.0356
14	GO:0051240	Positive regulation of multicellular organismal process	0.0014	3	hsa04390	Hippo signaling pathway	0.0356
13	GO:0045786	Negative regulation of cell cycle	1.11E-07	3	hsa04141	Protein processing in endoplasmic reticulum	0.0422
13	GO:1903047	Mitotic cell cycle process	2.48E-07	3	hsa05164	Influenza A	0.0422
13	GO:0080135	Regulation of cellular response to stress	1.29E-06	3	hsa05152	Tuberculosis	0.0428
13	GO:0010564	Regulation of cell cycle process	1.67E-06	Reactome Pathways			
13	GO:0014070	Response to organic cyclic compound	1.08E-05	# genes	term name	description	FDR value
13	GO:0009894	Regulation of catabolic process	3.90E-05	16	HSA-162582	Signal Transduction	0.0269
13	GO:1902533	Positive regulation of intracellular signal transduction	3.98E-05	15	HSA-74160	Gene expression (Transcription)	2.50E-04
13	GO:0012501	Programmed cell death	4.41E-05	13	HSA-212436	Generic Transcription Pathway	7.20E-04
13	GO:0051130	Positive regulation of cellular component organization	1.60E-04	8	HSA-2559583	Cellular Senescence	6.34E-06
13	GO:0009719	Response to endogenous stimulus	8.60E-04	8	HSA-3700989	Transcriptional Regulation by TP53	6.60E-04
13	GO:0043085	Positive regulation of catalytic activity	0.0011	7	HSA-109581	Apoptosis	1.40E-04
13	GO:0016070	RNA metabolic process	0.0019	7	HSA-69278	Cell Cycle, Mitotic	0.0185
13	GO:0009888	Tissue development	0.0045	6	HSA-2559585	Oncogene Induced Senescence	7.93E-07
13	GO:0022607	Cellular component assembly	0.0441	5	HSA-109606	Intrinsic Pathway for Apoptosis	1.40E-04
12	GO:0045787	Positive regulation of cell cycle	3.10E-08	5	HSA-2559580	Oxidative Stress Induced Senescence	7.90E-04
12	GO:2001233	Regulation of apoptotic signaling pathway	4.71E-08	4	HSA-5633008	TP53 Regulates Transcription of Cell Death Genes	8.70E-04
12	GO:0008283	Cell population proliferation	2.48E-07	4	HSA-6791312	TP53 Regulates Transcription of Cell Cycle Genes	0.0012
12	GO:0010638	Positive regulation of organelle organization	2.16E-06	4	HSA-69231	Cyclin D associated events in G1	0.0012
12	GO:0031329	Regulation of cellular catabolic process	4.41E-05	4	HSA-6785807	Interleukin-4 and Interleukin-13 signaling	0.013
12	GO:0006915	Apoptotic process	6.66E-05	4	HSA-5633007	Regulation of TP53 Activity	0.0376
12	GO:0003006	Developmental process involved in reproduction	8.70E-05	3	HSA-2559584	Formation of Senescence-Associated Heterochromatin Foci (SAHF)	0.0017
12	GO:0009887	Animal organ morphogenesis	1.10E-04	3	HSA-6803204	TP53 Regulates Transcription of Genes Involved in Cytochrome C Release	0.0024
12	GO:1901701	Cellular response to oxygen-containing compound	2.30E-04	3	HSA-114452	Activation of BH3-only proteins	0.0062
12	GO:0044092	Negative regulation of molecular function	5.50E-04	2	HSA-6804754	Regulation of TP53 Expression	0.0041
12	GO:0009056	Catabolic process	0.0402	2	HSA-111457	Release of apoptotic factors from the mitochondria	0.0116
11	GO:1901990	Regulation of mitotic cell cycle phase transition	4.77E-07	2	HSA-8941855	RUNX3 regulates CDKN1A transcription	0.0181
11	GO:0048608	Reproductive structure development	4.96E-07	2	HSA-111453	BH3-only proteins associate with and inactivate anti-apoptotic BCL-2 members	0.0208
11	GO:0040008	Regulation of growth	3.00E-05	2	HSA-196791	SHC-related events triggered by IGF1R	0.0244
11	GO:0035295	Tube development	1.90E-04	2	HSA-68689	Vitamin D (calciferol) metabolism	0.032
11	GO:0008284	Positive regulation of cell population proliferation	3.70E-04	2	HSA-6803211	CDC6 association with the ORC:origin complex	0.032
11	GO:0051093	Negative regulation of developmental process	6.20E-04	2	HSA-6804116	TP53 Regulates Transcription of Death Receptors and Ligands	0.0327
11	GO:0009790	Embryo development	7.30E-04	2		TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest	0.0404
11	GO:0060341	Regulation of cellular localization	8.80E-04				
11	GO:0051338	Regulation of transferase activity	9.40E-04				
11	GO:0071495	Cellular response to endogenous stimulus	0.0025				
11	GO:0031401	Positive regulation of protein modification process	0.0039				
11	GO:0044271	Cellular nitrogen compound biosynthetic process	0.015				
11	GO:0048699	Generation of neurons	0.0172				
11	GO:0048468	Cell development	0.0241				
11	GO:0042592	Homeostatic process	0.0287				
11	GO:0051049	Regulation of transport	0.042				
10	GO:0010821	Regulation of mitochondrion organization	1.60E-08				
10	GO:1901991	Negative regulation of mitotic cell cycle phase transition	3.51E-08				
10	GO:0097190	Apoptotic signaling pathway	2.48E-07				

10	GO:0090068	Positive regulation of cell cycle process	2.99E-07				
10	GO:0045936	Negative regulation of phosphate metabolic process	4.41E-05				
10	GO:0031400	Negative regulation of protein modification process	7.43E-05				
10	GO:0006325	Chromatin organization	2.80E-04				
10	GO:0034654	Nucleobase-containing compound biosynthetic process	0.0029				
10	GO:0045597	Positive regulation of cell differentiation	0.0029				
10	GO:0001934	Positive regulation of protein phosphorylation	0.0035				
10	GO:0051241	Negative regulation of multicellular organismal process	0.012				
10	GO:0051336	Regulation of hydrolase activity	0.0156				
9	GO:2001242	Regulation of intrinsic apoptotic signaling pathway	5.61E-08				
9	GO:0070482	Response to oxygen levels	1.97E-05				
9	GO:0007005	Mitochondrion organization	6.59E-05				
9	GO:1901652	Response to peptide	9.46E-05				
9	GO:0001501	Skeletal system development	1.30E-04				
9	GO:0071900	Regulation of protein serine/threonine kinase activity	1.80E-04				
9	GO:0006351	Transcription, DNA-templated	3.30E-04				
9	GO:0035239	Tube morphogenesis	8.60E-04				
9	GO:0051129	Negative regulation of cellular component organization	0.0015				
9	GO:0045596	Negative regulation of cell differentiation	0.0017				
9	GO:0030162	Regulation of proteolysis	0.0019				
9	GO:0060322	Head development	0.0028				
9	GO:0043086	Negative regulation of catalytic activity	0.0032				
9	GO:0032880	Regulation of protein localization	0.0078				
9	GO:0044087	Regulation of cellular component biogenesis	0.0097				
9	GO:0044703	Multi-organism reproductive process	0.0097				
9	GO:0007417	Central nervous system development	0.0108				
9	GO:0022603	Regulation of anatomical structure morphogenesis	0.0196				
9	GO:0034097	Response to cytokine	0.0203				
9	GO:0048878	Chemical homeostasis	0.023				
8	GO:0009411	Response to UV	3.62E-07				
8	GO:0097193	Intrinsic apoptotic signaling pathway	4.81E-07				
8	GO:2001235	Positive regulation of apoptotic signaling pathway	1.33E-06				
8	GO:0043523	Regulation of neuron apoptotic process	3.98E-06				
8	GO:2001020	Regulation of response to DNA damage stimulus	5.97E-06				
8	GO:2001234	Negative regulation of apoptotic signaling pathway	7.39E-06				
8	GO:0045165	Cell fate commitment	1.10E-05				
8	GO:0007568	Aging	2.16E-05				
8	GO:0001666	Response to hypoxia	8.43E-05				
8	GO:0010639	Negative regulation of organelle organization	2.10E-04				
8	GO:0001933	Negative regulation of protein phosphorylation	2.70E-04				
8	GO:1903827	Regulation of cellular protein localization	0.0019				
8	GO:0019221	Cytokine-mediated signaling pathway	0.005				
8	GO:0030155	Regulation of cell adhesion	0.0066				
8	GO:0007167	Enzyme linked receptor protein signaling pathway	0.007				
8	GO:0007420	Brain development	0.0084				
8	GO:0061024	Membrane organization	0.0122				
8	GO:0048609	Multicellular organismal reproductive process	0.0131				
8	GO:0033993	Response to lipid	0.0179				
8	GO:0044403	Symbiotic process	0.0188				
8	GO:0018193	Peptidyl-amino acid modification	0.019				
8	GO:0072359	Circulatory system development	0.0195				

8	GO:0048646	Anatomical structure formation involved in morphogenesis	0.021				
8	GO:0019725	Cellular homeostasis	0.0226				
8	GO:0051050	Positive regulation of transport	0.0265				
8	GO:0030182	Neuron differentiation	0.0437				
7	GO:0090199	Regulation of release of cytochrome c from mitochondria	1.89E-08				
7	GO:0008637	Apoptotic mitochondrial changes	4.07E-08				
7	GO:0046902	Regulation of mitochondrial membrane permeability	1.60E-07				
7	GO:2000134	Negative regulation of G1/S transition of mitotic cell cycle	1.02E-06				
7	GO:0007093	Mitotic cell cycle checkpoint	8.88E-06				
7	GO:0043281	Regulation of cysteine-type endopeptidase activity involved in apoptotic process	5.44E-05				
7	GO:0001503	Ossification	1.70E-04				
7	GO:0071214	Cellular response to abiotic stimulus	4.30E-04				
7	GO:0033044	Regulation of chromosome organization	8.90E-04				
7	GO:0022411	Cellular component disassembly	0.0015				
7	GO:0048732	Gland development	0.0018				
7	GO:0001558	Regulation of cell growth	0.002				
7	GO:0022407	Regulation of cell-cell adhesion	0.0021				
7	GO:0031667	Response to nutrient levels	0.0028				
7	GO:0009896	Positive regulation of catabolic process	0.0034				
7	GO:1902532	Negative regulation of intracellular signal transduction	0.0052				
7	GO:0002694	Regulation of leukocyte activation	0.0065				
7	GO:0044089	Positive regulation of cellular component biogenesis	0.0072				
7	GO:0007423	Sensory organ development	0.0086				
7	GO:0048598	Embryonic morphogenesis	0.0093				
7	GO:0071417	Cellular response to organonitrogen compound	0.0109				
7	GO:0051223	Regulation of protein transport	0.0136				
7	GO:0043408	Regulation of MAPK cascade	0.0289				
7	GO:0006259	DNA metabolic process	0.0337				
7	GO:0055082	Cellular chemical homeostasis	0.0342				
7	GO:0016032	Viral process	0.0396				
6	GO:0097192	Extrinsic apoptotic signaling pathway in absence of ligand	1.11E-07				
6	GO:2001244	Positive regulation of intrinsic apoptotic signaling pathway	1.15E-06				
6	GO:0008630	Intrinsic apoptotic signaling pathway in response to DNA damage	2.57E-06				
6	GO:0034644	Cellular response to UV	5.76E-06				
6	GO:0071158	Positive regulation of cell cycle arrest	6.07E-06				
6	GO:0010822	Positive regulation of mitochondrion organization	3.64E-05				
6	GO:0045931	Positive regulation of mitotic cell cycle	1.20E-04				
6	GO:0031497	Chromatin assembly	1.90E-04				
6	GO:1901796	Regulation of signal transduction by p53 class mediator	2.50E-04				
6	GO:0071453	Cellular response to oxygen levels	5.80E-04				
6	GO:0045926	Negative regulation of growth	0.0011				
6	GO:0007548	Sex differentiation	0.0013				
6	GO:0033157	Regulation of intracellular protein transport	0.0016				
6	GO:0044772	Mitotic cell cycle phase transition	0.0019				
6	GO:0051348	Negative regulation of transferase activity	0.0023				
6	GO:0062197	Cellular response to chemical stress	0.0023				
6	GO:0071496	Cellular response to external stimulus	0.0029				
6	GO:1903037	Regulation of leukocyte cell-cell adhesion	0.0032				
6	GO:1901653	Cellular response to peptide	0.004				
6	GO:1903829	Positive regulation of cellular protein localization	0.0045				
6	GO:0051052	Regulation of DNA metabolic process	0.0057				

6	GO:0051098	Regulation of binding	0.0067				
6	GO:0045862	Positive regulation of proteolysis	0.007				
6	GO:0048589	Developmental growth	0.008				
6	GO:0006979	Response to oxidative stress	0.0083				
6	GO:0031331	Positive regulation of cellular catabolic process	0.0086				
6	GO:0006366	Transcription by RNA polymerase II	0.0096				
6	GO:0045785	Positive regulation of cell adhesion	0.0115				
6	GO:0007389	Pattern specification process	0.0126				
6	GO:0051249	Regulation of lymphocyte activation	0.0143				
6	GO:0048568	Embryonic organ development	0.0146				
6	GO:0043254	Regulation of protein-containing complex assembly	0.0149				
6	GO:0034248	Regulation of cellular amide metabolic process	0.0157				
6	GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	0.0267				
6	GO:0006281	DNA repair	0.0276				
6	GO:0032990	Cell part morphogenesis	0.0283				
6	GO:0048729	Tissue morphogenesis	0.0369				
6	GO:0006875	Cellular metal ion homeostasis	0.0381				
6	GO:0030097	Hemopoiesis	0.0395				
6	GO:0010608	Posttranscriptional regulation of gene expression	0.0407				
5	GO:0001836	Release of cytochrome c from mitochondria	6.02E-07				
5	GO:0090200	Positive regulation of release of cytochrome c from mitochondria	1.43E-06				
5	GO:0070059	Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	3.14E-06				
5	GO:1901030	Positive regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway	4.44E-06				
5	GO:0007569	Cell aging	4.45E-05				
5	GO:2000106	Regulation of leukocyte apoptotic process	1.40E-04				
5	GO:0006919	Activation of cysteine-type endopeptidase activity involved in apoptotic process	1.60E-04				
5	GO:2001243	Negative regulation of intrinsic apoptotic signaling pathway	2.30E-04				
5	GO:0044773	Mitotic DNA damage checkpoint	2.50E-04				
5	GO:0072331	Signal transduction by p53 class mediator	5.60E-04				
5	GO:0071901	Negative regulation of protein serine/threonine kinase activity	8.30E-04				
5	GO:0048634	Regulation of muscle organ development	8.60E-04				
5	GO:0007050	Cell cycle arrest	9.50E-04				
5	GO:0043524	Negative regulation of neuron apoptotic process	0.0012				
5	GO:0048863	Stem cell differentiation	0.0014				
5	GO:0007088	Regulation of mitotic nuclear division	0.0016				
5	GO:0001763	Morphogenesis of a branching structure	0.0019				
5	GO:0007565	Female pregnancy	0.0026				
5	GO:0030308	Negative regulation of cell growth	0.0028				
5	GO:0071456	Cellular response to hypoxia	0.0029				
5	GO:1902275	Regulation of chromatin organization	0.0035				
5	GO:1905475	Regulation of protein localization to membrane	0.0035				
5	GO:0031669	Cellular response to nutrient levels	0.0039				
5	GO:0006338	Chromatin remodeling	0.004				
5	GO:0009612	Response to mechanical stimulus	0.0045				
5	GO:0050670	Regulation of lymphocyte proliferation	0.0049				
5	GO:0034599	Cellular response to oxidative stress	0.0076				
5	GO:0031334	Positive regulation of protein-containing complex assembly	0.0081				
5	GO:0031330	Negative regulation of cellular catabolic process	0.0092				
5	GO:0022409	Positive regulation of cell-cell adhesion	0.0113				
5	GO:0032259	Methylation	0.0162				

5	GO:0050863	Regulation of T cell activation	0.0226				
5	GO:0010721	Negative regulation of cell development	0.0228				
5	GO:0003002	Regionalization	0.0232				
5	GO:0002521	Leukocyte differentiation	0.0249				
5	GO:0050678	Regulation of epithelial cell proliferation	0.0252				
5	GO:0010506	Regulation of autophagy	0.0254				
5	GO:0071902	Positive regulation of protein serine/threonine kinase activity	0.0274				
5	GO:0016570	Histone modification	0.0282				
5	GO:0051222	Positive regulation of protein transport	0.0284				
5	GO:0001701	In utero embryonic development	0.038				
5	GO:0046649	Lymphocyte activation	0.0409				
5	GO:0048514	Blood vessel morphogenesis	0.049				
4	GO:0090343	Positive regulation of cell aging	8.93E-06				
4	GO:0035794	Positive regulation of mitochondrial membrane permeability	2.95E-05				
4	GO:1902108	Regulation of mitochondrial membrane permeability involved in apoptotic process	3.34E-05				
4	GO:0032469	Endoplasmic reticulum calcium ion homeostasis	4.19E-05				
4	GO:0045736	Negative regulation of cyclin-dependent protein serine/threonine kinase activity	6.59E-05				
4	GO:2000108	Positive regulation of leukocyte apoptotic process	7.28E-05				
4	GO:0090398	Cellular senescence	9.88E-05				
4	GO:2000772	Regulation of cellular senescence	2.30E-04				
4	GO:0051402	Neuron apoptotic process	2.90E-04				
4	GO:1904645	Response to amyloid-beta	3.60E-04				
4	GO:0002260	Lymphocyte homeostasis	4.60E-04				
4	GO:0002931	Response to ischemia	5.50E-04				
4	GO:0070228	Regulation of lymphocyte apoptotic process	5.50E-04				
4	GO:0010332	Response to gamma radiation	6.10E-04				
4	GO:0031507	Heterochromatin assembly	6.10E-04				
4	GO:0043525	Positive regulation of neuron apoptotic process	6.10E-04				
4	GO:0045840	Positive regulation of mitotic nuclear division	6.10E-04				
4	GO:0006977	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	6.40E-04				
4	GO:0010660	Regulation of muscle cell apoptotic process	9.10E-04				
4	GO:0051881	Regulation of mitochondrial membrane potential	0.0014				
4	GO:1903747	Regulation of establishment of protein localization to mitochondrion	0.0014				
4	GO:0010507	Negative regulation of autophagy	0.0018				
4	GO:0048145	Regulation of fibroblast proliferation	0.0019				
4	GO:2001021	Negative regulation of response to DNA damage stimulus	0.0019				
4	GO:0009791	Post-embryonic development	0.002				
4	GO:2001237	Negative regulation of extrinsic apoptotic signaling pathway	0.0039				
4	GO:0030183	B cell differentiation	0.0044				
4	GO:0046660	Female sex differentiation	0.0044				
4	GO:0010906	Regulation of glucose metabolic process	0.0054				
4	GO:0000082	G1/S transition of mitotic cell cycle	0.007				
4	GO:1905477	Positive regulation of protein localization to membrane	0.0072				
4	GO:0048754	Branching morphogenesis of an epithelial tube	0.0075				
4	GO:0008584	Male gonad development	0.0078				
4	GO:0016202	Regulation of striated muscle tissue development	0.0078				
4	GO:0006479	Protein methylation	0.008				
4	GO:0060968	Regulation of gene silencing	0.0085				
4	GO:2001251	Negative regulation of chromosome organization	0.0085				
4	GO:0033135	Regulation of peptidyl-serine phosphorylation	0.0103				

4	GO:0031056	Regulation of histone modification	0.0115				
4	GO:0001890	Placenta development	0.0122				
4	GO:0034249	Negative regulation of cellular amide metabolic process	0.0122				
4	GO:0051147	Regulation of muscle cell differentiation	0.0122				
4	GO:0007369	Gastrulation	0.0135				
4	GO:0006367	Transcription initiation from RNA polymerase II promoter	0.0143				
4	GO:0042129	Regulation of T cell proliferation	0.0149				
4	GO:0051100	Negative regulation of binding	0.0172				
4	GO:0090316	Positive regulation of intracellular protein transport	0.0208				
4	GO:0050731	Positive regulation of peptidyl-tyrosine phosphorylation	0.0252				
4	GO:0000302	Response to reactive oxygen species	0.0259				
4	GO:0042594	Response to starvation	0.0259				
4	GO:0060348	Bone development	0.0263				
4	GO:0071695	Anatomical structure maturation	0.0266				
4	GO:0009952	Anterior/posterior pattern specification	0.0321				
4	GO:1902749	Regulation of cell cycle G2/M phase transition	0.0324				
4	GO:0009636	Response to toxic substance	0.0342				
4	GO:0061448	Connective tissue development	0.0362				
4	GO:1903039	Positive regulation of leukocyte cell-cell adhesion	0.0387				
4	GO:0042110	T cell activation	0.047				
3	GO:0035986	Senescence-associated heterochromatin focus assembly	1.95E-05				
3	GO:0001844	Protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	8.72E-05				
3	GO:1903896	Positive regulation of IRE1-mediated unfolded protein response	1.20E-04				
3	GO:0032471	Negative regulation of endoplasmic reticulum calcium ion concentration	1.50E-04				
3	GO:2000774	Positive regulation of cellular senescence	3.30E-04				
3	GO:0070231	T cell apoptotic process	5.40E-04				
3	GO:0070230	Positive regulation of lymphocyte apoptotic process	7.80E-04				
3	GO:0034391	Regulation of smooth muscle cell apoptotic process	0.0012				
3	GO:1903798	Regulation of production of miRNAs involved in gene silencing by miRNA	0.0012				
3	GO:0001782	B cell homeostasis	0.0013				
3	GO:0010661	Positive regulation of muscle cell apoptotic process	0.0017				
3	GO:1900740	Positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	0.0017				
3	GO:0097300	Programmed necrotic cell death	0.0019				
3	GO:0033280	Response to vitamin D	0.002				
3	GO:0048147	Negative regulation of fibroblast proliferation	0.002				
3	GO:1902230	Negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage	0.0025				
3	GO:1902253	Regulation of intrinsic apoptotic signaling pathway by p53 class mediator	0.0025				
3	GO:0043029	T cell homeostasis	0.0029				
3	GO:0060969	Negative regulation of gene silencing	0.0029				
3	GO:0043516	Regulation of DNA damage response, signal transduction by p53 class mediator	0.0031				
3	GO:1900117	Regulation of execution phase of apoptosis	0.0045				
3	GO:0046825	Regulation of protein export from nucleus	0.0046				
3	GO:0048806	Genitalia development	0.0065				
3	GO:0072332	Intrinsic apoptotic signaling pathway by p53 class mediator	0.0074				
3	GO:0001541	Ovarian follicle development	0.008				
3	GO:0010823	Negative regulation of mitochondrion organization	0.0116				
3	GO:0022617	Extracellular matrix disassembly	0.0134				
3	GO:0045844	Positive regulation of striated muscle tissue development	0.0143				
3	GO:0040014	Regulation of multicellular organism growth	0.0147				
3	GO:0007492	Endoderm development	0.0183				

3	GO:0034103	Regulation of tissue remodeling	0.0189				
3	GO:0046651	Lymphocyte proliferation	0.022				
3	GO:0008286	Insulin receptor signaling pathway	0.0252				
3	GO:0001892	Embryonic placenta development	0.0259				
3	GO:2000177	Regulation of neural precursor cell proliferation	0.0259				
3	GO:0110110	Positive regulation of animal organ morphogenesis	0.0265				
3	GO:0016571	Histone methylation	0.029				
3	GO:0048771	Tissue remodeling	0.0311				
3	GO:0035282	Segmentation	0.0319				
3	GO:0043255	Regulation of carbohydrate biosynthetic process	0.0319				
3	GO:0022612	Gland morphogenesis	0.0333				
3	GO:0010522	Regulation of calcium ion transport into cytosol	0.0347				
3	GO:0051153	Regulation of striated muscle cell differentiation	0.0357				
3	GO:0032091	Negative regulation of protein binding	0.0391				
3	GO:0021761	Limbic system development	0.0417				
3	GO:0003279	Cardiac septum development	0.0436				
3	GO:0051781	Positive regulation of cell division	0.0492				
2	GO:0002352	B cell negative selection	0.0013				
2	GO:0090402	Oncogene-induced cell senescence	0.0013				
2	GO:0006808	Regulation of nitrogen utilization	0.0019				
2	GO:0048597	Post-embryonic camera-type eye morphogenesis	0.0019				
2	GO:0060058	Positive regulation of apoptotic process involved in mammary gland involution	0.0019				
2	GO:0070561	Vitamin D receptor signaling pathway	0.0019				
2	GO:0033088	Negative regulation of immature T cell proliferation in thymus	0.0035				
2	GO:0060011	Sertoli cell proliferation	0.0035				
2	GO:0070242	Thymocyte apoptotic process	0.0035				
2	GO:1902262	Apoptotic process involved in blood vessel morphogenesis	0.0035				
2	GO:0002326	B cell lineage commitment	0.0045				
2	GO:0070245	Positive regulation of thymocyte apoptotic process	0.0045				
2	GO:0008635	Activation of cysteine-type endopeptidase activity involved in apoptotic process by cytochrome c	0.0055				
2	GO:0010587	miRNA catabolic process	0.0055				
2	GO:0048102	Autophagic cell death	0.0055				
2	GO:0001783	B cell apoptotic process	0.0066				
2	GO:0048087	Positive regulation of developmental pigmentation	0.0078				
2	GO:1905459	Regulation of vascular associated smooth muscle cell apoptotic process	0.0078				
2	GO:0060068	Vagina development	0.0089				
2	GO:1903800	Positive regulation of production of miRNAs involved in gene silencing by miRNA	0.0103				
2	GO:2001269	Positive regulation of cysteine-type endopeptidase activity involved in apoptotic signaling pathway	0.0103				
2	GO:0035404	Histone-serine phosphorylation	0.0117				
2	GO:1902510	Regulation of apoptotic DNA fragmentation	0.0117				
2	GO:0051988	Regulation of attachment of spindle microtubules to kinetochore	0.0132				
2	GO:0090399	Replicative senescence	0.0132				
2	GO:0010248	Establishment or maintenance of transmembrane electrochemical gradient	0.0146				
2	GO:0031054	pre-miRNA processing	0.0146				
2	GO:0034393	Positive regulation of smooth muscle cell apoptotic process	0.0146				
2	GO:1903799	Negative regulation of production of miRNAs involved in gene silencing by miRNA	0.0146				
2	GO:0008053	Mitochondrial fusion	0.0161				
2	GO:0042359	Vitamin D metabolic process	0.0161				
2	GO:0010225	Response to UV-C	0.0177				

2	GO:0045725	Positive regulation of glycogen biosynthetic process	0.0177				
2	GO:0070102	interleukin-6-mediated signaling pathway	0.0177				
2	GO:1900119	Positive regulation of execution phase of apoptosis	0.0177				
2	GO:1902166	Negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	0.0194				
2	GO:0002902	Regulation of B cell apoptotic process	0.0211				
2	GO:2000811	Negative regulation of anoikis	0.0211				
2	GO:0006337	Nucleosome disassembly	0.023				
2	GO:0008340	Determination of adult lifespan	0.023				
2	GO:0002360	T cell lineage commitment	0.0266				
2	GO:0009303	rRNA transcription	0.0266				
2	GO:0021854	Hypothalamus development	0.0266				
2	GO:2000178	Negative regulation of neural precursor cell proliferation	0.0266				
2	GO:0090201	Negative regulation of release of cytochrome c from mitochondria	0.0284				
2	GO:0007094	Mitotic spindle assembly checkpoint	0.0305				
2	GO:0009651	Response to salt stress	0.0305				
2	GO:0070266	Necroptotic process	0.0305				
2	GO:1901522	Positive regulation of transcription from RNA polymerase II promoter involved in cellular response to chemical stimulus	0.0305				
2	GO:0042104	Positive regulation of activated T cell proliferation	0.0322				
2	GO:0048643	Positive regulation of skeletal muscle tissue development	0.0342				
2	GO:2000679	Positive regulation of transcription regulatory region DNA binding	0.0387				
2	GO:0033137	Negative regulation of peptidyl-serine phosphorylation	0.0409				
2	GO:0033032	Regulation of myeloid cell apoptotic process	0.0457				
2	GO:0048873	Homeostasis of number of cells within a tissue	0.0457				
2	GO:0006356	Regulation of transcription by RNA polymerase I	0.0481				
2	GO:2001032	Regulation of double-strand break repair via nonhomologous end joining	0.0481				

Supplementary Table 9 – GO, KEGG pathways and Reactome pathways enrichment analysis for terms with FDR P value <0.05 related to the cluster derived from the targets of the upregulated miRNAs in PC-3's response to G-AgNPs

GO Biological Process				GO Cellular Component			
# genes	description	FDR value	term name	# genes	term name	description	FDR value
37	Regulation of programmed cell death	9.47E-16	GO:0043067	17	GO:1902494	Catalytic complex	0.0027
37	Regulation of cell differentiation	7.25E-14	GO:0045595	4	GO:0017053	Transcription repressor complex	0.0235
36	Response to endogenous stimulus	9.47E-16	GO:0009719	GO Molecular Function			
34	Regulation of phosphate metabolic process	2.92E-12	GO:0019220	# genes	term name	description	FDR value
34	Negative regulation of cellular metabolic process	2.22E-08	GO:0031324	27	GO:0140096	Catalytic activity, acting on a protein	1.51E-05
32	Establishment of localization	0.0093	GO:0051234	26	GO:0042802	Identical protein binding	1.05E-05
27	Regulation of cellular component movement	1.44E-12	GO:0051270	24	GO:0097367	Carbohydrate derivative binding	6.40E-04
27	Positive regulation of cell communication	1.55E-07	GO:0010647	21	GO:0019900	Kinase binding	6.79E-09
24	Regulation of cell cycle	1.13E-08	GO:0051726	18	GO:0044877	Protein-containing complex binding	2.60E-04
23	Regulation of growth	1.97E-12	GO:0040008	15	GO:0019904	Protein domain specific binding	4.45E-05
23	Positive regulation of cell population proliferation	3.47E-10	GO:0008284	14	GO:0016773	Phosphotransferase activity, alcohol group as acceptor	1.10E-04
23	Positive regulation of cellular component organization	3.93E-08	GO:0051130	9	GO:0140297	DNA-binding transcription factor binding	0.0015
23	Cellular response to stress	1.42E-05	GO:0033554	8	GO:0031625	Ubiquitin protein ligase binding	0.0022
23	Response to external stimulus	0.001	GO:0009605	6	GO:0042826	Histone deacetylase binding	9.00E-04
21	Tube development	3.92E-09	GO:0035295	5	GO:0001085	RNA polymerase II transcription factor binding	5.50E-04
21	Programmed cell death	1.09E-07	GO:0012501	5	GO:0019838	Growth factor binding	0.0149

21	Positive regulation of transcription by RNA polymerase II	1.54E-06	GO:0045944	4	GO:0008013	Beta-catenin binding	0.0234
20	Animal organ morphogenesis	1.53E-07	GO:0009887	3	GO:0048156	Tau protein binding	0.0437
19	Regulation of cellular response to stress	1.73E-08	GO:0080135	KEGG Pathways			
19	Cellular response to cytokine stimulus	1.51E-06	GO:0071345	# genes	term name	description	FDR value
18	Hematopoietic or lymphoid organ development	9.31E-09	GO:0048534	28	hsa05200	Pathways in cancer	3.29E-21
18	Central nervous system development	5.06E-06	GO:0007417	18	hsa04151	PI3K-Akt signaling pathway	5.52E-13
18	Neuron differentiation	7.60E-06	GO:0030182	15	hsa05206	MicroRNAs in cancer	6.48E-14
17	Response to peptide	1.89E-09	GO:1901652	14	hsa05205	Proteoglycans in cancer	6.40E-12
17	Response to growth factor	6.83E-09	GO:0070848	13	hsa05215	Prostate cancer	6.48E-14
17	Positive regulation of apoptotic process	1.35E-07	GO:0043065	13	hsa05202	Transcriptional misregulation in cancer	1.90E-11
17	Homeostatic process	0.0079	GO:0042592	12	hsa05203	Viral carcinogenesis	5.29E-10
16	Negative regulation of cell population proliferation	1.52E-06	GO:0008285	11	hsa05212	Pancreatic cancer	2.14E-12
16	Positive regulation of transport	4.50E-05	GO:0051050	11	hsa05218	Melanoma	2.14E-12
16	Movement of cell or subcellular component	0.0072	GO:0006928	11	hsa05160	Hepatitis C	1.59E-09
15	Regulation of apoptotic signaling pathway	1.81E-08	GO:2001233	10	hsa04510	Focal adhesion	1.48E-07
15	Regulation of MAPK cascade	1.34E-05	GO:0043408	9	hsa04068	FoxO signaling pathway	5.99E-08
15	Regulation of cell development	2.60E-04	GO:0060284	8	hsa04934	Cushing syndrome	2.97E-06
14	Response to oxygen levels	5.92E-08	GO:0070482	8	hsa05166	Human T-cell leukemia virus 1 infection	2.28E-05
14	Gland development	1.42E-07	GO:0048732	7	hsa04928	Parathyroid hormone synthesis, secretion and action	3.10E-06
14	Cellular response to organic cyclic compound	2.64E-06	GO:0071407	7	hsa04926	Relaxin signaling pathway	1.03E-05
14	Cellular response to hormone stimulus	5.06E-06	GO:0032870	7	hsa04390	Hippo signaling pathway	2.94E-05
14	Mitotic cell cycle	4.28E-05	GO:0000278	7	hsa04630	JAK-STAT signaling pathway	3.73E-05
14	Regulation of cell adhesion	5.47E-05	GO:0030155	7	hsa04024	cAMP signaling pathway	1.60E-04
14	Symbiotic process	3.70E-04	GO:0044403	6	hsa05217	Basal cell carcinoma	3.15E-06
14	Cell activation	0.0028	GO:0001775	6	hsa05230	Central carbon metabolism in cancer	5.10E-06
14	Proteolysis	0.0105	GO:0006508	6	hsa01521	EGFR tyrosine kinase inhibitor resistance	9.75E-06
13	Ossification	1.20E-08	GO:0001503	6	hsa04670	Leukocyte transendothelial migration	5.01E-05
13	Cellular response to abiotic stimulus	6.39E-08	GO:0071214	6	hsa04722	Neurotrophin signaling pathway	6.15E-05
13	Regulation of epithelial cell proliferation	1.50E-07	GO:0050678	6	hsa04140	Autophagy - animal	1.20E-04
13	Regulation of binding	3.92E-07	GO:0051098	6	hsa04530	Tight junction	2.80E-04
13	Reproductive structure development	1.35E-06	GO:0048608	6	hsa05014	Amyotrophic lateral sclerosis	0.0126
13	Cell population proliferation	6.81E-06	GO:0008283	5	hsa04137	Mitophagy - animal	5.54E-05
13	Negative regulation of phosphate metabolic process	2.49E-05	GO:0045936	5	hsa05142	Chagas disease	3.50E-04
13	Chordate embryonic development	8.93E-05	GO:0043009	5	hsa04071	Sphingolipid signaling pathway	6.90E-04
13	Epithelial cell differentiation	1.40E-04	GO:0030855	5	hsa04932	Non-alcoholic fatty liver disease	0.002
13	Membrane organization	6.60E-04	GO:0061024	5	hsa04360	Axon guidance	0.004
12	Positive regulation of protein serine/threonine kinase activity	1.52E-06	GO:0071902	4	hsa04340	Hedgehog signaling pathway	2.90E-04
12	Response to oxidative stress	4.98E-06	GO:0006979	4	hsa04917	Prolactin signaling pathway	0.0011
12	Negative regulation of intracellular signal transduction	5.46E-05	GO:1902532	4	hsa04912	GnRH signaling pathway	0.0026
12	Negative regulation of cellular component organization	9.90E-04	GO:0051129	4	hsa04350	TGF-beta signaling pathway	0.0028
12	Peptidyl-amino acid modification	0.0047	GO:0018193	4	hsa04668	TNF signaling pathway	0.0056
12	Multi-organism process	0.0179	GO:0051704	4	hsa04371	Apelin signaling pathway	0.0089
11	Regulation of epithelial cell migration	2.69E-07	GO:0010632	4	hsa05130	Pathogenic Escherichia coli infection	0.027
11	Regulation of leukocyte differentiation	1.98E-06	GO:1902105	3	hsa05231	Choline metabolism in cancer	0.027
11	Regulation of protein-containing complex assembly	1.10E-04	GO:0043254	Reactome Pathways			
11	Heart development	3.50E-04	GO:0007507	# genes	term name	description	FDR value
11	Sensory organ development	6.20E-04	GO:0007423	28	HSA-212436	Generic Transcription Pathway	3.63E-11
11	Protein modification by small protein conjugation or removal	0.0402	GO:0070647	22	HSA-168256	Immune System	5.30E-04
10	Regulation of cell cycle G1/S phase transition	3.19E-07	GO:1902806	15	HSA-5663202	Diseases of signal transduction by growth factor receptors and second messengers	4.54E-08
10	Response to mechanical stimulus	1.51E-06	GO:0009612	12	HSA-2262752	Cellular responses to stress	1.70E-04
10	Angiogenesis	3.69E-05	GO:0001525	10	HSA-8878166	Transcriptional regulation by RUNX2	4.70E-08

10	Regulation of developmental growth	5.67E-05	GO:0048638	10	HSA-8939211	ESR-mediated signaling	2.19E-06
10	Regulation of vasculature development	6.10E-05	GO:1901342	9	HSA-6785807	Interleukin-4 and Interleukin-13 signaling	3.23E-07
10	Regulation of autophagy	6.63E-05	GO:0010506	9	HSA-3700989	Transcriptional Regulation by TP53	0.001
10	Positive regulation of cell activation	7.57E-05	GO:0050867	7	HSA-157118	Signaling by NOTCH	0.0011
10	Regulation of Wnt signaling pathway	1.30E-04	GO:0030111	7	HSA-76002	Platelet activation, signaling and aggregation	0.0042
10	Blood circulation	2.00E-04	GO:0008015	7	HSA-5683057	MAPK family signaling cascades	0.0112
10	Cell part morphogenesis	0.0016	GO:0032990	6	HSA-8878159	Transcriptional regulation by RUNX3	2.10E-04
10	Response to inorganic substance	0.0019	GO:0010035	5	HSA-198323	AKT phosphorylates targets in the cytosol	2.65E-06
10	Regulation of system process	0.0037	GO:0044057	5	HSA-9614085	FOXO-mediated transcription	5.70E-04
10	Regulation of ion transport	0.0107	GO:0043269	4	HSA-8940973	RUNX2 regulates osteoblast differentiation	3.10E-04
9	Response to amino acid	1.53E-07	GO:0043200	4	HSA-8848021	Signaling by PTK6	0.0035
9	Regulation of reactive oxygen species metabolic process	6.04E-06	GO:2000377	4	HSA-70171	Glycolysis	0.0084
9	Skeletal system morphogenesis	3.63E-05	GO:0048705	4	HSA-3371556	Cellular response to heat stress	0.015
9	Negative regulation of growth	4.47E-05	GO:0045926	4	HSA-187037	Signaling by NTRK1 (TRKA)	0.0288
9	Positive regulation of growth	7.62E-05	GO:0045927	3	HSA-8934593	Regulation of RUNX1 Expression and Activity	0.0031
9	Aging	9.07E-05	GO:0007568	3	HSA-3232118	SUMOylation of transcription factors	0.0034
9	Negative regulation of cellular component movement	2.20E-04	GO:0051271	3	HSA-182971	EGFR downregulation	0.011
9	Response to wounding	0.0071	GO:0009611	3	HSA-8864260	Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors	0.0133
9	Cell morphogenesis involved in differentiation	0.0103	GO:0000904	3	HSA-76009	Platelet Aggregation (Plug Formation)	0.0166
9	Regulated exocytosis	0.0322	GO:0045055	3	HSA-9678108	SARS-CoV-1 Infection	0.0267
8	Regulation of smooth muscle cell proliferation	7.02E-06	GO:0048660	3	HSA-6796648	TP53 Regulates Transcription of DNA Repair Genes	0.0421
8	Regulation of generation of precursor metabolites and energy	2.12E-05	GO:0043467	2	HSA-416550	Sema4D mediated inhibition of cell attachment and migration	0.0176
8	Regulation of cysteine-type endopeptidase activity involved in apoptotic process	1.40E-04	GO:0043281	2	HSA-2465910	MASTL Facilitates Mitotic Progression	0.0203
8	Myeloid cell differentiation	1.60E-04	GO:0030099	2	HSA-8875555	MET activates RAP1 and RAC1	0.0267
8	Developmental growth	0.0046	GO:0048589	2	HSA-113501	Inhibition of replication initiation of damaged DNA by RB1/E2F1	0.0328
8	Positive regulation of cell projection organization	0.0047	GO:0031346				
8	Regulation of DNA-binding transcription factor activity	0.0089	GO:0051090				
8	Positive regulation of catabolic process	0.0124	GO:0009896				
7	Positive regulation of neuron apoptotic process	6.11E-07	GO:0043525				
7	Animal organ regeneration	2.57E-06	GO:0031100				
7	Regulation of neural precursor cell proliferation	6.58E-06	GO:2000177				
7	Regulation of DNA replication	2.50E-05	GO:0006275				
7	Regulation of fat cell differentiation	7.05E-05	GO:0045598				
7	Regulation of muscle cell differentiation	1.60E-04	GO:0051147				
7	Response to corticosteroid	2.20E-04	GO:0031960				
7	Cartilage development	2.80E-04	GO:0051216				
7	Regulation of cell division	6.00E-04	GO:0051302				
7	Gliogenesis	0.0013	GO:0042063				
7	Ras protein signal transduction	0.0014	GO:0007265				
7	Cell fate commitment	0.0021	GO:0045165				
7	Embryonic organ morphogenesis	0.0055	GO:0048562				
7	Epithelial tube morphogenesis	0.0061	GO:0060562				
7	Negative regulation of catabolic process	0.0071	GO:0009895				
7	Regulation of MAP kinase activity	0.0104	GO:0043405				
6	Response to amyloid-beta	5.30E-06	GO:1904645				
6	Cellular response to UV	7.10E-05	GO:0034644				
6	Regulation of fibroblast proliferation	7.10E-05	GO:0048145				
6	Tissue remodeling	1.40E-04	GO:0048771				
6	Regulation of protein localization to cell periphery	5.00E-04	GO:1904375				
6	G1/S transition of mitotic cell cycle	5.50E-04	GO:0000082				
6	Cellular response to transforming growth factor beta stimulus	0.0011	GO:0071560				

6	Regulation of epithelial cell differentiation	0.0012	GO:0030856				
6	Lung development	0.0021	GO:0030324				
6	Response to carbohydrate	0.0023	GO:0009743				
6	Epithelial cell development	0.0039	GO:0002064				
6	Positive regulation of supramolecular fiber organization	0.0041	GO:1902905				
6	Anatomical structure maturation	0.0043	GO:0071695				
6	Regulation of cell-substrate adhesion	0.0056	GO:0010810				
6	Regulation of response to DNA damage stimulus	0.0073	GO:2001020				
6	Cellular response to tumor necrosis factor	0.0105	GO:0071356				
6	Rhythmic process	0.0161	GO:0048511				
6	Positive regulation of cell-cell adhesion	0.0164	GO:0022409				
6	Striated muscle tissue development	0.0184	GO:0014706				
6	Regulation of ERK1 and ERK2 cascade	0.0217	GO:0070372				
5	Cellular response to epidermal growth factor stimulus	6.39E-05	GO:0071364				
5	Exocrine system development	8.27E-05	GO:0035272				
5	Regulation of release of cytochrome c from mitochondria	1.10E-04	GO:0090199				
5	Negative regulation of myeloid leukocyte differentiation	1.40E-04	GO:0002762				
5	Regulation of extrinsic apoptotic signaling pathway via death domain receptors	2.20E-04	GO:1902041				
5	Female gonad development	0.0012	GO:0008585				
5	Positive regulation of endothelial cell proliferation	0.0014	GO:0001938				
5	Regulation of nucleocytoplasmic transport	0.0031	GO:0046822				
5	Regulation of transforming growth factor beta receptor signaling pathway	0.0035	GO:0017015				
5	Regulation of stem cell differentiation	0.0035	GO:2000736				
5	Endocrine system development	0.0036	GO:0035270				
5	Negative regulation of epithelial cell proliferation	0.005	GO:0050680				
5	Interaction with host	0.0087	GO:0051701				
5	Gastrulation	0.0104	GO:0007369				
5	Regulation of mitotic nuclear division	0.0111	GO:0007088				
5	Regulation of microtubule cytoskeleton organization	0.0205	GO:0070507				
5	Viral life cycle	0.0242	GO:0019058				
5	Tissue homeostasis	0.0316	GO:0001894				
4	Histone H3 deacetylation	1.00E-04	GO:0070932				
4	Leukocyte apoptotic process	2.30E-04	GO:0071887				
4	Endochondral ossification	3.30E-04	GO:0001958				
4	Positive regulation of G1/S transition of mitotic cell cycle	6.30E-04	GO:1900087				
4	Neuron projection organization	8.20E-04	GO:0106027				
4	Negative regulation of Notch signaling pathway	0.0011	GO:0045746				
4	Regulation of pri-miRNA transcription by RNA polymerase II	0.002	GO:1902893				
4	Telencephalon cell migration	0.0026	GO:0022029				
4	Forebrain generation of neurons	0.0028	GO:0021872				
4	Positive regulation of DNA binding	0.003	GO:0043388				
4	Cellular response to drug	0.0032	GO:0035690				
4	Regulation of lipid kinase activity	0.0042	GO:0043550				
4	Regulation of sprouting angiogenesis	0.0055	GO:1903670				
4	Positive regulation of lipase activity	0.0064	GO:0060193				
4	Regulation of intracellular steroid hormone receptor signaling pathway	0.0066	GO:0033143				
4	Phosphatidylinositol-mediated signaling	0.0074	GO:0048015				
4	Canonical Wnt signaling pathway	0.0093	GO:0060070				
4	Positive regulation of protein binding	0.0096	GO:0032092				
4	Regulation of epidermal growth factor receptor signaling pathway	0.01	GO:0042058				
4	Midbrain development	0.0103	GO:0030901				

4	Response to cAMP	0.0143	GO:0051591				
4	Positive regulation of lymphocyte differentiation	0.016	GO:0045621				
4	Cerebellum development	0.0179	GO:0021549				
4	Response to alkaloid	0.0179	GO:0043279				
4	Limbic system development	0.0194	GO:0021761				
4	Spinal cord development	0.0199	GO:0021510				
4	Mesoderm development	0.0235	GO:0007498				
4	Odontogenesis	0.0253	GO:0042476				
4	Osteoblast differentiation	0.0296	GO:0001649				
4	Regulation of protein dephosphorylation	0.0347	GO:0035304				
4	Positive regulation of JNK cascade	0.0364	GO:0046330				
4	T cell differentiation	0.0401	GO:0030217				
4	Regulation of cold-induced thermogenesis	0.0418	GO:0120161				
4	Negative regulation of ion transport	0.0468	GO:0043271				
4	Eye morphogenesis	0.0477	GO:0048592				
3	Glial cell apoptotic process	1.40E-04	GO:0034349				
3	Hepatocyte apoptotic process	6.80E-04	GO:0097284				
3	Positive regulation of production of miRNAs involved in gene silencing by miRNA	6.80E-04	GO:1903800				
3	Negative regulation of vascular associated smooth muscle cell proliferation	0.0021	GO:1904706				
3	Establishment of apical/basal cell polarity	0.0023	GO:0035089				
3	Positive regulation of extracellular matrix organization	0.0029	GO:1903055				
3	Response to hyperoxia	0.0033	GO:0055093				
3	Tongue development	0.0036	GO:0043586				
3	Macrophage differentiation	0.0048	GO:0030225				
3	Negative regulation of ATP metabolic process	0.0052	GO:1903579				
3	Membrane depolarization during action potential	0.0067	GO:0086010				
3	Cell fate determination	0.0145	GO:0001709				
3	Positive regulation of epithelial to mesenchymal transition	0.0185	GO:0010718				
3	Negative regulation of I-kappaB kinase/NF-kappaB signaling	0.0185	GO:0043124				
3	Positive regulation of organ growth	0.0203	GO:0046622				
3	Negative regulation of muscle cell differentiation	0.025	GO:0051148				
3	Cellular response to glucocorticoid stimulus	0.0303	GO:0071385				
3	Positive regulation of macroautophagy	0.0396	GO:0016239				
3	Positive regulation of fat cell differentiation	0.042	GO:0045600				
3	Pancreas development	0.0476	GO:0031016				
3	Response to estrogen	0.0491	GO:0043627				
3	Regulation of protein acetylation	0.0491	GO:1901983				
2	Negative regulation of helicase activity	0.007	GO:0051097				
2	Regulation of phosphate transport	0.0131	GO:0010966				
2	Regulation of hydrogen peroxide-mediated programmed cell death	0.0154	GO:1901298				
2	Pyrimidine dimer repair	0.0179	GO:0006290				
2	Negative regulation of granulocyte differentiation	0.0179	GO:0030853				
2	Chondrocyte proliferation	0.0179	GO:0035988				
2	Response to muscle inactivity	0.0203	GO:0014870				
2	Regulation of peroxisome proliferator activated receptor signaling pathway	0.0283	GO:0035358				
2	White fat cell differentiation	0.0283	GO:0050872				
2	Golgi localization	0.0283	GO:0051645				
2	Myelin maintenance	0.0338	GO:0043217				
2	Positive regulation of vascular endothelial cell proliferation	0.0338	GO:1905564				
2	Positive regulation of oxidative stress-induced cell death	0.0368	GO:1903209				
2	Regulation of mitophagy	0.04	GO:1901524				

2	Negative regulation of cellular senescence	0.0467	GO:2000773				
2	Melanocyte differentiation	0.05	GO:0030318				

GENERAL DISCUSSION

Despite the investment in the oncology field, cancer is expected to become the major impediment to life expectancy raise during the present century, with the incidence doubling by 2070 and an estimated global economic cost of \$25.2 trillion in international dollars [1, 2]. Even though there have been major developments in the comprehension of cancer biology and in the development of new anticancer therapies, two major concerns can be identified: development of treatment resistance and the lack of biomarkers that can predict it, complicating personalized patients' follow-up approaches.

Indeed, the new generation of anticancer therapies, the targeted therapies, have brought a new way to think cancer treatment with the ability to direct the drug to the deregulated tumor pathway, to reduce side effects and to eliminate macroscopic tumors at primary and metastatic sites [3]. However, patients end up relapsing, due to treatment resistance acquisition. Nowadays, it has been proposed that cellular plasticity can contribute to this resistance, as cancer cells play "hide and seek" by constant phenotypic switching to evade the targeted therapy. Both a passive Darwinian selection and enrichment triggered by drug exposure, and an active adaptation to this stimulus (Lamarckian induction) have been proposed to explain cellular plasticity [4]. Inevitably, the resistance to targeted therapy will modulate tumor cells to a more aggressive phenotype, with multiple signaling pathways promoting proliferation and invasiveness and inhibiting apoptosis.

PCa follows this progression scheme, showing a different molecular profile in primary PCa and metastatic and hormone resistant PCa. In a therapy naïve setting, PCa cells show a major dependence on the androgens pathways to proliferate and evade apoptosis [5]. However, in metastatic PCa and CRPC, defects in DNA repair genes and alteration in PI3K/mTOR, MAPK and Wnt signaling pathways, which are associated with aggressiveness features are commonly reported [6]. Indeed, to CRPC patients, the available therapies are limited and show low impact on patients' OS [7]. Therefore, the question to which is the best way to surpass resistance remains: through the specific target of a resistance mechanism or through a multi-targeted approach.

The major challenge in a multi-targeted approach is the high toxicity observed. The introduction of nanomedicine in the biomedical field and drug design allows the surpass of that challenge with the ability to design drug carriers that specifically target the cancer cell and that encapsulate drugs that can multi-target different pathways [8]. AgNPs arise as a promising approach since their physical-chemical properties and the ability to chemistry functionalize their surface give them antitumor properties demonstrated in different tumor models [9].

The modulation of AgNPs uptake impacts their cytotoxic effect.

In this study, we synthesized AgNPs functionalized with glucose that showed a selective effect to the CRPC cell lines (PC-3 and Du-145 cells) and no effect to the hormone sensitive ones (LNCaP cells). We further proved that this selective effect was due to the uptake mechanism of AgNPs, since LNCaP cells do not express Cav1 nor Cav2, therefore not showing any caveolae-dependent endocytosis, which is their key entrance pathway. Moreover, we proved that functionalization of AgNPs with glucose enhanced the cytotoxic effect, mostly due to an increased uptake in consequence of metabolic specificities of advanced tumor cells.

Indeed, AgNPs can be directed to tumor cells through two step mechanisms. On one hand, the EPR effect leads to an increased uptake and retention of AgNPs by tumor tissues due to the irregular neovascularization and inefficient drainage of lymphatic system [10]. On the other hand, their physical characteristics, such as size and shape, and chemical characteristics, namely their surface functionalization, can impact the selective uptake of AgNPs through different endocytic mechanisms. The spherical shape and AgNPs small size in our study can explain the caveolae-dependent endocytosis as entry pathway and the glucose functionalization can increase this uptake [11, 12]. When modulating the caveolae-dependent endocytic pathway we were able to influence the cytotoxic capacity of AgNPs, increasing the cytotoxic effect in LNCaP cells by inducing caveolae-dependent endocytosis and inhibiting the cytotoxic effect in Du-145 and PC-3 by blocking this pathway. This further validated the impact of selective targeting in increasing the cytotoxic capacity of AgNPs.

Our results hint two main hypotheses. On one hand, the modulation of drug uptake can be one key strategy to achieve a selective effect, which goes in accordance with previous studies that reported that the entry pathway and intracellular location of NPs is determinant for induction of toxicity [13-15]. On the other hand, they highlight the importance of exploring the molecular profile of tumor cells' membrane and endocytic pathways, since they can help design a drug that selectively targets tumor cells. In PCa, caveolins expression is correlated with tumor progression and therapy resistance, therefore, targeting this endocytic pathway can help enhance the cytotoxic effect of the drug in the most advanced stages [16-18].

AgNPs are able to multi-target the major pathways deregulated in cancer

One of the goals of this project was to address the CRPC lack of therapies problem through G-AgNPs and fully comprehend their phenotypic and molecular mechanism.

Several different authors have shown the anti-tumor properties of different AgNPs against PCa. Most researchers focus exclusively in PC-3 cells, showing that differently

capped AgNPs, most of them synthesized through biological methods, have a cytotoxic effect against that cell line, through apoptosis induction with upregulation of apoptotic genes such as Bax, Caspases and p53 and downregulation of Bcl-2 [19-29]. Zhang and collaborators showed that silver nanoparticles (AgNPs) synthesized from leaf extract of *Salvia miltiorrhiza* showed cytotoxic effect against LNCaP cells through an increase of ROS and apoptosis [30]. Additionally, Reddy showed that AgNPs synthesized through *Perilla frutescens* Leaf Extract were cytotoxic to LNCaP cells, inducing cell shrinkage, membrane blebbing, chromatin condensation, nuclei fragmentation and apoptotic bodies formation [31]. Shing and collaborators, on the other hand synthesized AgNPs using *Carica papaya* Leaf Extract and significantly inhibited cell growth of Du-145 with an increase in ROS production and a cell cycle arrest at G2/M phase at 24 h, and a G1 and G2/M phase arrests at 48 h [32]. Both Gavamukulyaa and Kumari studies showed that AgNPs did not have a cytotoxic effect against normal prostate cell line (PNT1A) and human peripheral blood lymphocytes, respectively, while showing cytotoxic effect against PC-3 with increased ROS generation, mitochondrial depolarization, apoptosis induction and DNA fragmentation [33, 34]. El-Sheikh conducted an *in vivo* study with AgNPs loaded with cisplatin which showed an enhanced effect in PC-3 cells with a decreased nephrotoxic and hematotoxic effect in adult male rats [35]. However, to the best of our knowledge, no author had yet compared the effect of AgNPs in the three cell lines PC-3, Du-145 and LNCaP cells and fully explored their phenotypic and molecular effect.

Still, the mechanism of action of the AgNPs synthesized in our study follows the general mechanism described for AgNPs antitumor capacities [9]. After uptake through endocytosis, AgNPs are trapped into lysosomes where the “lysosome-enhanced Trojan horse effect” will induce their cytotoxicity [36]. The lysosome acidic environment and its enzymes will degrade the AgNPs capping and Ag^+ will induce an increase of ROS production that will damage different cellular organelles, such as mitochondria, which in fact exhibited membrane depolarization. Moreover, these alterations lead to a cell cycle blockage in the S-phase, which is responsible for checking DNA replication reliability and confirms there are no areas of unrepaired DNA damage [37]. Inevitably, there is a proliferation blockage and cell death induction through apoptosis. This mechanism hints a generalized cellular effect by AgNPs, where the generation of ROS and its spread through the cell, will damage all the organelles rather than selectively targeting a specific molecular pathway.

We further explored this hypothesis in our study through a wide-screening approach (799 miRNAs) and an *in silico* analysis that revealed which molecular pathways are deregulated in response to AgNPs and G-AgNPs. Firstly, the *in silico* analysis highlighted the same pathways in both AgNPs and G-AgNPs and in both cell lines, confirming that G-

AgNPs increased cytotoxicity is due to an increased uptake and not a different cytotoxic mechanism. This is in accordance with a previous study of Komuro and co-workers who demonstrated that GLUT-1 had an involvement in the internalization process of hydroxyapatite NPs modified with glucose by caveolae-mediated endocytosis [38]. The *in silico* analysis revealed the involvement of multiple pathways directly or indirectly related to the oxidative stress induced by an increase of ROS production, in the response of cells to AgNPs. Moreover, pathways such as AGE-RAGE, PI3K-Akt and mTOR, FOXO, JAK-STAT and HIF1 are reported to be disrupted in the most advanced forms of PCa [6].

Our study strengthens the hypothesis that a multi-targeted approach can be a possibility in the most advanced forms of cancer, where multiple pathways are deregulated and are used by the tumor as a way to raise resistance to multiple targeted therapies. By simultaneously targeting these pathways, we can reduce the possibility of cell survival and adaptation through therapy resistance. This approach has been widely discussed in the use of natural compounds, computational strategies for drug discoveries or through the combination of different drugs to achieve synergic effects [39-42]. One of the main concerns regarding multi-targeted therapy are the side effects. However, the selective targeting of tumor tissue and ability to direct the drug to the tumor cells, through a mechanism similar to what we showed in our study, can help circumvent the side effects while more effectively targeting the deregulated pathways of the tumor.

miRNAs can be a potential strategy of response biomarker profile

The establishment of a biomarker profile of therapy response in a pre-clinical stage of drug development can help reduce costs and increase the success rate of a future therapeutic approach [43]. In the present study, we propose the usage of miRNAs as potential biomarkers of response of AgNPs and G-AgNPs and performed a wide-screening of 799 miRNAs in PC-3 and Du-145 cells after NPs administration. ROS has been shown to interfere in miRNA biogenesis and function which gives strength to the hypothesis that a deregulation in miRNA's expression patterns can reflect the effect of AgNPs and G-AgNPs [44].

When comparing the deregulated miRNAs after AgNPs or G-AgNPs administration to PC-3 or Du-145 cells, there were 2 miRNAs downregulated and 92 miRNAs upregulated. This great overlap is consistent with the overlap of genes in the *in silico* analysis. From these 94 miRNAs, we selected the ones present in the top 10 deregulated miRNAs of each panel, because they show the higher differential expression. Therefore, we achieved a miRNA profile composed by 2 downregulated miRNAs (hsa-miR-1283 and hsa-let-7i-5p) and 16 upregulated miRNAs (hsa-miR-1-3p, hsa-miR-1200, hsa-miR-1228-3p, hsa-miR-

1272, hsa-miR-187-3p, hsa-miR-203a-5p, hsa-miR-494-5p, hsa-miR-495-5p, hsa-miR-517c-3p, hsa-miR-519a-3p, hsa-miR-518e-3p, hsa-miR548g-3p, hsa-miR-572, hsa-miR-590-3p, hsa-miR-637 and hsa-miR-648). Being systematically deregulated among cell lines and between AgNPs and G-AgNPs, we believe this miRNA profile will have a high accuracy in predicting response to this potential future therapeutic strategy. Among these miRNAs, most have been shown deregulated in therapy resistance [45-50]. Hsa-let-7i-5p has been associated with resistance to cisplatin chemoradiotherapy in larynx and hypopharynx cancer, while overexpression of hsa-miR-1-3p was able to restore the sensitivity of lung cancer cells to gefitinib [45, 46]. In gastric cancer, inhibition of hsa-miR-1200 and hsa-miR-637 enhanced cisplatin resistance and, in glioblastoma, inhibition of hsa-miR-519a-3p contributed to resistance to temozolomide [47, 48, 50]. However, exosomal delivery of hsa-miR-590-3p from cancer-associated fibroblasts increased radio-resistance in colorectal cancer [49]. Indeed, the expression of miRNAs and their oncogenic or tumor suppressor roles is known to be tumor specific [51]. Nevertheless, hsa-miR-1-3p, hsa-miR-1272, hsa-miR-187-3p and hsa-miR-590-3p, which are upregulated after NPs administration, have been reported to be tumor suppressor miRNAs in PCa, with their downregulation being associated with metastatic disease and poor prognosis [52-58]. Ultimately, the stability and easy quantification of miRNAs, aligned with the deregulation observed in the two CRPC cell lines after the AgNPs and G-AgNPs administration, the *in silico* analysis and the existing literature data, make the miRNA profile that we propose, promising biomarkers candidates to assess a future response to an AgNPs therapeutic approach.

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CONCLUDING REMARKS

The presents study represents a thorough *in vitro* analysis of G-AgNPs application in an advanced PCa model, from synthesis and functionalization of AgNPs to their uptake mechanisms, phenotypic and molecular mechanisms, and screening of potential biomarkers of response. Through this study, we propose that drug development should focus on a specific targeting of tumor cells allied to a multiple target of deregulated pathways in cancer, to achieve better therapeutic results and overcome resistance. We showed that the employment of G-AgNPs as a future therapeutic approach in CRPC is promising, since they can be selectively uptaken through caveolae-dependent endocytosis and this uptake can be enhanced through glucose functionalization. Moreover, they are able to target multiple pathways that are known to be deregulated in CRPC. However, in the future, *in vivo* studies are needed to confirm and validate these results in more advanced cancer models. Moreover, we showed that miRNAs can be potential biomarkers of response of PCa cells to this therapeutic approach and future 3D and *in vivo* studies should focus on narrowing the profile and proving its capability to predict response to G-AgNPs.