UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

The “ins and outs” of prostate metabolism towards carcinogenesis: the case of methoxychlor

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Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

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Covilhã, outubro de 2017

Dedicatória

Quero dedicar este trabalho, em primeiro lugar, aos meus pais porque sem eles nada disto seria possível, sem eles nada disto teria acontecido. Este projeto não é so fruto do meu trabalho e da minha dedicação, é fruto do vosso esforço e capacidade de sofrimento.

E por último, quero deixar uma dedicatória especial a duas pessoas que por forças maiores não poderam ver com os seus próprios olhos a minha ascenção nem o meu percurso ao longo destes últimos anos. Querido Tio, querido Avô, estiveram sempre no meu pensamento e foram fonte de parte da energia que dispendi para este trabalho. A vossa presença pode não ser física, mas a vossa alma e energia enche o meu coração cada vez que penso em vós. Dedico-vos este meu esforço durante este ano porque vocês realmente o merecem, estarão sempre comigo vá para onde for.

Agradecimentos

Queria começar por agradecer em primeiro lugar à minha orientadora Professora Doutora Sílvia Socorro por ter confiado em mim este projecto e pela oportunidade que me proporcionou em demonstrar o meu valor e o meu trabalho. Agradecer também por todo o tempo que dispensou em prol da minha orientação e por todos os conselhos e revisões que ajudaram a melhorar consideravelmente esta dissetação. Foi um prazer enorme trabalhar sob a sua orientação e sem dúvida que tomei a decisão certa em trabalhar consigo e com o grupo fantástico que conseguiu reunir ao longo destes anos.

Agradecer também à minha co-orientadora Doutora Cátia Vaz, por toda a paciência que demonstrou ter em aturar todas as minhas dúvidas e em me acompanhar mais de perto na maioria dos meus passos no laboratório. Obrigado pelos fins de semana e alguns feriados que te fiz perder, que tão prontamente dispensaste para me ajudar. Obrigado pelo apoio, pela motivação, por me encaminhares sempre na melhor direção, pelos conselhos e pela tua boa disposição e amizade.

Não esquecendo também os meus colegas de trabalho, começando pela Mariana Feijó sem dúvida que foste um pilar importante e que foi muito devido à tua motivação e à boa disposição que fui conseguindo prosseguir de cabeça erguida, obrigado por isso e pela amizade de longa data que construímos. Obrigado também pela tua ajuda em algumas situações no trabalho prático que foram bastante valiosas. Henrique Cardoso, foste uma das pessoas que me levou a integrar este grupo, a única que conhecia, ainda que pouco, à prior e a qual tenho que agradecer por me teres ajudado nessa decisão. Desde então a nossa amizade tem estado sempre em crescimento. Obrigado pela paciência, pelos conselhos, por me ajudares a espairecer quando mais precisava, pelo apoio incondicional, pelas palhaçadas e por me fazeres rir. Agradeço-te imenso pela tua ajuda crucial principalmente nesta recta final, que não foi nada curta. Quanto a ti Marília Figueira, obrigado pela atenção, pela tua enorme paciência, pelo apoio, pela ajuda e pela confiança, boa disposição e força que me transmites. Faltam-me palavras para descrecer o que fizeste por mim este tempo todo, nunca me deixaste desistir e quando tudo parecia estar perdido tu levantaste-me sempre a cabeça e orientaste-me para a solução. Obrigado pelas boleias que me deste e que me proporcionou estar mais tempo em casa na companhia da minha família e por todos os momentos contigo que me levavam a esquecer o trabalho. Agradecer também aos utilizadores do gabinete nº D+1 047 por ter permitido que este fosse a minha segunda casa na Covilhã e pelos bons momentos que lá passei, quando por vezes era necessária uma pausa no trabalho.

Resta-me também agradecer à Ana Silva, Margarida Grilo, Ana Oliveira, Sara Correira e Sara Gonçalves por todo o apoio e palavras de motivação ao longo deste ano.

Quero agradecer às pessoas mais importantes na minha vida, a minha família. Pai, Mãe, obrigado! Obrigado por tudo o que têm feito por mim, por tudo o que têm sacrificado por mim e eu sei que não foi pouco. Obrigado pelo ser humano que moldaram em mim, por todos os valores e educação que me transmitiram e pelo apoio incondicional a tempo inteiro. Tudo o que sou e tenho devo-o a vocês. Eu não me esqueço… e espero em breve compensar-vos por tudo. Mano, miúdo, obrigado por me aturares ao fim de semana quando eu já ia insuportável para casa por vezes. Obrigado pela preocupação que sempre tiveste, obrigado pelos momentos parvos que me fazem rir, obrigado pela companhia e por me ouvires quando por vezes preciso e me aturares quando mais ninguém tem capacidade para isso. Não há nada neste mundo que eu defenda com mais vigor do que a ti. Não quero passar sem agradecer também aos meus avós, à minha tia, ao meu padrinho e aos meus primos por toda a força, preocupação e carinho que me transmitiram para que conseguisse acabar com sucesso esta etapa.

Ao meu grupo de amigos de Belmonte, eles sabem quem são, quero também deixar aqui o meu agradecimento pelo apoio que foi imenso e pela teimosia em me fazerem lembrar que a vida não é só trabalho. A vossa força e motivação também pesou para a coragem necessária na escrita deste documento. Queria só destacar alguns nomes Carlos, Barroso, Débora e Guilherme.

Bruno, Gonçalo, Pimentel, Coelho e Sérgio só para vos recordar que os irmãos não precisam de partilhar do mesmo sangue. E mais não é preciso dizer… há coisas que não precisam de ser ditas. Há momentos que valem a pena e convosco já são muitos que temos para contar. Obrigado por me fazerem lembrar que a vida é feita de grandes e longas amizades e de apesar de a distância por vezes ser traiçoeira, que estas perduram fortes e sólidas. Sérgio, tenho de te dar uma frase de destaque não por teres sido o meu colega de casa fixo neste último ano, que nos levou até ao nosso limite, mas sim pelas sidras que te tirava do frigorífco, sem tu dares conta, para minha refastelação. Não posso passar sem mencionar nomes que ao longo destes anos mudaram a minha vida… Ana Laura, Catarina Chendo, Ângela, Jéssica, Midões, Pereirinha e tantos outros. Obrigado pela vossa amizade e pelo vosso apoio incondicional. As amizades da faculdade são para a vida e as vossas são exemplo disso.

Deixar também um obrigado ao João e Rita pela companhia e pelos bons momentos passados em casa.

E com receio de me estar a esquecer de alguém importante, resta-me desejar um grande e sincero obrigado a todos os que de certa forma contribuíram para que tudo isto fosse possível. OBRIGADO!

Okay I'm going to say something now,
This year was not the easiest, I get that
And most of the time it was tough and tiring
I might have been suffering, but I was in an excellent team
They are among the strongest men and women that I know
That is what it is to be one of us
To be an investigator
To be able to change the faith of the people
That means when some people give up from themselves
We work until we find a little hope to provide them
The job we've to take on is to make a difference

What some of the people of this group have done this year
Sacrificing their own time, their own work
For that of another
That is the true meaning of teamwork
If we quit because one day that goes wrong
We quit on all the people we can help in the future
Sometimes it's better to live with the failure for a little while
Please don't give up on them

Family, community, solidarity
Those are the things that sustain us through hard times
They are also the things that make our group strong

There's a family out there, and they're just like mine and they are counting on our work
Help them, it's what I signed up for, it's why I do this
"What am I doing?
What you were born to do"
I like these appearance
I like to be an investigator of this research group

Resumo

A capacidade de reprogramação metabólica apresentada pelas células tumorais tem emergido, nos últimos anos, como um dos "Hallmarks" do cancro. As alterações metabólicas típicas das células cancerígenas caracterizam-se essencialmente pela utilização da via glicolítica anaeróbia em detrimento da fosforilação oxidativa, o que culmina no aumento da produção de lactato, e na, consequente, acidificação do microambiente tumoral, o que favorece a progressão do cancro. Estudos anteriores do nosso grupo de investigação e outros demonstraram que as hormonas esteróides desempenham um papel relevante no estabelecimento das alterações metabólicas associadas ao desenvolvimento e progressão do cancro da próstata. Contudo, a panóplia completa dos potenciais (des)reguladores metabólicos relacionados com o cancro da próstata é ainda desconhecida. Os disruptores endócrinos são um grupo de compostos que interfere na síntese, secreção e metabolismo das hormonas naturais, os quais têm igualmente sido implicados na carcinogénese. O metoxicloro (MXC) é um pesticida organoclorado largamente disseminado no ambiente através do seu uso na atividade agrícola, tendo vários estudos demonstrado as suas propriedades estrogénicas. No entanto, os efeitos do MXC na indução de alterações metabólicas em células da próstata são totalmente desconhecidos. A presente dissertação tem como objetivo analisar o efeito do MXC na viabilidade celular, apoptose e metabolismo glicolítico de células da próstata humana, neoplásicas (LNCaP e PC3) e não neoplásicas (PNT1A). Com este propósito, as células LNCaP, PC3 e PNT1A foram mantidas em cultura na presença ou ausência de MXC (0, 0.1, 1, 10 and 100 μM) durante 48 e 72 horas. Os ensaios de (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) realizados para todas as condições experimentais demonstraram que o MXC diminui a viabilidade das células da próstata neoplásicas, assim como das não neoplásicas, e de uma forma dependente do tempo de exposição e da concentração. A concentração de 100 μM e o tempo de tratamento de 48 horas foram as condições selecionadas para avaliação dos efeitos do MXC na apoptose e no metabolismo glicolítico de todas as linhas celulares em estudo. A expressão proteica e a atividade de moduladores alvo destes processos foram analisadas por Western Blot e ensaios bioquímicos. Os resultados obtidos revelaram que o tratamento com MXC diminuiu a taxa de apoptose das células PNT1A, apesar da diminuição da proliferação celular observada. Curiosamente, nas células LNCaP e PC3 tratadas com MXC verificou-se o oposto, ou seja, o aumento da atividade da caspase-3, a proteína efetora da apoptose, bem como da expressão de reguladores apoptóticos.

No que diz respeito ao metabolismo, a medição dos níveis de glicose e de lactato por ensaios espectrofotométricos mostrou que o MXC estimulou o fluxo glicolítico quer nas linhas celulares de próstata neoplásicas quer nas não neoplásicas, como indicado pelos aumentos observados no consumo de glicose e na produção de lactato. Esta resposta metabólica foi

suportada pelo aumento da expressão dos transportadores de glicose e pela atividade de enzimas glicolíticas.

Os resultados obtidos demonstram que o MXC pode ter um papel no desenvolvimento e na progressão do cancro da próstata, suprimindo a apoptose nas células epiteliais de próstata não neoplásicas e estimulando a via glicolítica quer nas células não neoplásicas quer nas neoplásicas. Para além disso, estas evidências alertam para o impacto que o MXC também pode ter noutras doenças igualmente associadas a alterações na apoptose e metabolismo, como, por exemplo, a diabetes, a obesidade e a infertilidade.

Palavras-chave

Apoptose, cancro da próstata, LNCaP, metabolismo glicolítico, metoxicloro, PC3, PNT1A.

Resumo Alargado

A capacidade de reprogramação metabólica apresentada pelas células tumorais tem emergido, nos últimos anos, como um dos "Hallmarks" do cancro. Esta mudança característica das células neoplásicas visa assegurar as elevadas necessidades energéticas associadas ao aumento da sobrevivência e intensa proliferação celular, e confere uma vantagem adaptativa no contexto do ambiente tumoral. As alterações metabólicas típicas das células cancerígenas caracterizam-se essencialmente pela utilização da via glicolítica anaeróbia em detrimento da fosforilação oxidativa, ainda que em situações de níveis normais de oxigénio. O processo glicolítico depende da incorporação de glicose mediada pelos transportadores de glicose (GLUTs) localizados na membrana celular, e inicia-se através da ação da enzima hexocinase, a qual é responsável pela conversão da glicose em glicose-6-fosfato. Em seguida, decorre toda uma sequência de reações químicas que produzem como produto final o piruvato. Na glicólise anaeróbia, este piruvato é utilizado preferencialmente para formar lactato através da ação da enzima lactato desidrogenase (LDH), o que leva a um aumento da produção de lactato, e à consequente acidificação do microambiente tumoral, o que favorece a progressão do cancro e contribui para o escape do mesmo ao sistema imunitário. Existem várias vias de sinalização molecular envolvidas na reprogramação do metabolismo celular nas células tumorais, tais como, a via da fosfoinositídeo 3-cinase (PI3K), a via do fator indutor de hipóxia 1 (HIF1), a via da proteína cinase ativada pela AMP (AMPK). A própria proteína supressora tumoral p53 também tem um papel importante como reguladora metabólica. Estudos anteriores do nosso grupo de investigação e outros demonstraram que, para além das vias de sinalização referidas acima, as hormonas esteróides, nomeadamente os androgénios, também desempenham um papel relevante no estabelecimento das alterações metabólicas associadas ao desenvolvimento e progressão do cancro da próstata. Os androgénios estimularam o consumo de glicose e a produção de lactato pelas células de cancro da próstata, efeitos que foram mais pronunciados nos modelos celulares que mimetizam estados mais avançados da doença. Contudo, a panóplia completa dos potenciais (des)reguladores metabólicos relacionados com o cancro da próstata é ainda desconhecida. Os disruptores endócrinos são um grupo de compostos que interfere na síntese, secreção e metabolismo das hormonas naturais, que são responsáveis pela manutenção da homeostase, reprodução e desenvolvimento, e têm igualmente sido implicados na carcinogénese. Estes químicos podem estar presentes no ar que respiramos, na água que bebemos e no solo onde cultivamos os alimentos que consumimos, o que faz com que a exposição aos seus efeitos seja uma constante no nosso quotidiano. Os efeitos adversos para a saúde humana intimamente associados a estes compostos são descritos no contexto de doenças como a diabetes, a obesidade, a infertilidade e, também o cancro. O metoxicloro (MXC) é um pesticida organoclorado, sintetizado pela primeira vez em 1893, e largamente disseminado no ambiente através do seu uso na atividade agrícola, principalmente contra pestes tais como

moscas, mosquitos, baratas e vários artrópodes. Apesar de vários estudos terem demonstrado as suas propriedades estrogénicas, os efeitos do MXC na indução de alterações metabólicas em células da próstata são totalmente desconhecidos. A presente dissertação tem como objetivo analisar o efeito do MXC na viabilidade celular, apoptose e metabolismo glicolítico de células da próstata humana, neoplásicas (LNCaP e PC3) e não neoplásicas (PNT1A), visando identificar uma possível relação entre o MXC e o desenvolvimento e progressão do cancro da próstata. Com este propósito, as células LNCaP, PC3 e PNT1A foram mantidas em cultura na presença ou ausência de MXC (0, 0.1, 1, 10 and 100 μM) durante 48 e 72 horas. Os ensaios de MTT realizados para todas as condições experimentais demonstraram que o MXC diminui a viabilidade das células da próstata neoplásicas, assim como das não neoplásicas, e de uma forma dependente do tempo de exposição e da concentração. A concentração de 100 μM e o tempo de tratamento de 48 horas foram as condições selecionadas para avaliação dos efeitos do MXC na apoptose e no metabolismo glicolítico de todas as linhas celulares em estudo. A expressão proteica e a atividade de moduladores alvo destes processos foram analisadas por Western Blot e ensaios bioquímicos. Os resultados obtidos revelaram que o tratamento com MXC diminuiu a taxa de apoptose das células PNT1A através da supressão da via extrínseca, o que foi suportado pela diminuição do receptor Fas (FasR), assim como do seu ligando (FasL), apesar da diminuição da proliferação celular observada. Curiosamente, nas células LNCaP e PC3 tratadas com MXC verificou-se o oposto, ou seja, o aumento da atividade da caspase-3, a proteína efetora da apoptose, bem como da expressão de reguladores apoptóticos, tais como p53, Bax, bcl-2 e caspase-9. No caso das LNCaP, a alteração mais notória observada foi na proteína supressora tumoral p53, cuja expressão aumentou enormemente após o tratamento com MXC. Por fim, no que diz respeito às células PC3, o aumento da taxa apoptótica ocorreu devido à estimulação da via intrínseca, o que foi corroborado pelo aumento do rácio Bax (proapoptotica)/Bcl-2 (antiapoptótica) e da expressão da caspase-9.

No que diz respeito ao metabolismo, a medição dos níveis de glicose e de lactato por ensaios espectrofotométricos mostrou que o MXC estimulou o fluxo glicolítico quer nas linhas celulares de próstata neoplásicas quer nas não neoplásicas, como indicado pelos aumentos observados no consumo de glicose e na produção de lactato. Esta resposta metabólica foi suportada pelo aumento da expressão dos GLUTs e pela atividade de enzimas glicolíticas, no entanto com algumas variações nas diferentes linhas celulares. Nas células PNT1A, a expressão do GLUT1, da fosfofrutocinase-1 (PFK1) e a atividade da LDH aumentaram após o estímulo com MXC. Quanto às linhas celulares neoplásicas, verificou-se um aumento nos níveis de GLUT3 e na atividade da LDH nas células LNCaP, enquanto que nas PC3 foi observado um aumento na expressão dos GLUT1 e GLUT3, e na atividade da LDH.

Os resultados obtidos demonstram que o MXC pode ter um papel no desenvolvimento e na progressão do cancro da próstata, suprimindo a apoptose nas células epiteliais de próstata não neoplásicas e estimulando a via glicolítica quer nas células não neoplásicas quer nas neoplásicas. Para além disso, estas evidências alertam para o impacto que o MXC também

pode ter noutras doenças igualmente associadas a alterações no metabolismo e apoptose, como, por exemplo, a diabetes, a obesidade e a infertilidade.

Abstract

The last years have witnessed the emergence of metabolic reprogramming as a hallmark of cancer. The changes in cancer cell metabolism include, among others, a shift in glucose metabolism from oxidative phosphorylation to aerobic glycolysis, which culminates in an increased lactate production and acidification of microenvironment favoring tumor progression. We and others previous work have shown that steroid hormones play a relevant role driven the metabolic changes associated with development and progression of prostate cancer. Nevertheless, the panoply of metabolic (de)regulators linked with prostate cancer remains poorly known. Endocrine-disrupting chemicals are a group of compounds that interfere with the synthesis, secretion, and metabolism of natural hormones, which have also been implicated in carcinogenesis. Methoxychlor (MXC) is a chlorinated pesticide widely dispersed in the environment by its use in agricultural activities, and several reports have demonstrated its estrogenic properties. However, the MXC effects inducing metabolic alterations in prostate cells are largely unknown. This study aimed to analyze the effect of MXC on cell viability, apoptosis and glycolytic metabolism of neoplastic (LNCaP and PC3) and non-neoplastic (PNT1A) human prostate cells. For this purpose, LNCaP, PC3, and PNT1A cells were cultured in the presence or absence of a range of MXC concentrations (0, 0.1, 1, 10 and 100 μM) for 48 and 72 hours. MTT assays performed for all experimental conditions demonstrated that MXC diminished the viability of both neoplastic and non-neoplastic prostate cells in a time- and concentration-dependent manner. The 100 μM concentration and a treatment period of 48 hours were the conditions selected for evaluation of the effect of MXC on apoptosis and glycolytic metabolism of all cell lines under study. Protein expression and activity of target modulators of these biological processes were assessed by means of Western blot analysis and biochemical assays. The obtained results showed that MXC-treatment decreased the apoptotic rate of PNT1A cells, despite the observed decrease in cell proliferation. Curiously, in LNCaP- and PC3-treated cells MXC had an opposite effect increasing caspase-3 activity, the effector protein of apoptosis, and up-regulating the expression of apoptotic regulators.

Regarding metabolism, measurement of glucose and lactate levels by spectrophotometric assays showed that MXC stimulated the glycolytic flux in both non-neoplastic and neoplastic human prostate cell lines, as indicated by the enhanced glucose consumption and lactate production. This metabolic response was underpinned by the increased expression of glucose transporters and activity of glycolytic enzymes.

The present findings demonstrated that MXC may have a role in the development and progression of prostate cancer by suppressing apoptosis in non-neoplastic prostate epithelial cells and stimulating the glycolytic pathway in both non-neoplastic and neoplastic cells. Moreover, the evidence gathered herein highlights for the impact of MXC in other human

diseases, such as diabetes, obesity, and infertility, since they are all associated with alterations in apoptosis and metabolism also.

Keywords

Apoptosis, glycolytic metabolism, LNCaP, methoxychlor, PC3, PNT1A, prostate cancer.

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List of Abbreviations

AFS	Anterior fibromuscular stroma
AhR	Aryl hydrocarbon receptor
ALT	Alanine transaminase
AMPK	AMP-activated protein kinase
AR	Androgen receptor
AREs	Androgen-response elements
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BPA	Bisphenol-A
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAR	Constitutive androstane receptor
CS-FBS	Charcoal-stripped FBS
CYP	Cytochromes P450
CZ	Central zone
DDT	4,4'-(2,2,2-trichloroethane-1,1-diyl)bis(chlorobenzene)
DES	Diethylstilbestrol
DHT	5-α-Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DR	Death receptor
EDCs	Endocrine-disrupting chemicals
EGF	Epidermal growth factor
ERRs	Estrogen-related receptors
ERs	Estrogen receptors
F-1,6-P	Fructose 1,6-biphosphate

F-2,6-P	Fructose-2,6-biphosphate
F-6-P	Fructose-6-phosphate
FBS	Fetal bovine serum
G-6-P	Glucose-6-phosphate
GLUTs	Glucose transporters
GPER	G protein-coupled estrogen receptor
HIF	Hypoxia-inducible factor
HK2	Hexokinase 2
IGF-1	Insulin-like growth factor 1
KGF	Keratinocyte growth factor
LDH	lactate dehydrogenase
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
MCTs	Monocarboxylate transporters
mTOR	mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MXC	Metoxychlor
NRs	Nuclear receptors
PAP	Prostatic acid phospathase
PBS	Phosphate buffer saline
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PFK1	Phosphofructokinase-1
PI3K	Phosphoinositide-3-kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PKM2	Pyruvate kinase M2
PMSF	Phenylmethylsulfonyl fluoride
pNA	p-Nitro-Aniline

PPAR	Peroxisome-proliferator activated receptor
PSA	Prostate-specific antigen
PTEN	Tumor-suppressor phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
PXR	Pregnane X receptor (PXR)
PZ	Peripheral zone
RAR	Retinoid acid receptor
RIPA	Radioimmunoprecipitation assay buffer
SCO2	Synthesis of cytochrome c oxidase subunit 2
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
SHBG	Sex-hormone-binding globulin
TIGAR	Tp53-induced glycolysis and apoptosis regulator
TR	Thyroid receptor
TRAIL	TNF-related apoptosis-inducing ligand
TZ	Transition zone
VEGF	Vascular endothelial growth factor

I. INTRODUCTION

1. General description of prostate anatomy and physiology

The prostate gland is the largest accessory gland of the male reproductive system. It is located anterior to the rectum, posterior to the lower portion of the symphysis pubis and inferior to the urinary bladder in the subperitoneal compartment between the pelvic diaphragm and the peritoneal cavity (1, 2). The anatomic description of prostate includes a base, an apex, and the anterior, posterior, and inferior-lateral surfaces. The nerve supply to the gland arises from the prostatic plexus and the arterial supply is given by branches of the internal iliac artery (2).

Regarding prostate embryology, during the third month of gestation, the prostate gland develops from epithelial invaginations of the posterior urogenital sinus. Androgenic stimulation is absolutely required for prostatic development, namely by the presence of 5α-dihydrotestosterone (DHT), which is synthesized from fetal testosterone by the activity of 5α-reductase (3). At birth, prostate only weights a few grams and enlarges to reach the average adult weight of around 20 g by 25-30 years of age (1). At puberty, the prostate undergoes a phase of exponential growth, which corresponds to the rise in serum testosterone to adult levels. Mean prostatic weight then stabilizes and remains almost constant until the end of the third decade of life when mean prostatic weight begins to rise slowly (4). This effect reflects the onset of benign prostatic hyperplasia (BPH), a condition that affects approximately one quarter of men aged around 50, one third of men aged around 60, and approximately fifty percent of all men aged equally or above 80 (5).

Human prostate is composed of glandular and stromal elements, which are tightly fused within a pseudocapsule. The inner layer of the prostate capsule is composed of smooth muscle with an out layer covering of collagen. The glandular tissue presents a differential distribution in the gland. The prostate is divided into four major regions (Fig. 1), the peripheral zone (PZ), which is the larger one comprising around 70% of the glandular tissue and the most susceptible to carcinoma, chronic prostatitis and postinflammatory atrophy; the transition zone (TZ), which includes only 5% of the glandular tissue and consists of two small lobules of glandular tissue and represents the portion of the gland tissue that enlarges due to BPH; the central zone (CZ), which is located at the base of the prostate between the PZ and TZ and includes approximately 25% of the glandular tissue and, lastly, the anterior fibromuscular stroma (AFS) that forms the convexity of the anterior external surface and is devoid of glandular tissue and is composed of fibrous and smooth muscular elements (1, 2, 6, 7).

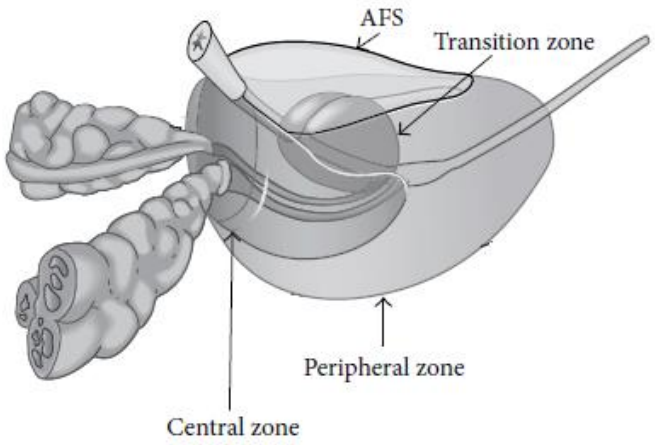

Figure 1. Zonal anatomy of the human prostate. The prostate is divided in four zones, peripheral zone that consists in about 70% of prostate, transition zone that surrounds proximal prostatic urethra, central zone that surrounds the ejaculatory duct and the anterior fibromuscular stroma (AFS), which allows the connection between anterior and apical surfaces (adapted from (1)).

These zones have different embryologic origins and can be distinguished by their anatomic landmarks, appearance, biological functions and susceptibility to pathology (Table 1) (6, 7). Around 70% of all prostate cancers are derived from the PZ, which primarily arises from the urogenital sinus. On the other hand, a very low incidence of prostate cancer is found in the CZ, which is derived from the Wolffian duct. Despite TZ shares a similar embryologic origin with the PZ, the percentage of prostate cancer arising from this zone is lower, approximately 25%. This disparity may be explained by the differences in the stromal component of these two regions. The stroma of the TZ is more fibromuscular, and it has been postulated that BPH, which mainly arises in TZ, is a pathology of the fibromuscular stroma (1, 2).

Table 1. Histological composition and embryologic origins of the several zones of the prostate gland (adapted from (1)).

	Central zone (CZ)	**Transistion zone (TZ)**	**Peripheral zone (PZ)**
Volume of normal prostate (%)	25	5	70
Embryologic origin	Wolffian duct	Urogenital sinus	Urogenital sinus
Stroma	Compact	Compact	Loose
Origin of prostatic adenocarcinoma (%)	5	25	70
Benign prostatic hyperplasia (%)	-	100	-

The glandular prostatic epithelium is constituted by the secretory epithelial cells, basal cells, stem cells and neuroendocrine cells (8). The majority of epithelial cells are columnar luminal cells and are responsible for production of prostatic secretions (9). The most well-known human prostatic secretory protein is the prostate-specific antigen (PSA), used as an indicator of prostate cancer. It is normally secreted apically into ductal lumina and is removed by ejaculation (10). Normally, this protein does not cross the epithelial basement membrane and is not found in the bloodstream of adult males. However, under conditions where the prostatic architecture is disrupted, such as chronic inflammation and cancer, PSA can leak back into the prostatic stroma and can be detected in the bloodstream (10, 11). Basal cells are present on the base of prostate gland in contact with the basement membrane (9). Prostate stem cells are confined to the basal compartment and represents a quiescent reserve that can divide originating basal or luminal epithelial-like stem cells (12). The last cell type in the prostatic epithelium are the neuroendocrine cells, which can secret neurosecretory products that promote prostate growth (13, 14).

The prostate contributes to about 30% of the volume of ejaculate (8, 10). In general, the major functions of the prostatic secretions relate to semen gelation, coagulation and liquefaction (15). The normal action of prostate gland is crucial to ensure sperm motility because this gland produces secretory proteins that are involved in the coating and uncoating of spermatozoa (15). The milky secretions of the prostate have a basic pH, which contributes to neutralize the acidic environment of duct deferent and female vagina. There are other factors produced by prostate epithelial cells for proper sperm function such as citric acid and proteolytic enzymes (16).

2. Prostate cancer: brief overview of aetiology and progression mechanisms

2.1 Epidemiological notes on prostate cancer

Prostate cancer is the second commonest diagnosed malignancy and the fifth leading cause of cancer mortality in men, representing a substantial public health burden (17). The incidence and prevalence of prostate cancer vary in different parts of the world, and is significantly affected by implementation of health care resources and epidemiologic resources (18-20).It is estimated that 1.1 million men were diagnosed with prostate cancer worldwide in 2012, 70% of them (795 000 cases) in developed countries and with 307 000 estimated deaths (17). The highest incidence rates of prostate cancer are found in the developed regions including Australia/New Zeeland, Western and Northern Europe and Northern America whereas Asian and African countries have lower rates of incidence (21, 22). This discrepancy in incidence rates may be partially explained by the practice of PSA testing,

which detects even the asymptomatic tumours, and subsequent biopsy has become available for prostate cancer screening in developing countries and those regions have experienced an increase in prostate cancer incidence. In Portugal, according to the most recent published data, prostate cancer was the most frequent cancer among men with 5433 new cases each year, representing 22.4% of the overall cancers cases. The estimates indicate that by 2020 there will be an expected occurrence of 8600 new cases and 1700 deaths due to prostate cancer (23). Although the incidence in Portugal is increasing, the mortality associated with prostate cancer seems to be steadily decreasing over the time (Fig. 2) (24).

Figure 2. Incidence and mortality associated with prostate cancer in Portugal. Data are expressed as the age-standardized rate per 100 000 habitants. Blue line refers to incidence and red line mortality (24).

2.2 Risk factors for prostate cancer

Several research efforts have given insight into the causes and risk factors for prostate cancer. Although the specific causes remain unknown, several risk factors have been identified, namely, genetic and environmental, which may contribute to the initiation and progression of pathology. The major risk factor for prostate cancer is age. Prostate cancer has a low overall incidence in men younger than 50 years old, representing only less than 0.1 % of all affected patients, and about 85% of cases of prostate cancer are diagnosed after the age of 65 (25). However, the emergence of PSA screening caused an age deviation, whereby the incidence of prostate cancer in men aged 50-59 years has increased (26). In addition to age, ethnicity is referred as a prostate cancer risk factor, since incidence of disease varies between different ethnic groups (Fig. 3). These differences in prostate cancer risk may be a reflection of some factors, such as differences in environmental exposure including dietary habits, variations in detection and genetic background (27).

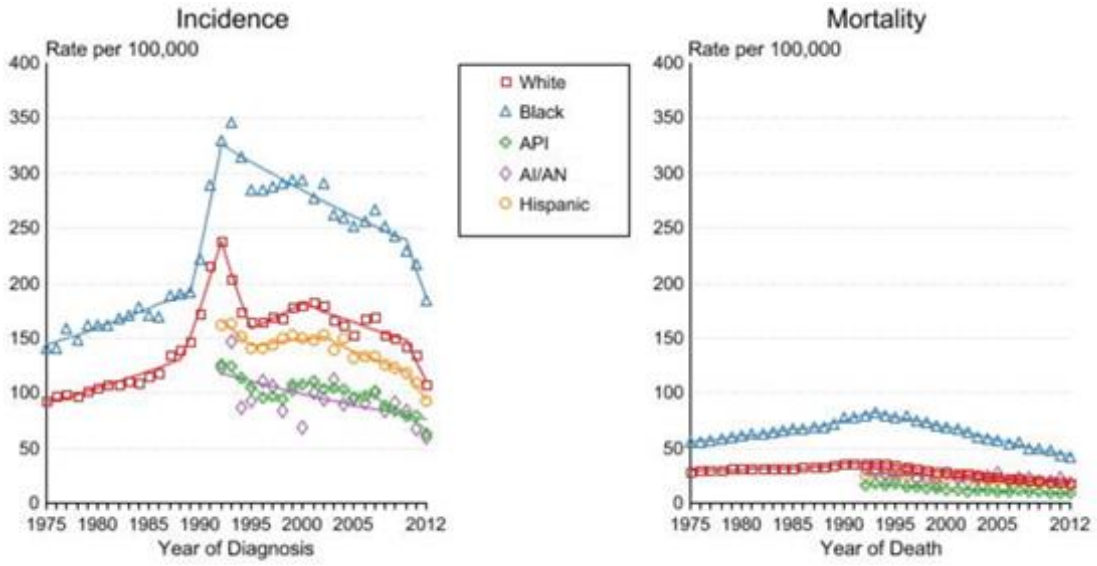

Figure 3. Differences in incidence and mortality of prostate cancer among ethnic groups. Rates of incidence and mortality of prostate cancer in USA from 1975 to 2012. The raise in number of diagnosis per year verified between 1986 and 1990 (approximately) coincide with emergence of PSA testing. API – Asian/Pacific Islander; AI/NA- American Indian/ Alaska Native (adapted from (27)).

Other risk factor lies in familial and genetic history, with several reports suggesting that risk of prostate cancer is increased in men with an affected first-degree relative (28, 29). The risk of an individual to suffer from prostate cancer increases with the number of affected family members and the degree of relatedness, and is inversely related to the age at which the family members were affected (30). Several prostate cancer susceptibility genes have been identified, such as RNASEL, ELAC2, MSR1, OGG1, CHEK2, BRCA2, PON1 and GDF15 (31). Many of these genes encode proteins with roles in cellular defence against inflammation and oxidative stress, which can lead to increase in susceptibility to tumour formation if these proteins had functional defects (32). The deletion of tumour suppressor genes (tumor-supressor phosphatase and tensin homolog (PTEN) and TP53) and the activation of oncogenes (MYC) are also linked with prostate carcinogenesis (33). The combination of increased proliferation, disrupted apoptosis and altered metabolic profile contribute to uncontrolled cancer (34, 35).

Inflammation and infection are other features that may contribute to malignant transformation in the prostate, as the prostate is exposed to infectious agents via the urine and sexual activity and the hypothesis that infectious agents might initiate inflammatory processes and lead to cancer is reasonable (36, 37). Epidemiologic studies suggest that infection-associated inflammation and hyperproliferation can lead to the development of prostate cancer. Findings that are corroborated by laboratorial studies. The hyperproliferative state in proliferative inflammatory atrophy can result in mutations in rapidly dividing cells, which may lead to cancer (38). Normally, adjacent to high-grade prostate intraepithelial neoplasia (PIN) is identified the proliferative inflammatory atrophy state (39). Concerning molecular pathways, PIN and prostate cancer tissue all have low levels

of cytoplasmic protein p27, which is an inhibitor of cell-cycle progression (40-42). Superoxide, hydrogen peroxide and nitric oxide are examples of highly reactive molecular species released from inflammatory cells and can damage the DNA of epithelial cells, which results in cell death (43, 44). Progenitor cells then differentiate to replace the lost epithelial cells, however, epithelial cell DNA synthesis in the setting of oxidative or nitrative stress increases the risk of mutation (39).

Lastly, diet and life style are two aspects that might contribute to prostate cancer risk (45). Prostate cancer incidence and mortality worldwide both correlate with the average intake of fat (46, 47). Obesity has shown to have a relationship with the progression and aggressiveness of prostate cancer (48). Also, the intake of red meat might be associated with increased risk of prostate cancer (49). On the other hand, vitamin E, selenium and lycopene can be beneficial in order to reduce the risk of prostate cancer (50, 51). The alcohol consumption might have a dose-dependent relationship with prostate cancer risk (52). Sexual activity might expose the prostate to infectious agents, which in turn can lead to malignant transformation (53, 54).

2.3 Diagnosis options for prostate cancer

Prostate cancer has an asymptomatic nature that only manifest in latent stages, thus an early detection of this disease is essential. The first serum biomarker used for prostate cancer screening was the human prostatic acid phosphatase (PAP), however it showed insufficient sensitivity (55). Later on, PSA was considered the optimal biomarker for screening of prostate cancer and it is still used as a diagnosis biomarker (56). Nowadays, there are two principal methods of diagnosis, digital rectal examination, which is a physical exam, and a biochemical examination of the serum content of PSA (57, 58). Concerning PSA, it is also known that 15% of men with normal or low levels of PSA had prostate cancer and high levels of PSA are not necessarily associated with prostate cancer (59, 60). Thus, a biopsy is crucial for diagnosis, allowing the elimination of false positives and false negatives of the PSA test (61). New biochemical approaches are being tested, such as the 4kscore blood examination that tests four biomarkers instead of just one, however all these techniques are new and producing only preliminary results (62).

2.4 Development process of prostate cancer

As above mentioned, the epithelium of prostate can be damaged by inflammation, infection and/or carcinogens, which can evolve to a state called proliferative inflammatory atrophy (PIA) (63). Alterations at this point can lead to the formation of histological lesions also known as PIN (64). PIN is characterized by the appearance of dysplasia of prostate luminal epithelial cells and an initiation of loss of distinct basal and secretory layers (65, 66). Finally, high-grade PIN can be considered as the precursor of prostate cancer and its invasive state (Fig. 4) (65).

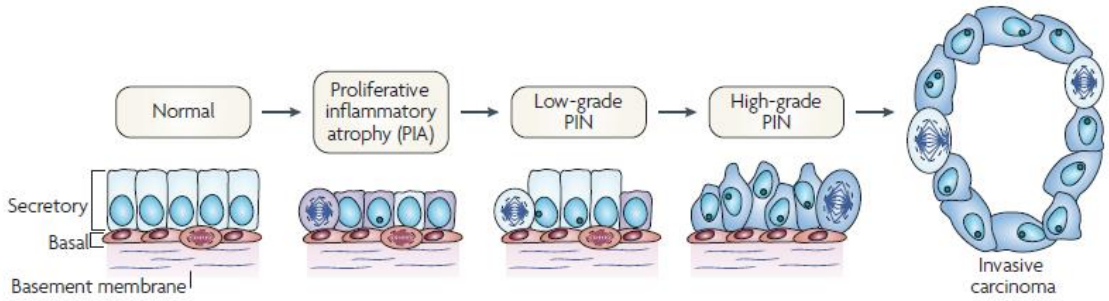

Figure 4. Cellular model of early prostate neoplasia progression. This process is characterized by the infiltration of lymphocytes, macrophages and neutrophils. Phagocytes release reactive oxygen species causing DNA damage, cell injury and cell death, which trigger the epithelial regeneration. The downregulation of some genes such as p27 and PTEN in some luminal cells stimulates cell-cycle progression. This alteration lead to genetic instability and the continued proliferation of genetically unstable luminal cells and the further accumulation of genomic changes lead to progression towards invasive carcinomas. PIN – prostatic intraepithelial neoplasia (Adapted from (39)).

Initially, prostate cancer growth depends on the androgens action, which can regulate the ratio of cells proliferating to those dying by both stimulating proliferation and inhibiting apoptosis (67). Testosterone, the principal circulating androgen, is mainly secreted by the testes and circulates in the blood bound to albumin and sex-hormone-binding globulin (SHBG) (68). When free testosterone enters prostate cells, approximately 90% is converted to DHT by the enzyme 5α-reductase. This androgen is the more active hormone, having a higher affinity (5 fold) for the androgen receptor (AR) than does testosterone. AR is a member of the nuclear receptor superfamily that, like other nuclear receptors, in the basal state is bound to heat-shock proteins in a conformation that prevents DNA binding. A conformational change in the AR is induced by binding of androgens and leads to dissociation from the heat-shock proteins and receptor phosphorylation (69). The ligand-induced conformational change facilitates the formation of homodimer complexes that bind androgen-response elements (AREs) in the promoter regions of target genes, which are responsible for growth and survival of prostate cells (69). For this reason, androgen ablation is the principal therapy for progressive prostate cancer, leading regression of androgen-dependent tumours (70, 71). However, more advanced cases of prostate cancer acquire androgen-independent growth progressing independently of the androgen suppression (Fig. 5). This causes the failure of androgen ablation therapies and represents a usually lethal form of prostate cancer that progresses and metastasizes, and for which at present there is no effective therapy (72, 73).

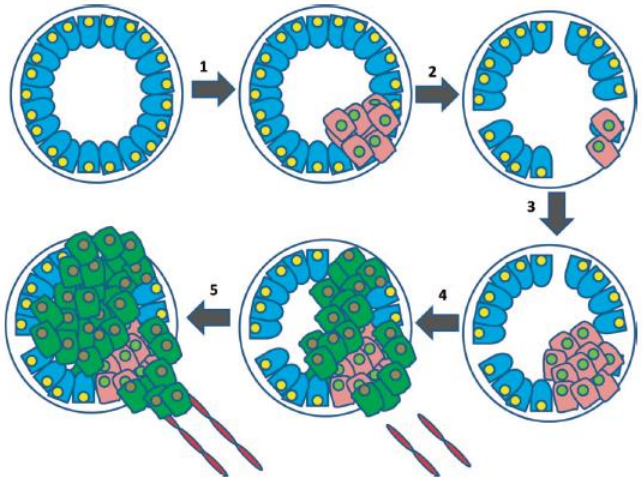

Figure 5. Malignant transformation of prostate and progression from androgen-dependent to androgen-independent prostate cancers. 1. Several carcinogenic alterations occur and some prostate cells proliferate out of control. 2. Prostate cancer cells are initially androgen-dependent, thus the androgen-deprivation therapy is successful in destroying cancer cells. 3. However, some cells can survive to this treatment and continue proliferating. 4. These cells became androgen-independent and acquire subsequent changes resulting in increased angiogenesis. 5. At this stage, prostate cancer starts to metastasize to distant sites through blood circulation *(74)*.

2.5 Mechanisms underlying the acquisition of independence from androgens

There are several mechanisms underlying the development of androgen-independent prostate cancer, which are summarized in Table 2.

Table 2. The different pathways involved in the development of androgen-resistant prostate cancer (Adapted from (75)).

Number	Pathway	Ligand Dependence	AR Dependence	Mechanism
1	Hypersensitive AR	Androgen dependent	AR dependent	Amplified AR; Sensitive AR; Increased DHT;
2	Promiscuous AR	Pseudo-androgens Androgen antagonists Corticosteroids	Dependent on a mutant AR	Antagonist acting as agonists;
3	Outlaw AR	Androgen independent Ligand independt	AR dependent	Mutant *PTEN*; Activated *PI3K*; Activated *MAPK*;
4	Bypass AR	Androgen independent	AR independent	Parallel or alternative survival pathways
5	Lurker cells	Androgen independent	AR independent	Malignant epithelial stem cells

The first pathway by which prostate cancer bypass the effects of androgen ablation therapy is through increasing its sensitivity to low levels of androgens. This is the so-called **hypersensitive pathway**, and cells are not entirely androgen-independent, since their responses depend on AR and androgens, but the threshold for androgenic responses has lowered (75), which can be achieved by distinct mechanisms. One possibility is by increasing the expression of the AR itself, which will lead to enhanced ligand-occupied receptor content, even in the case of reduced level of androgens (76). About 30% of tumours that become androgen-independent after ablation therapy have the AR gene amplified, whereas none of the primary tumours before androgen ablation had this amplification (77). A second mechanism consists of increased AR sensitivity and results from high expression level of the AR and enhanced nuclear localization of AR in cancer cells. Thus, the levels of DHT required for growth in these androgen-independent cells are much lower than the required by the androgen-dependent cells (78). Lastly, the third mechanism arises by increasing the androgen levels to compensate the overall decline in circulating testosterone. By increasing the 5α-reductase activity, prostate cancer cells could increase the conversion of testosterone to DHT. Thus, continued AR signalling occurs even with lower levels of serum testosterone. In support of this theory, are the finding showing that after androgen ablation therapy, serum testosterone levels decrease by 95%, but the DHT in prostate tissue is only reduced about 60% (79).

The **promiscuous pathway** defends that some cases of androgen independence develop from the acquisition of genetic changes, which lead to aberrant activation of the androgen signalling (80). These changes are usually missense mutations in the AR gene that decrease the specificity of ligand binding and allow inappropriate activation by other ligands (androgen antagonists and various non-androgen steroids). Some reports indicate that there is an increased incidence of somatic AR mutations in metastatic samples (81, 82). The frequency of mutation in the AR is significantly higher in tumours after androgen ablation comparing with the AR mutations in primary tumours before therapy (81-83). The T7877A substitution is an example of how a single mutation allows alterations of AR specificity since, in LNCaP cells, it permits cell growth in response to androgens and also to non-androgenic steroids (84). Co-activators of AR recruit other transcription factors to induce transactivation of AR-regulated genes, whereas co-repressors inhibit the transcription of AR-regulated genes. These kinds of proteins can also be modified in androgen-independent prostate cancer. The ARA70, Tip60, TIF2 and SCR1 are examples of increased co-activators in androgen-independent prostate cancer (85-87). On the other hand, the SMRT is a co-repressor altered that may lead to progression of prostate cancer for advanced stages (88).

The third is the so-called **outlaw pathway** and depends on the AR activation by ligand-independent mechanisms. Some growth factors such as insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF), can activate the AR, leading to an outlaw receptor, thus inducing expression of AR target genes in the absence of androgens (89). These growth factors are ligands for receptor tyrosine kinases and initiate

complex intracellular signalling. IGF-1, the most powerful of the factors tested, induced a fivefold increase in PSA secretion in LNCaP cells (89). HER-2/neu, a member of the EGF receptor family of receptors tyrosine kinases, is overexpressed in androgen-independent cell lines arisen from xenografts implanted in castrated mice, and this overexpression can activate AR-dependent genes in the absence of AR ligand (90, 91). Another mechanism of action is that HER-2/neu can act through the mitogen-activated protein kinase (MAPK) pathway and activate the AR. MAPK can phosphorylate the AR and leads to AR activation (91). The last pathway involved is the AKT pathway responsible for cell survival, and AKT activity is increased in androgen-independent cell lines when compared with androgen-dependent (92). Moreover, it was found that overexpression of AKT in LNCaP xenograft tumours accelerated tumour growth and downregulated the expression of p27, a cell cycle regulator, in these cells (92, 93).

The fourth case is the **bypass pathway** and is also a possible pathway to give the cells ability to survive independently of AR activation. B-cell lymphoma 2 (Bcl-2) is considered the obvious candidate gene that allows the blocking of apoptosis and it is not normally expressed in prostate (94). Several reports have shown that Bcl-2 is overexpressed in androgen-independent stages of prostate cancer when compared to early stages of the disease (95, 96). Moreover, the blockage of Bcl-2 actions induces apoptosis in LNCaP cells, which suggests that upregulation of Bcl-2 could bypass the signal for apoptosis that is normally generated by androgen ablation and contribute to arisen of androgen-independent stages of the disease (95, 97).

The fifth and last mechanism is called the **lurker cell pathway**, which defends that androgen ablation fails due the presence of a subpopulation of tumour cells that are not dependent on androgen and was present even before the therapy was initiated. The putative epithelial stem cells among the basal cells of the prostate are believed to be androgen-independent, since their rates of proliferation and death are no affected by androgen ablation therapy (67). Moreover, a minority sub-population of cells in prostate cancer that not express AR, has been identified as prostate cancer stem or progenitor cells, and these cells can sustain their proliferation ability even during the ablation therapy, producing a cancer cell selection for cells capable of self-renewal and drug resistant (98).

These are the different possible pathways already described for the transformation of early stages of the prostate cancer (androgen-dependent phase) to more aggressive and lethal forms of prostate cancer, the androgen-independent stage (Fig. 6). Increasing the understanding of the active players in the different pathways, and of the factors that may stimulate androgen-independency are important research that aims for the discovery of an effective therapy for this stage of the disease (99).

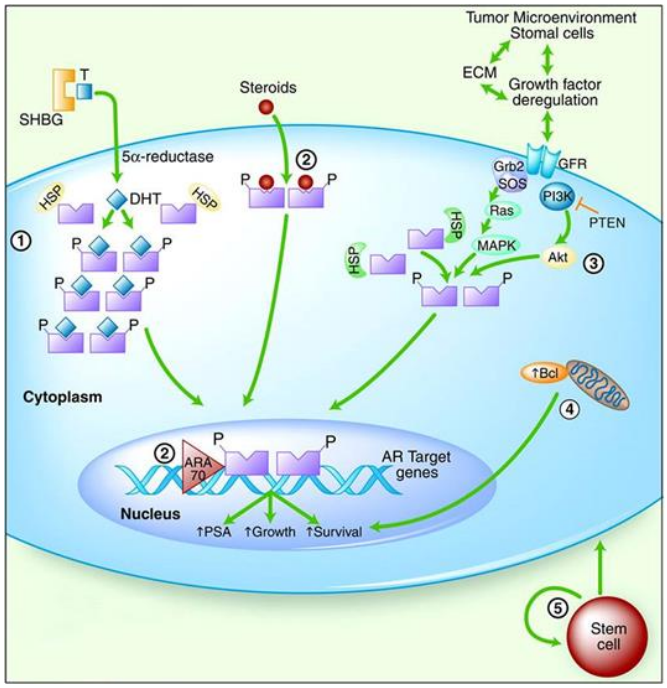

Figure 6. Mechanisms underlying the development of androgen-independent prostate cancer. 1. Hypersensitive pathway where prostate cancer cells acquire the ability to use low levels of androgen for growth and survival by increasing the production of AR (gene amplification), increasing the sensitivity of the receptor to androgens or by increasing local conversion of testosterone to DHT. 2. Promiscuous pathway where mutations on the AR allow nonandrogenic steroid molecules present in the circulation as well as antiandrogens to bind and activate the AR. Also, alteration in the balance of coactivators and corepressors influence the activation of AR. 3. Outlaw pathway where nonsteroid molecules activate the AR by ligand-dependent binding or downstream signalling of the AR by ligand-independent mechanisms. 4. Bypass pathway where prostate cancer develops the ability of survive independently of AR. The best-known mechanism is through modulation of Bcl-2 by androgen-independent prostate cancer cells, which confers protection from apoptosis upon testosterone withdrawal. 5 Lurker cell pathway where prostate cancer stem cells, which do not depend of AR to survive, continually resupply the tumour cell population despite therapy (Adapted from (99)).

2.6 Therapeutic options for prostate cancer

Nowadays, there are a wide variety of possible therapies for the treatment or management of prostate cancer that are dependent on the disease’s phase of progression. If the tumour is small, local and has not spread beyond the gland, it is recommended a monitoring strategy called “Watchful Waiting” (100). The options for pre-metastatic stages include androgen-deprivation therapy, radical prostatectomy, brachytherapy and external beam radiation therapy (101). In the case of metastasis, more advanced stages, it may be used chemotherapy, radiotherapy and hormonal therapy (102). Besides the existence of all these options, there is a need for discovery of innovative therapies due to the limitations of the existing ones.

3. Metabolic reprogramming of prostate cancer cells

The chronic and uncontrolled cell proliferation, that represents a crucial feature of neoplastic diseases, involves deregulated control of cell proliferation and the respective necessary adjustments of energy metabolism to fuel cell growth, division and survival (103). The first anomalous characteristic of cancer cell energy metabolism was reported by Otto Warburg, which observed that even in the presence of oxygen, cancer cells can reprogram glucose metabolism by limiting their energy metabolism largely to glycolysis, leading to a state that has been called “aerobic glycolysis” (104). Several molecular mechanisms are responsible for altering cellular metabolism and support the crucial needs of a dividing cell: rapid adenosine triphosphate (ATP) generation to ensure energy, increased biosynthesis of macromolecules and maintenance of cellular redox status (105). More recently, it was found that to meet these necessities cancer cells develop alterations in the metabolism of all four major classes of macromolecules: carbohydrates, proteins, lipids and nucleic acids (106). These occur in the “hostile” and changeable microenvironment of the solid tumour, where concentrations of key nutrients such as glucose and oxygen are spatially and temporally heterogenous (107).

From now on, and following the aim of the thesis, the glycolytic metabolism will be the focus. In addition to the energy that is required to support rapid division of tumour cells, they also must evade the checkpoint controls that would normally block proliferation under the stressful metabolic conditions that are characteristic of the abnormal tumour microenvironment (Fig. 7) (103). Cancer cells reprogramme their metabolism to full fill these needs, and the Warburg effect is the metabolic phenotype best characterized in tumour cells, i.e., a change from ATP generation through oxidative phosphorylation to ATP generation through glycolysis, even in normoxia (108). Although ATP production by glycolysis is faster than by oxidative phosphorylation, it is much less efficient in terms of the ATP generated per glucose molecule consumed, which demands that tumour cells implement an abnormally high rate of glucose uptake for satisfying their needs (109, 110).

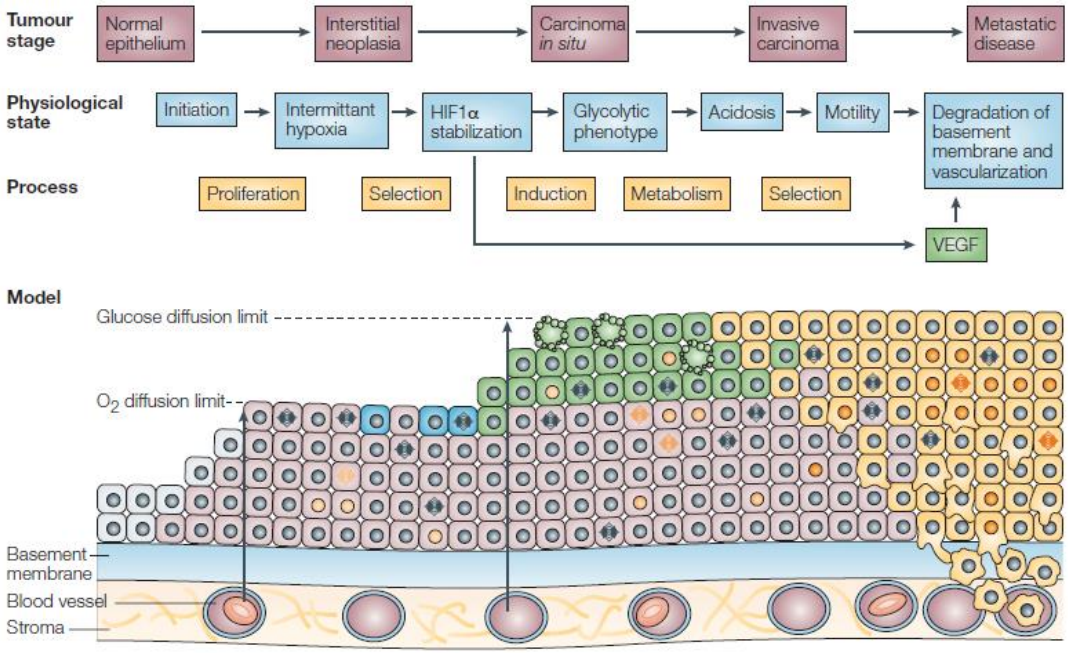

Figure 7. Model for heterogenous cell-microenvironment interactions in carcinogenesis and associated metabolic changes. The phases of tumour growth and their respective physiological states are represented, demonstrating that progression from one stage to the next is governed by distinct processes. Briefly, normal epithelial cells (grey) become hyperproliferative (pink) following induction. At the point that they reach the oxygen diffusion limit, they become hypoxic (blue), which can lead to cell death (blebbing) or adaptation of a glycolytic phenotype (green), which ensures cell survival. By using glycolysis, lesions become acidic, which selects for motile cells (yellow) that may eventually break the basement membrane. (The nuclei shown as light orange for one mutation and darker for more mutations). VEGF- vascular endothelial growth factor; HIF1α – hypoxia-inducible factor (111).

Briefly, in a healthy cell, the glycolytic process is strictly regulated and begins with the glucose uptake from extracellular space. This process occurs via the glucose transporters (GLUTs) family members, which are responsible for the transport of glucose across the membrane. The major isoforms associated with cancer cells are GLUT1 and GLUT3, which are responsible for a substantial amount of glucose uptake (112-115). Once inside of cell, glucose is converted into pyruvate by a chain of reactions designed by glycolysis. The first step of glycolysis is driven by the action of hexokinase, an enzyme responsible for the conversion of glucose into glucose 6-phospate (116). The main limiting step of this metabolic cascade is the phosphorylation of fructose-6-phosphate (F-6-P) into fructose 1,6-biphosphate (F-1,6-P) through the action of the enzyme phosphofructokinase-1 (PFK1) (112, 117). The activity of PFK1 can be stimulated due the action of the PFK2, an enzyme that produces fructose-2,6-biphosphate (F-2,6-P), which is an allosteric activator of PFK1 (118). The final product of the entire glycolytic process is pyruvate (112). Then, pyruvate can be used for numerous other metabolic pathways, such as the citric acid cycle, which is the common via for cells undergoing aerobic respiration and in normal cellular function it is the pathway more used due to its higher ATP yield (119). The other principal possible pathway is the anaerobic pathway, which produce less amount of ATP and normally occurs in conditions of hypoxia and/or cellular stress. Lastly, there are other alternative pathway to pyruvate called the alanine cycle, which recycles pyruvate back into glucose by shuttling it to the liver (119).

Regarding the anaerobic pathway and cancer cells glycolytic features, pyruvate is converted into lactate by the enzyme lactate dehydrogenase (LDH) and then is exported from the cell by the monocarboxylate transporters (MCTs). The principal isoform responsible for lactate export is the MCT4 family member (120, 121).

As mentioned above, cancer cells, including the case of prostate cancer cells, tend to prioritize the anaerobic pathway over the transportation of pyruvate into the mitochondria, and rely on aerobic glycolysis (Fig. 8) (104).

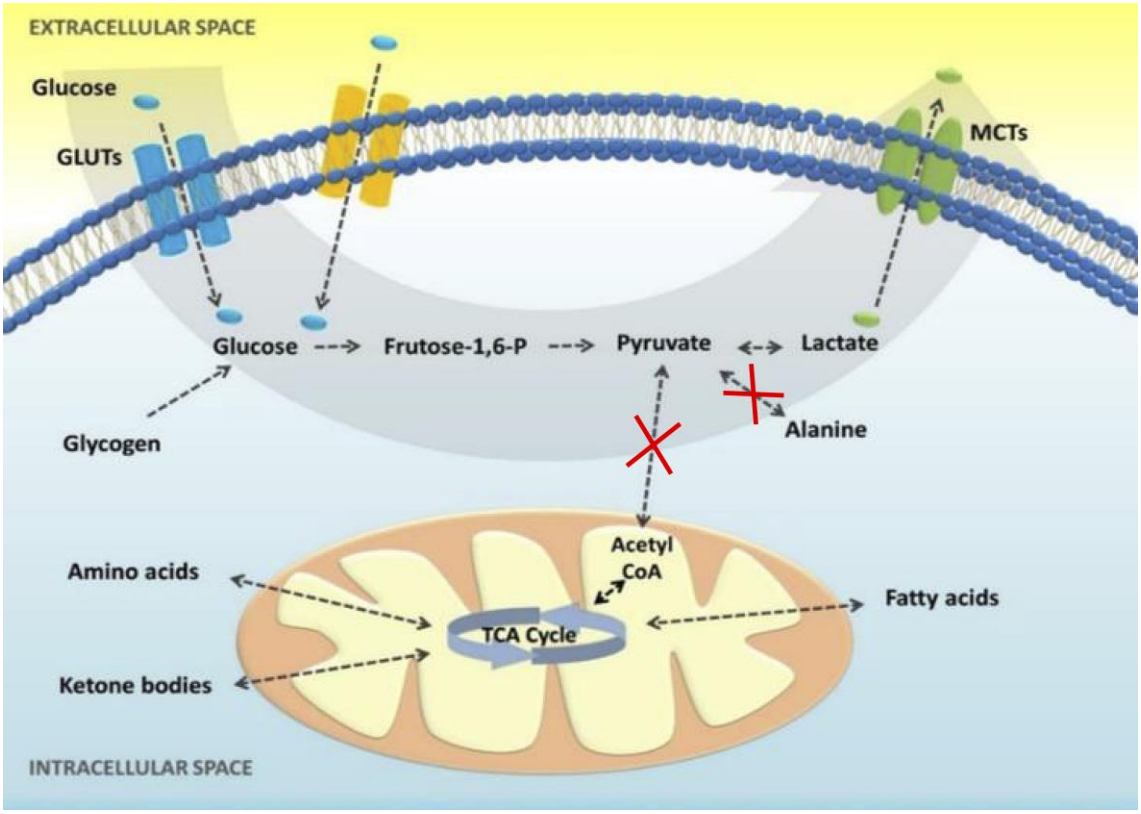

Figure 8. Warburg effect in prostate cancer cells (122).

There are several signalling pathways that are altered in cancer cells, which seems to have effects in metabolism and may be responsible for some alterations in cancer cells metabolism (Fig. 9).

Firstly, the phosphoinositide 3-kinase (PI3K) pathway is one of the most commonly altered signalling pathways in human cancers (123). Usually, this pathway is activated by mutations in tumour suppressor genes, such as PTEN, mutations in the components of PI3K complex or by aberrant signalling from receptor tyrosine kinases (124). The well-studied effector downstream of this pathway is AKT1, also called PKB. AKT1 is a crucial driver of the tumour glycolytic phenotype and stimulates ATP generation by several mechanisms, certifying that cells have the bioenergetic capacity necessary to respond to growth signals (125, 126). It stimulates glycolysis by increasing the expression of GLUTs and by phosphorylating key glycolytic enzymes, such as hexokinase and PFK2 (127). It also stimulates the kinase

mammalian target of rapamycin (mTOR) by phosphorylating and inhibiting its negative regulator (127). Activated mTOR stimulates lipid and protein biosynthesis and cell growth in response to sufficient nutrient and energy conditions (128). Also, causes indirectly other metabolic alterations by activating transcription factors such as the hypoxia-inducible factor 1 (HIF1) even under normoxic conditions.

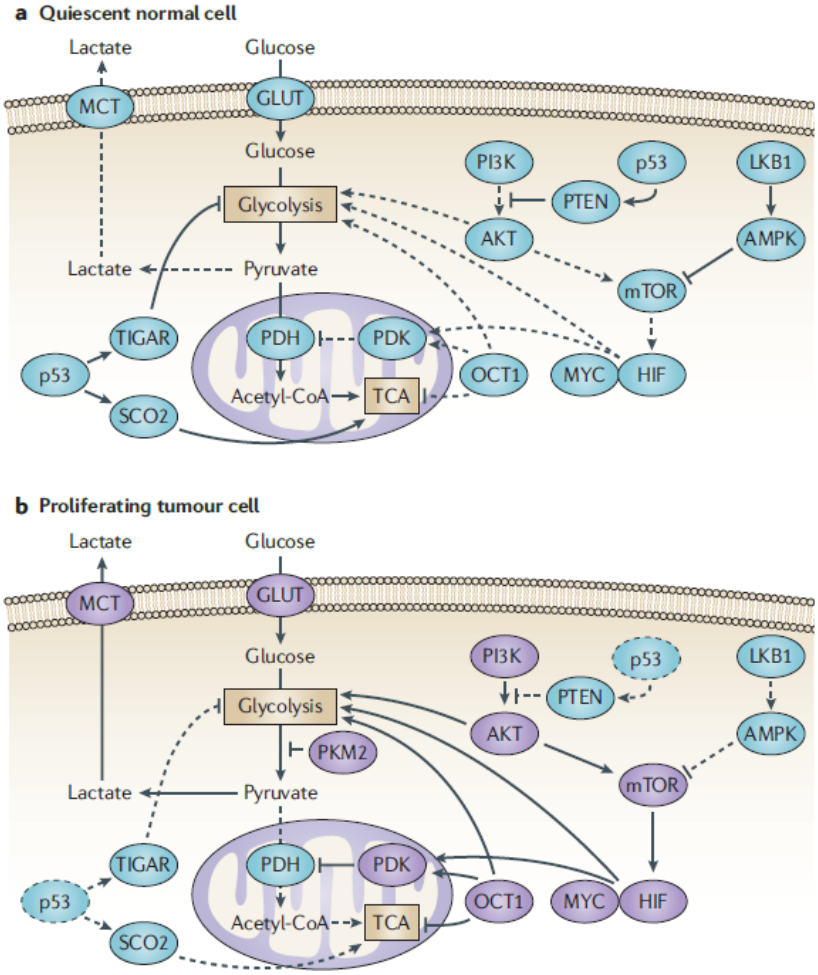

Figure 9. Signalling pathways driving metabolic alterations into glycolytic phenotype. Multiple oncogenic signalling pathways can drive the shift from normal cells (a) to aerobic glycolysis in tumour cells (b). PI3K activates AKT, which stimulates glycolysis by regulating glycolytic enzymes and activating mTOR. LKB1, a tumour suppressor, opposes the glycolytic phenotype by inhibiting mTOR, through activation of AMPK. mTOR alters metabolism by increasing HIF1 activity, which starts an hypoxia-adaptive transcriptional programme. MYC cooperates with HIF in activating several genes that encode glycolytic proteins. The tumour suppressor p53 counteracts the glycolytic phenotype by suppressing glycolysis, through the activation of TIGAR, an apoptosis regulator and increasing mitochondrial metabolism via SCO2 and controlling the expression of PTEN. OCT1 also acts in an opposite way, activates the transcription of genes responsible for glycolysis and suppresses the oxidative phosphorylation. The dashed lines indicate loss of p53 function. MCT – monocarboxylate transporter; GLUT – glucose transporters; TIGAR - Tp53-induced Glycolysis and *Apoptosis* Regulator; SCO2 – synthesis of cytochrome c oxidase subunit 2; PKM2 – pyruvate kinase M2; PDH – pyruvate dehydrogenase; PDK – pyruvate dehydrogenase kinase; LKB1 – liver kinase B1; AMPK – AMP-activated protein kinase; HIF – hypoxia-inducible factor; mTOR- mammalian target of rapamycin; PTEN – phosphatase and tensin homolog; PI3K – phosphoinositide 3-kinase (129).

Other altered pathways are the HIF1 and MYC-dependent signalling. The HIF1 is one of the major transcription factors responsible for the alterations in gene expression in response to low oxygen conditions. After activated, HIF1 amplifies the transcription of genes encoding GLUTs and most glycolytic enzymes (130). Besides that, HIF1 also activates the pyruvate dehydrogenase kinases (PDKs), which inactivates the mitochondrial pyruvate dehydrogenase complex and thereby reduce the flow of pyruvate into the mitochondria (131-133). This reduction in pyruvate flux decreases the rate of oxidative phosphorylation, reinforcing the glycolytic phenotype. Regarding MYC, an oncogenic transcription factor, it also has numerous important effects on cell metabolism. It seems that MYC can collaborate with HIF in the activation of several GLUTs, glycolytic enzymes, LDH and PDK1 (134).

Also, the AMP-activated protein kinase (AMPK) pathway is altered in cancer cells. AMPK is a key sensor of energy status and has a crucial role in cellular responses to metabolic stress. AMPK becomes activated, during periods of stress, in response to an increased AMP/ATP ratio, and is responsible for shifting cells to an oxidative metabolic phenotype and inhibit cell proliferation (135, 136). Thus, cancer cells need to overcome this checkpoint in order to proliferate in response to activated growth signalling. Many oncogenic mutations and pathways can suppress AMPK signalling, allowing cancer cells to divide under abnormal nutrient conditions (137). It seems that mutations on liver kinase B1 (LKB1), which is the upstream kinase necessary for AMPK activation, are tumorigenic due the resulting decrease in AMPK signalling and loss of mTOR inhibition (137). The AMPK loss allows the activation of mTOR and HIF1, which support the shift towards glycolytic metabolism.

Finally, p53 is also an important regulator of metabolism, since it activates the expression of hexokinase 2 (HK2), which converts glucose into glucose-6-phosphate (G-6-P) (138, 139). However, p53 inhibits the glycolytic pathway through upregulation of the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), enzyme that decreases the levels of the glycolytic activator F-2,6-P (140). In addition, p53 also can promote oxidative phosphorylation by activating the expression of synthesis of cytochrome c oxidase subunit 2 (SCO2), which is required for this process (141). Thus, the p53 loss might also play a crucial role in the metabolic alterations in cancer cells into glycolytic phenotype. OCT1, a ubiquitous transcript factor, initiated a transcriptional programme which can cooperate with the loss of p53 and supports the resistance to oxidative stress. OCT1 regulates several genes responsible for increasing glucose metabolism and reduce mitochondrial respiration (142).

In this context, it is expected that more aggressive, androgen-independent stages of prostate cancer produce more lactate when compared to the androgen-dependent phases of the disease with lower proliferative rates. This has been demonstrated by the findings showing increased activity of LDH and MCT4, which justify the higher lactate production observed in cell line models mimicking androgen-independent prostate cancer (35). Also, glycolytic metabolism itself was upregulated in prostate cancer cells, by the increased expression of the GLUTs and limiting glycolytic enzyme PFK1 (35).

4. Generalities on endocrine-disrupting chemicals (EDCs)

4.1 Classification

Endocrine-disrupting chemicals (EDCs) are defined by the U.S. Environmental Protection Agency as the compounds that can interfere with the synthesis, secretion, transport, binding or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour (143).

These agents can be classified into different categories according to their chemical origin, source or physiological mode of action. Regarding their chemical origin, EDCs can be separated in two groups, those that occur naturally, such as phytoestrogens (e.g. genistein) and natural estrogens (e.g. 17-β-estradiol), and those that are synthesized, such as bisphenol A, vinclozolin and dioxins (144).

Concerning their source, EDCs can be grouped in natural and artificial hormones (e.g. phytoestrogens and contraceptive pills, respectively), drugs with hormonal side effects (e.g. naproxen and metoprolol), and industrial and household chemicals (e.g. phthalates, fire retardants and plasticizers) (144, 145).

Finally, concerning the mode of action and physiological responses, these compounds can be grouped into four categories: xenoestrogens, xenoandrogens, antiestrogens and antiandrogens. However, it is important to emphasise that there are chemicals that can exhibit more than one of the previously mentioned behaviour. Xenoestrogens consist in a group of chemicals that interfere with the endocrine system by mimicking estrogens, thus, binding the estrogen receptors (ERs) as agonists and displaying estrogenic properties (146-148). On the other hand, xenoandrogens represent a set of substances that can disrupt the endocrine homeostasis mimicking androgens actions by its interaction with the AR as agonists (149, 150). Antiestrogens and antiandrogens are the class of EDCs, which antagonize ERs and AR, respectively. These groups of compounds act as antagonists by blocking or reducing ERs and AR activity deeply disturbing hormonal actions and affecting several physiological responses in a wide range of tissues (151-154).

Endocrine disruptors are present in the air that we breathe, the water that we drink and in the soil in which our food is cultivated (155). Thus, exposure to EDCs can occur by numerous ways, such as drinking contaminated water, breathing contaminated air, ingesting food or contacting contaminated soil that has been exposed to pesticides, plasticizers, and other compounds used in agriculture, industry and household applicants (156). Many of these EDCs end up in the aquatic ambient and make water a potential source of these chemicals, since the water treatments like flocculation, sedimentation, filtration and chlorination are not able to eliminate all the contaminants (157-160). Other major source of exposure are the industries due to their waste material and their potential adverse effects on environment (161). This material can contain pharmaceutical products such birth control pills and other

sources of EDCs from several detergents, soaps, plastics, food and some personal care products (162, 163). Lastly, also the use of pesticides, insecticides and herbicides in agriculture represents another source of exposure to these compounds. Some EDCs are degraded in the environment for example by sunlight, bacteria and chemical processes, while others persist in the environment with variable time range (164).

Besides inhalation and food consumption there are other routes of exposure, namely, the contact with skin, through which our body can contact with pesticides, brominated flame retardants and cosmetics, personal care products and sunscreens. Another case is the intravenous route, for which the main example are the phthalates, commonly present in intravenous tubing, and using this route to enter the human body. Lastly, human body can get exposed to these chemicals without being in direct contact with them, which encompasses biological transfer from placenta and maternal milk (165).

There are other relevant aspects to be considered for a better understanding of the endocrine disruption. Firstly, the age of exposure is crucial since EDCs may have different effects in adults when compared with the developing fetus or infants (145), since these chemicals have a greater risk in the early development period rather than in adulthood. Moreover, some studies have shown that the harmful effects of EDCs may be passed to subsequent generations. Latency from exposure is another important matter, since the disease resulting from endocrine disruption may not be manifested during the exposure period, it might be manifested in adulthood or during aging (145). Another important feature of endocrine disruption is the effects of different classes of EDCs at the same time, which can be additive or even synergistic since populations aren´t exposed only to a single compound (145). Lastly, the concentration of EDCs and the low dose effects are also very important since it has to be overcome the idea that EDCs only exert their effects at high concentrations (166-168).

4.2 Mechanism of action

EDCs can exert their effects through several mechanisms affecting directly endocrine organs and also at systemic level by altering endocrine homeostasis and the hormonal actions. Below, the evidence for the principal mechanisms underlying EDCs mode of action will be presented.

The most well-known mechanisms used by EDCs is the activation (e.g. xenoestrogens and xenoandrogens) or blocking (e.g. antiestrogens and antiandrogens) of nuclear receptors (NRs), a class of specific receptor proteins that act as transcription factors and playing a crucial role in the modulation of the gene expression network in target cells (169, 170). The effects mediated through NRs are sustained by receptor binding to the hormone-responsive elements, consensus DNA sequences in the promoter region of target genes, as well as, by the receptor interaction with other co-activators and co-repressors proteins (171, 172). The main NRs targeted by EDCs are the classical steroid NRs, ERs and AR. However, EDCs can also bind other

members of the NRs family, such as the subfamily of estrogen-related receptors (ERRs), the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), the peroxisome-proliferator activated receptor (PPAR), the retinoid acid receptor (RAR) and the thyroid receptor (TR) (173, 174). Activation of above-mentioned receptors by EDCs and the downstream up- or down-regulation of their target genes affect the normal action of endogenous hormones (estrogens and androgens), by interfering with their actions with target receptors, namely ER and AR, leading to consequences both at hormonal and reproductive levels (172). Finally, the (de)regulatory actions of EDCs over gene transcription have also been linked to their effects modulating the population of small non-coding RNAs (175-177).

More recently, rapid nongenomic actions of EDCs, by its interaction with plasma membrane receptors, and not depending on the regulation of gene transcription, also have been reported. Briefly, the mechanisms of rapid responses involve second messenger-triggered signal cascades, with the most typical example being the case of membrane-bound estrogen receptors: mERα, mERß and the G protein-coupled estrogen receptor (GPER or GPR30) (178). An example of a nongenomic mechanism mediated by mERα and mERß is the increase of intracellular calcium (Ca^{2+}) levels driven by a rapid increase in Ca^{2+} influx, which can promote changes in intra- and extra-cellular processes, cell motility and rapid hormone secretion (179). Concerning GPER, its activation by EDCs has been linked to the deregulation of the hormonal balance with impact in a broad range of tissues (180-182).

Besides directly interfering with NRs and affecting the array of genes expressed in a specific cell at a given moment, EDCs can also modulate the epigenetic panorama in target tissues and cells. The major epigenetic changes induced by EDCs are DNA methylation and histone modifications, leading, for example, to an altered expression of tumor suppressor genes and increased susceptibility to carcinogenesis (183-186). Moreover, it has been established that early life exposure to EDCs altering gene expression via epigenetic mechanisms is a feature that can be heritable in successive generations (187, 188).

Lastly, another mechanism through which EDCs can exert disruptive effects is by interfering with the hormone synthesis and metabolism. The activity of steroidogenic enzymes such as hydroxysteroid dehydrogenases, aromatase, sulfatase and sulfotransferases has been shown to be affected by xenoestrogens (189-195). Besides aromatase, EDCs can also inhibit other p450 enzymes that are involved in the metabolism of testosterone and estrone in the liver (196, 197). The major affected member of p450 enzymes family are cytochrome p450 (CYP)1, CYP2 and CYP3 which are responsible for drug and steroid metabolism (198-200).

From a physiological perspective, an EDC is an agent that through environmental or inappropriate developmental exposure, alters the hormonal and homeostatic systems. As major consequences of exposure to these chemicals are the effects perceived by the reproductive system, prostate, breast, lung, liver, and thyroid, as well as the metabolic changes and obesity (201). Epidemiological studies have provided evidence that the increase in the incidence and prevalence of some diseases are associated with EDCs, namely, breast, prostate, and testis cancer, as well as, diabetes, obesity and decreased fertility (201).

Metoxychlor (MXC), is a synthetic chemical with a double ring structure (Fig. 10), belonging to the group of organochlorine pesticides, which was first synthesized in 1893. The commercial production of MXC was first reported in 1946, and the compound was first registered in 1948, as a less persistent alternative pesticide to 4,4'-(2,2,2-trichloroethane-1,1-diyl)bis(chlorobenzene) (DDT) (202, 203). Since then, MXC has been used against pests such as houseflies, mosquitoes, cockroaches, and several arthropods normally found on field crops, vegetables and fruits (204). This chemical is less toxic than DDT and less persistent in the environment, however, its residue in soils and sediments is still of concern (205). It is likely that the widespread use of MXC during several decades has produced substantial environmental contamination and significant animal and human exposure to this pesticide and its transformation products (206). Classical receptor binding assays have shown that MXC can bind human ERα, though with weak affinity (207). By using recombinant human ERα, it was confirmed that MXC weakly competes with fluorescent nonsteroidal estrogen at 10-6M or higher concentrations (208). Thus, MXC is estimated to have approximately 1000- to 14000-fold lower binding affinity to human ERα compared to the natural ligand, 17β-estradiol (207). Concerning ERβ, MXC has a lower binding capability or no affinity to this ER subtype when compared with ERα (208).

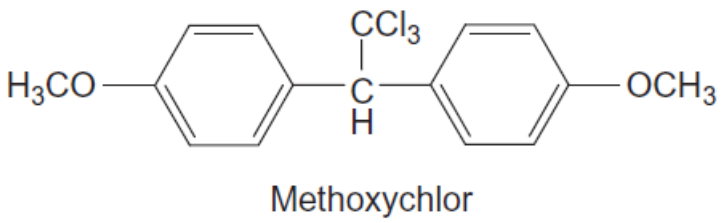

Figure 10. Structure of methoxychlor. (Adapted from (208)).

Due to the above mentioned activity, MXC has been studied for its toxicological effects, which includes carcinogenicity, reproductive and developmental toxicities, neurotoxicity, immunotoxicity and estrogenic activities (209-215). Moreover, exposure to MXC results in reduced fertility and ovulation with alterations in specific gene expression, and folliculogenesis in the ovary (216). In addition, MXC disturbs spermatogenesis and reduces the size of the testes, prostate, and seminal vesicles in males (217). The acute LD50 in rat is approximately 6000mg/kg body weight (218). At dose levels lower than LD50, MXC has been shown to affect the testes. Weanling rats fed with 1% of MXC displayed histopathological changes in the testes, accessory glands and kidneys (219). MXC can stimulate proliferation of ER-positive breast cancer MCF-7 cells by up-regulating genes linked with the progression of cell cycle whereas downregulating anti-proliferative genes (210). The cell-stimulating effect of MXC is thought to be ER-mediated since co-treatment with ICI 182720, a synthetic antiestrogen, clearly blocked the effect (211).

5. EDCs and prostate cancer

Nowadays, there is increasing evidence both from epidemiology and animal models studies that some EDCs may influence the development or progression of prostate cancer (220-222). These effects appear to be linked to the capability of these compounds to interfere with estrogen signalling, either through interacting with ERs or by influencing steroid metabolism and altering estrogen levels within the body (223). It has been found a link between prostate cancer rates and pesticides, where chronic or intermittent exposures to these chemicals may contribute to higher rates of this disease. Six pesticides (chlorpyriphos, fonofos, coumaphos, phorate, permethrin and butylate) of 45 commonly used in agricultural activities showed correlation with exposure and increased prostate cancer in men with a familial history, suggesting gene-environment interactions (221, 224). In addition, chlorpyriphos, fonofos and phorate strongly inhibit CYP1A2 and CYP3A4, which are responsible for metabolize estradiol, estrone and testosterone (196, 197). Moreover, these two enzymes are expressed by human prostate and responsible for intraprostatic metabolism of steroids, thus raising the possibility that exposure to these compounds may interfere with the steroid hormone metabolism and alter steroid balance and availability, which in turn may lead to increased prostate cancer risk (225, 226).

Maternal exposure to diethylstilbestrol (DES) during pregnancy was found to cause more extensive prostatic squamous metaplasia in human male offspring than observed with maternal estradiol alone (227). Thus, men exposed prenatally to DES may be at increased risk for development of prostate cancer later in life (228). Also in rodents, it was verified that marked abnormalities in the adult prostate including increased susceptibility to carcinogenesis occur with early exposure to DES (229-231).

Concerning bisphenol-A (BPA) a widely-distributed EDC, evidence from both rodent models and humans prostate cell lines indicate that this chemical influences carcinogenesis, modulate prostate cancer cell proliferation and in some tumours, stimulates their progression. Early life exposure to BPA may increase susceptibility to hormonal carcinogenesis in the prostate gland, possibly by developmentally reprogramming carcinogenic risk (184, 232). Some alterations in DNA methylation patterns in multiple cell signalling genes in BPA-exposed prostates have been identified, which suggests that environmentally doses of BPA “imprint” the developing prostate through epigenetic alterations (184, 232).

The effect of BPA on human prostate cancer cells harbouring an AR point mutation (AR-T877A) frequently found in advanced prostate cancers was investigated (233). It was observed that 1 nM of BPA activates AR-T877A in transcriptional assays and leads to cell cycle progression and cellular proliferation *in vitro* in the absence of androgen. As BPA had no effect on wild-type AR, these data indicate that this gain-of-function AR mutant attained the ability to use BPA as an agonist (234). Experiments performed in rats showed that even a brief neonatal exposure to a low dose of BPA significantly increased the incidence and grade of PIN lesions following adult estrogen exposure (235).

An epidemiologic study of capacitor manufacturing plant workers highly exposed to PCBs demonstrated a strong exposure-response relationship for prostate cancer mortality (236). This finding corroborates other previous correlations between PCBs 153 and 180 and prostate cancer risk in electric utility workers (237, 238). On rat prostate cells a chemical called Aroclor-1254, a mixture of 60 PCB pollutants, was tested and showed to disrupt gap junctions, expression of connexin 32 and 43 and increase double-stranded DNA breaks suggesting that PCBs might be able to transform prostate cell leading to carcinogenesis (239).

Some reports indicated that developmental exposure to UV filters can alter rat prostate gland development and estrogen target genes expression, thus, increasing the possibility that fetal prostate might be affected following maternal use of these compounds (240, 241). Also, cadmium, a metal ion known to bind ERs and mimic estrogens actions, was reported to have proliferative effects in human prostate cells *in vitro* through an ER-dependent mechanism. Cadmium exposure was also associated with the acquisition of androgen independence (242). Moreover, prostatic tumours have been shown to be experimentally induced by oral exposure to cadmium (243, 244). Another chemical associated with prostate cancer is arsenic, with some data showing an association between inorganic arsenic exposure from the environment and prostate cancer incidence and mortality in the human population (245). It is documented that arsenic mediated some of these effects specifically through interaction with ERs and activation of estrogen-regulated genes (246). A study conducted in prostate epithelial cells reported that arsenic can induce malignant transformation and drive them toward an androgen-independent state. It was shown that this effect might be mediated through Ras-MAPK pathways and it is possible that the membrane ERs may be involved in this mechanism (247).

Despite the above-mentioned reports demonstrating the strong relationship between prostate cancer and estrogen-like EDCs, together with the fact that MXC is one of the main EDCs with the ability to mimic estrogens, very few data are available concerning its liaison with the development and progression of prostate cancer.

II. AIM

Several reports have been showing significant variations in the incidence of prostate cancer between populations living in different areas. These differences can probably be associated with lifestyle habits, including the exposition to a set of environmental substances or compounds that may have a role in the aetiology of disease. A recent study has shown that the majority of cancers are a consequence of extrinsic factors (environment) instead of being just a question of “bad luck” (248), which has challenged the concept of cancer aetiology and highlighted the importance of environment in the development of oncological diseases. EDCs are a group of environmental chemicals that can mimic or alter hormone signalling being able to exert effects at very low doses, over the years, and even across generations, which also have been implicated in carcinogenesis. In the case of prostate, it is likely to assume that EDCs may interfere with estrogen signalling contributing to carcinogenesis since alterations in estrogens levels were shown to affect prostate cell survival and death, and prostate growth and development. Methoxychlor is a chlorinated pesticide widely dispersed in the environment because of its use in agricultural activities, and several reports have demonstrated its estrogenic properties.
Although it is well known that hormonally active compounds have the potential to affect human health adversely, and that the metabolic reprogramming is a known hallmark of cancer, the actions of these chemicals inducing metabolic alterations towards the malignant transformation of prostate remain to be clarified.

The present dissertation aims to clear up these questions by:

- Analysing the effects of methoxychlor on proliferation and apoptosis of cell line models mimicking different stages of prostate cancer;

- Studying the effect of methoxychlor as a (de)regulator of metabolism in distinct cell line models of prostate cancer, as a strategy to clarify the relationship between metabolism and prostate cancer;

- Evaluating the possible relationship between the action of methoxychlor and prostate carcinogenesis;

III. MATERIAL AND METHODS

1. Cell lines

The human non-neoplastic prostate epithelial cell line PNT1A, and the human prostate cancer cell lines LNCaP and PC3 were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The LNCaP cell line was derived from a lymph node metastasis of prostate cancer and is androgen-responsive (249). PC3 cells were derived from bone metastasis and are considered non-responsive to androgens (250). Thus, LNCaP and PC3 cells represent different stages of the disease and have been commonly used as *in vitro* models of androgen-dependent and androgen-independent phases of prostate cancer, respectively (35, 251).

2. Cell culture and methoxychlor treatment

Both neoplastic and non-neoplastic prostate cell lines were cultured and maintained in RPMI 1640 phenol red culture medium (Sigma-Aldrich, St.Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich), in an air incubator at 37°C equilibrated with 5% CO_2. At 60% of confluence, the culture medium was replaced by phenol red-free RPMI 1640 medium containing 5% charcoal-stripped FBS (CS-FBS) (Sigma-Aldrich), which is steroid hormones-free among other components. After maintenance for additional 24 hours in this culture medium, cells were exposed to different concentrations of the EDC methoxychlor (MXC) (Sigma-Aldrich). The MXC stock solution was prepared by dissolving 35 mg of MXC in 1 ml of dimethyl sulfoxide (DMSO) resulting in a 100mM MXC solution. All other MXC work solutions were prepared from the stock through 1:10 serial dilutions. Cells were stimulated or not with 0 µM, 0.1 µM, 1 µM, 10 µM and 100 µM of MXC for 48 and 72 hours through replacement of culture medium by CS-FBS alone or containing MXC. This range of experimental concentrations of MXC has already been used and described in the literature (210, 252). After treatment, cells were trypsinized and harvested for posterior analysis. Culture medium of MXC-treated and untreated cells was collected for measurement of extracellular metabolites.

3. Cell viability assay

PNT1A (10000 cells/well), LNCaP (15000 cells/well) and PC3 (4500 cells/well) cells were grown in 96-well plates with 100 µL of culture medium and cell viability was assessed by the MTT assay at 48 hours and 72 hours after treatment with MXC (0 µM, 0.1 µM, 1 µM, 10 µM and 100 µM). The MTT stock solution (5mg/ml) (Sigma-Aldrich) was prepared by dissolving the powder in phosphate buffer saline (PBS) at pH 7.4 and filtered. After MXC treatment, 10 µL of MTT stock solution was added and cells were incubated in the dark for 4 hours at 37°C. Briefly, MTT, a yellow tetrazole, is reduced to formazan in living cells, which allows to assess cell viability as indicated by its metabolic activity. After incubation, the medium and MTT solution were carefully removed. A solubilisation solution was added, 100 µL DMSO, to

dissolve the insoluble formazan into a coloured solution (purple). The absorbance was measured spectrophotometrically using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA) at 570nm and the value of absorbance is directly proportional to the number of viable cells.

4. Total protein extraction

Total proteins were isolated from human prostate cells using the radioimmunoprecipitation assay buffer (RIPA buffer) (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1% protease inhibitors cocktail (Applichem, Darmstadt, Germany) and 10% phenylmehtylsulfonyl fluoride (PMSF) (Fisher, Darmstadt, Germany). Samples were kept on ice (20 minutes) and occasionally mixed. Then, samples were centrifugated at 14,000 rpm for 20 minutes at 4°C, and total proteins (supernatant) were recovered to a fresh eppendorf tube. Total protein concentration was assessed using the Bradford method (253) with Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA). Briefly, in a 96-well plate, 1 µL of protein sample was mixed with 40 µL of Bradford reagent and 159 µL of milli-Q water to reach a total volume of 200 µL. In the blanks, 1 µL of RIPA buffer was added instead of 1 µL of protein sample. The absorbance was measured spectrophotometrically (xMark™ Spectrophotometer, Bio-Rad) at 595nm. The standard curve for protein quantification was obtained in the same way using serial concentrations of bovine serum albumin (BSA).

5. Western blot analysis

Total proteins of all cell lines (25µg) were heat-denatured at 100°C for 5 minutes and resolved on 12,5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed at 200 V for 50 minutes, approximately. Then, proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) at 750mA for 90 minutes. Membranes were blocked with 5% skimmed dried milk for 1 hour and then incubated overnight at 4°C with rabbit anti-Bax (1:1000, #2772, Cell Signalling Technology), anti-Bcl-2 (1:1000, #2876, Cell Signalling Technology), anti-caspase-9 (1:1000, p35, H-170, SC-8355, Santa Cruz Biotechnology), anti-FasR (1:500, A-20, SC-1023, Santa Cruz Biotechnology), anti-FasL (1:500, C-178, SC-6237, Santa Cruz Biotechnology), anti-p53 (1:1000, FL-393, SC-6243, Santa Cruz Biotechnology), anti-GLUT1 (1:1000, CBL242, Millipore), anti-GLUT2 (1:1000, SC-9117, Santa Cruz Biotechnology), anti-GLUT3 (1:1000, H-50, SC-30107, Santa Cruz Biotechnology), anti-LDH (1:10000, EP15664, Abcam), anti-PFK1 (1:1000, H-55, SC-67028, Santa Cruz Biotechnology), and anti-MCT4 (1:1000, H-90, SC-50329, Santa Cruz Biotechnology) primary antibodies. A mouse anti-β-actin antibody (1:10000, A5441, Sigma-Aldrich) was used for protein loading control in all analyses. After washing, the incubation of membranes with the goat anti-rabbit (1:40000, IgG-HRP, SC-2004, Santa Cruz Biotechnology) or goat anti-mouse (1:40000, IgG-HRP, SC-2005, Santa Cruz Biotechnology) secondary antibody proceeded

for 1 hour. At the end, membranes were washed, incubated with the ECL substrate (Bio-Rad) for 5 minutes, and scanned with ChemidocTM MP Imaging System (Bio-Rad). Band densities were obtained by the volumetric analysis tool from Bio-Rad Image Lab 5.1 software and normalized with the respective ß-actin band density.

6. Quantification of extracellular metabolites

The concentration of glucose and lactate in the culture medium of untreated and MXC-treated cells was assessed by means of spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain). For glucose quantification, 1 µL of cell culture medium from MXC-treated and nontreated cells was recovered at 0 hours and 48 hours after addition of MXC, and placed in a 96-well plate. Culture medium samples were mixed with 100 µL of a previously prepared kit reagent and incubated at 37°C for 10 minutes, and then the absorbance values were measured at 505nm (xMark™ Spectrophotometer, Bio-Rad). Briefly, this assay consists in the oxidation of the glucose present in the samples by the glucose oxidase present in the prepared reagent. This reaction will form hydrogen peroxide, which will react with phenol and aminophenazone (also present in the prepared reagent) to form quinone. The wells acquired a red/violet colour due to the presence of quinone and the development of colour was measured spectrophotometrically (505 nm, xMark™ Spectrophotometer, Bio-Rad).

Regarding lactate quantification, 1 µL of cell culture medium from MXC-treated and nontreated cells was placed in a 96 well-plate. Culture medium samples were mixed with 100 µL of a previously prepared kit reagent and incubated at 37°C for 5 minutes, and the absorbance values were read at 505nm (xMark™ Spectrophotometer, Bio-Rad). The principle of this assay is similar to the quantification of glucose and relies on the oxidation of lactate present in each sample by the lactate oxidase present in the prepared reagent, resulting in pyruvate and peroxide, the latter of which will be transformed in quinone by the action of peroxidase. The development of violet/red colour due to the presence of quinone was the measured parameter (absorbance at 505 nm, xMark™ Spectrophotometer, Bio-Rad).

The glucose consumption and lactate production by the non-neoplastic PNT1A cells, and LNCaP and PC3 prostate cancer cell models in response to MXC were determined comparatively with the culture medium samples collected at 0 hours, and normalized for the total number of cells in each experimental group.

7. LDH activity assay

The enzymatic activity of LDH in all human prostate cell lines was measured using a commercial kit (Spinreact, Girona, Spain). 1 µL of prostate cells protein extract was added to 150 µL of a previously prepared kit reagent in a 96-well plate and incubated for 1 minute at 37°C in the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). At this time-point, the initial absorbance was acquired, followed by subsequent readings every minute for 3 minutes. All readings were taken at 340 nm with a constant temperature of 37°C. The variation of absorbance along three minutes at 37°C is directly proportional to the activity of LDH in each sample. The obtained activities were calculated by µg of protein and expressed as fold variation relative to the control group.

8. Caspase-3 activity assay

The enzymatic activity of caspase-3 in human prostate cell lines was measured by a colorimetric method. 5 µL of total protein extracts from each cell line were mixed with the appropriated volume of assay buffer (20mM HEPES, pH 7.4, 2mM EDTA, 0.1% CHAPS, 5mM DTT) and 200 µM of caspase-3 substrate (Ac-DEVD-pNA) (Sigma-Aldrich) in a 96-well plate. Blank samples were performed without protein. Briefly, the reactions were left to proceed overnight at 37°C, and after caspase cleavage of Ac-DEVD-pNA, the p-nitro-aniline (pNA) (Sigma-Aldrich) product is released producing a yellow colour, which was determined spectrophotometrically at 405nm (Bio-Rad). The amount of generated pNA was calculated by extrapolation using a standard curve of free pNA, and is directly proportional to the activity of caspase-3.

9. Statistical Analysis

Statistical significance of differences between experimental groups was evaluated by unpaired T-test with Welch’s correction or one-way ANOVA, followed by Tukey post-test, using GraphPad Prism v6.01 (GraphPad Software). Significant differences were considered when p-values <0.05. All experimental data are shown as mean ± SEM.

IV. RESULTS

1. Methoxychlor treatment diminished the viability of human prostate cells

The viability of non-neoplastic, PNT1A, and neoplastic, LNCaP and PC3, human prostate cells in response to several concentrations (0.1, 1, 10 and 100 µM) of MXC was evaluated by the MTT assay.

A diminished viability of PNT1A cells (Fig. 11A) was observed after exposure to MXC. Although the results obtained for 48 hours of treatment with 0.1 and 1 µM MXC showed no significant differences when compared to control, the highest concentrations (10 and 100 µM) caused a significant decrease in cell viability, approximately 84% and 66%, respectively. For 72 hours of treatment, no significant difference was observed using 0.1, 1 or 10 µM of MXC, when compared with control group. However, a noticeable decrease of PNT1A proliferation of nearly 64% was obtained in the group treated with 100 µM of MXC.

In the case of LNCaP cells (Fig. 11B), treatment with MXC for 48 hours showed no significant differences between the 0.1 µM and the control group. This was followed by a remarkable decrease in viability of about 75%, 69% and 60% in the MXC-treated groups, 1, 10 and 100 µM, respectively. 72 hours of treatment, did not affect the viability of LNCaP cells with 0.1 and 1 µM concentrations of MXC when compared with non-treated group. However, the highest concentrations (10 and 100 µM) caused a significant decrease in cell viability around 82% and 44%, respectively.

Lastly, concerning the PC3 cells (Fig. 11C), 48 hours after administration, the lowest concentrations of MXC (0.1 and 1 µM) showed no significant differences when compared to non-treated group. On the other hand, the highest concentrations (10 and 100 µM) caused a notorious decrease in cell viability of approximately 82% and 53%, respectively. Finally, for the 72 hours, it was also verified that the 0.1 and 1 µM concentrations of MXC displayed no significant differences, whereas 10 and 100 µM induced a significant reduction in cell viability about 83% and 55%, respectively.

Overall, in all cell lines under study, MXC decreased cell viability in a concentration and time-dependent manner.

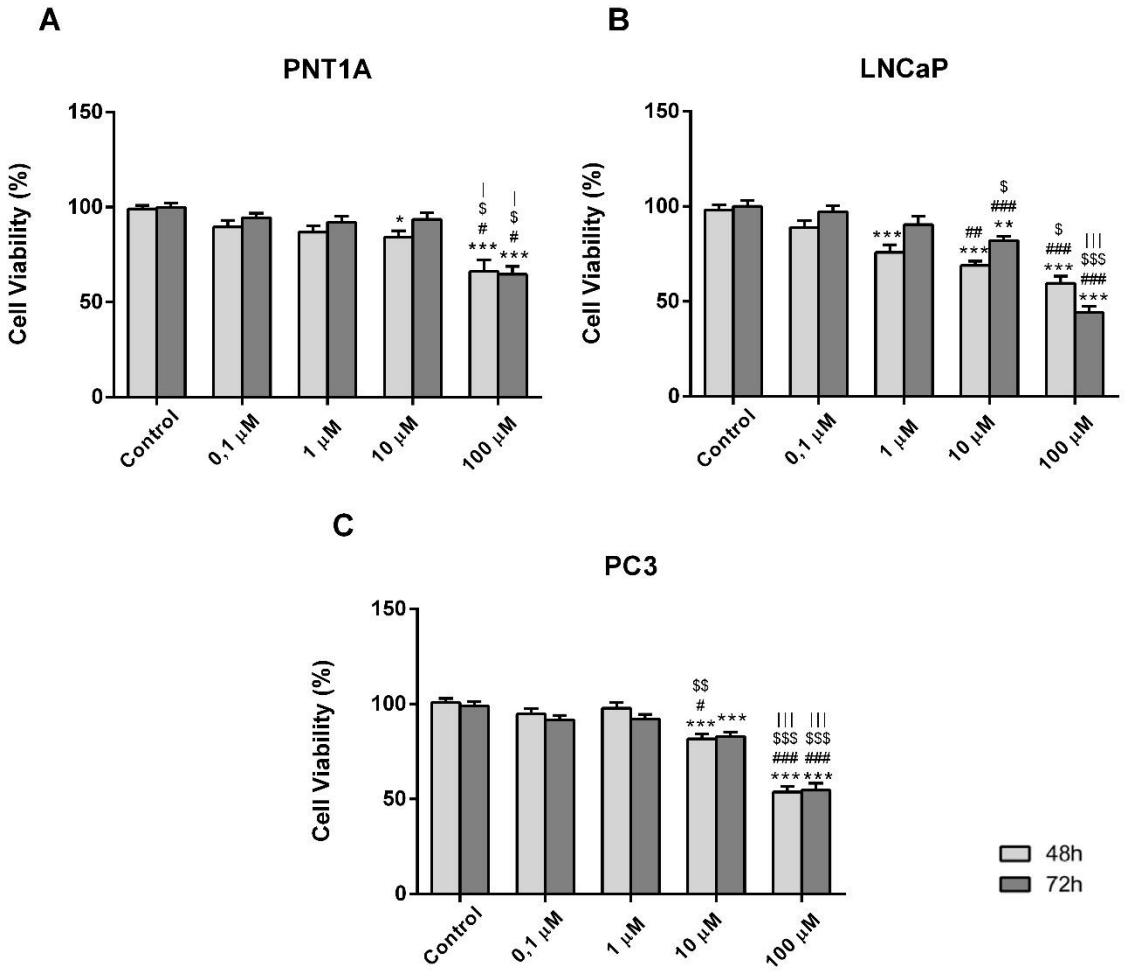

Figure 11. Percentage of viable non-neoplastic, PNT1A (A), and neoplastic, LNCaP (B) and PC3 (C) human prostate cells after exposure to several concentrations of MXC (0.1, 1, 10 and 100 μM) for 48 and 72 hours evaluated by the MTT assay. Results are expressed as % of control group. Error bars indicate mean ± S.E.M (n≥6). * p<0.05; ** p<0.01; *** p<0.001 when compared with the control group. # p<0.05; ## p<0.01; ### p<0.001 when compared with 0.1 μM-treated group. $ p<0.05; $$ p<0.01; $$$ p<0.001 when compared with 1 μM-treated group. | p<0.05; ||| p<0.001 when compared with 10 μM-treated group.

After analysis and integration of the obtained results on cell viability for the three human prostate cell lines under study, the 100 μM concentration of MXC and the incubation time of 48 hours were selected for the subsequent analysis of the influence of this compound on apoptosis and glycolytic metabolism.

2. Apoptosis mechanisms of human prostate cells are modulated by methoxychlor

The activity of caspase-3, a main indicator of apoptosis driven by both the intrinsic and extrinsic pathways (Fig.12 A), was evaluated in oth non-neoplastic PNT1A epithelial cells and neoplastic LNCaP and PC3 human prostate cancer cells through a biochemical assay (Fig. 12 B). In the case of PNT1A treated cells, the activity of caspase-3 was significantly decreased upon treatment with MXC (about 40% relatively to control, p=0.0005, Fig. 12). Oppositely, in LNCaP and PC3-treated cells, the activity of caspase-3 was significantly increased in response to MXC (over than 2.0-fold, p=0.0003 and 1.3-fold variation relatively to control, p=0.0058, respectively) (Fig. 12).

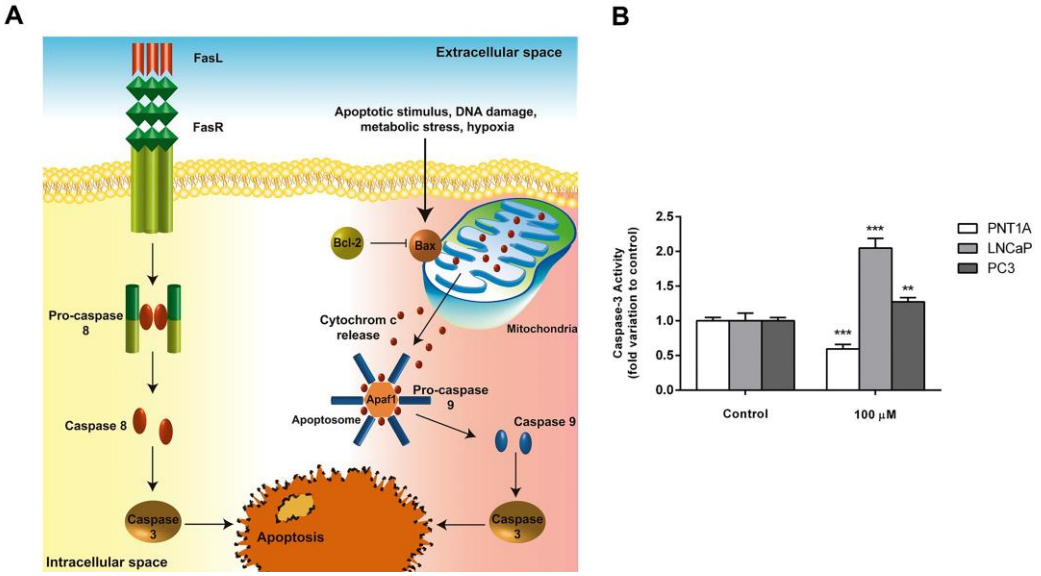

Figure 12. Apoptosis pathways (A) and activity of caspase-3 (B) in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after treatment with 100 µM of MXC for 48 hours, determined by a spectrophotometric assay kit. Errors bars indicate mean ± S.E.M (n=6). ** p<0.01; *** p<0.001. Caspase-3 can be activated by internal or external stimuli, activating the intrinsic or mitochondrial pathway, or by several receptors at cell membrane, activating the activating extrinsic pathway. (A) In the extrinsic pathway of apoptosis, the activation of death receptors at cell membrane (Fas R) will lead to the cleavage of pro-caspase 8 into its active form caspase-8, that is the executioner caspase of this pathway. In turn, caspase-8 will induce the activation of caspase-3, which is recognized as the end point of apoptosis. In the intrinsic pathway, several stimuli such as DNA damage and metabolic stress are responsible for the activation of proapoptotic protein Bax by inhibiting the anti-apoptotic Bcl-2, which induces the release of cytochrome c from the mitochondria. This results in the formation nof the apoptosime and activation of caspase-9, the executioner caspase of the intrinsic pathway, which will activate caspase-3 promoting apoptosis (adapted from (254)).

The expression of the pro-apoptotic protein Bax was determined in all cells lines under study (Fig. 13A). Following the results obtained on caspase-3 activity, Bax expression was significantly decreased in PNT1A-treated cells (~ 50% reduction in the MXC group vs. control, p=0.0257, Fig. 13A) and in PC3-treated cells (~ 40% reduction in the MXC group vs. control, p=0.0338, Fig. 13A). In the case of LNCaP-treated cells, despite the significantly increased

caspase-3 activity, no significant alterations were perceived on the expression of Bax protein when compared with the non-treated group (p=0.6134, Fig. 13A).

The expression of Bcl-2, an anti-apoptotic protein known to supress the activity of Bax, was also measured. In PNT1A- and PC3-treated cells, the expression of this anti-apoptotic protein was significantly decreased (approximately 43% of reduction, p=0.0415 and over than 50% of reduction, p=0.0048, respectively, Fig. 13B) when compared with untreated groups. Concerning the MXC-treated LNCaP cells, the expression of Bcl-2 was slightly increased with no significance (p=0.5085, Fig. 13B) when compared with control.

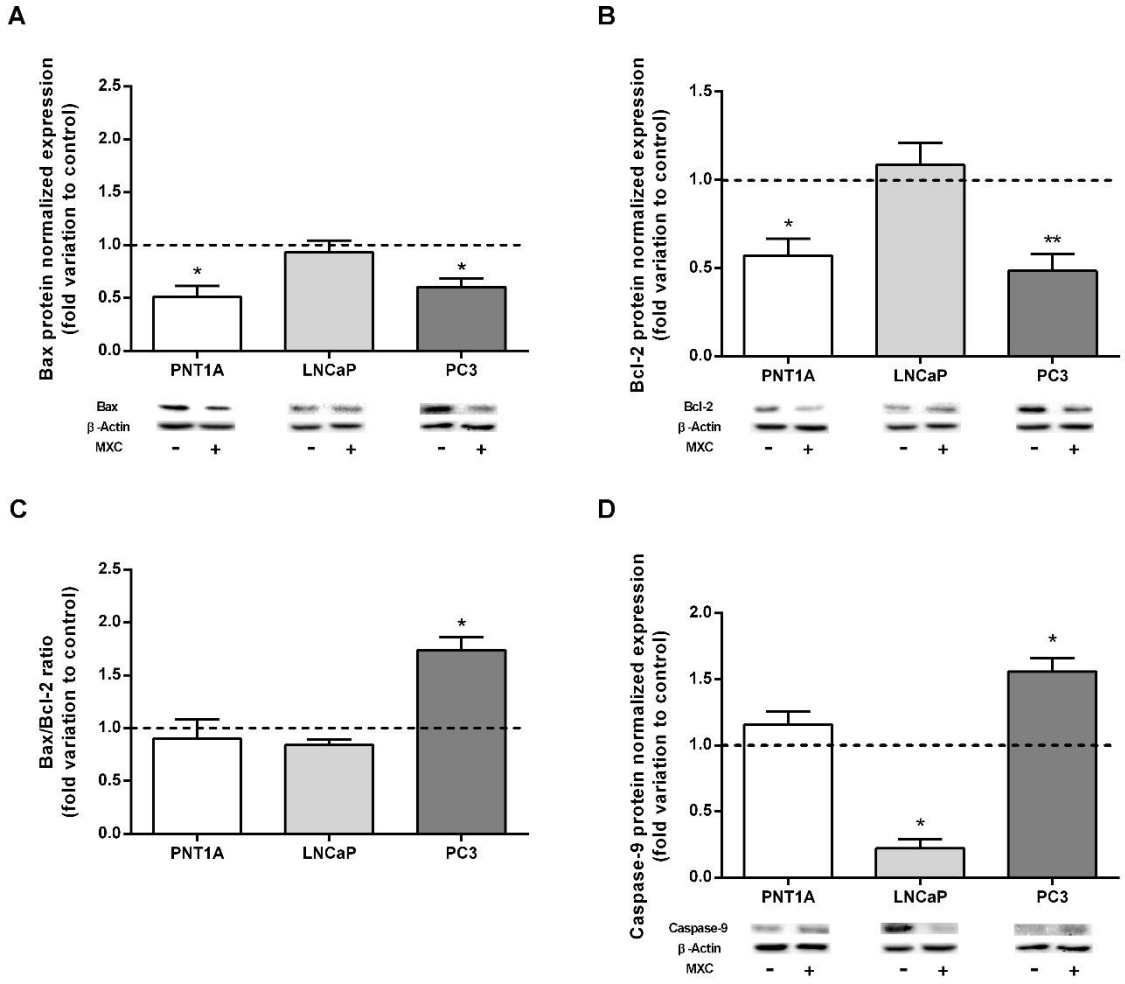

Figure 13. Expression of proteins associated with the intrinsic pathway of apoptosis, pro-apoptotic protein Bax (A), anti-apoptotic protein Bcl-2 (B), Bax/Bcl-2 ratio (C) and the initiator caspase-9 (D) in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after stimulation with 100 µM of MXC for 48 hours, obtained by Western blot analysis after normalization with ß-actin. Results are expressed as fold-variation comparatively to control (dashed line). Error bars indicate mean ± S.E.M (n=6). * p<0.05; ** p<0.01. Representative blots are shown below the respective graph.

After analysis of the expression of Bax and Bcl-2 apoptotic markers, the Bax/Bcl-2 ratio, used as an indicator of a pro- or anti-apoptotic cell state was calculated (Fig. 13C). Regarding PNT1A and LNCaP-treated cells, the Bax/Bcl-2 ratio was slightly decreased with no significant alterations (p=0.6567 and p=0.0550, respectively, Fig. 13C) when compared with the non-exposed group. Finally, the Bax/Bcl-2 ratio was significantly increased (over than 1.7-fold, p=0.0276, Fig 13C) in MXC-stimulated PC3 cells when compared with untreated group.

Lastly, quantification of the expression of caspase-9 active form, the initiator caspase associated with the intrinsic pathway of apoptosis was also performed. Exposure of PNT1A cells to MXC showed no significant alterations in the expression of caspase-9 (p=0.3966, Fig. 13D) when compared to the non-treated group. In the case of LNCaP-treated cells, MXC caused a significant decrease in caspase-9 expression, approximately 78% of reduction relatively to control group (p=0.0131, Fig. 13D). Accordingly with the observed increase in caspase-3 activity, exposure of neoplastic PC3 cells to MXC induced a significant increase in caspase-9 expression, approximately 1.56-fold variation relatively to control (p=0.0355, Fig. 13D).

The expression of FAS receptor (FasR) and Fas-ligand (FasL), the two proteins that trigger the activation of the extrinsic pathway of apoptosis, were also quantified (Fig. 14). In the case of PNT1A-treated cells, FasR expression was significantly decreased (~ 60% of reduction, p=0.0193, Fig. 14A) when compared with non-treated group. On the other hand, despite the decreased observed in expression of FasR in PC3-stimulated cells, no significance was found when compared to control group (p=0.1121, Fig. 14A). Concerning LNCaP-treated cells, no alterations were obtained in the expression of FasR when compared with the untreated group (p=0.7845, Fig. 14A).

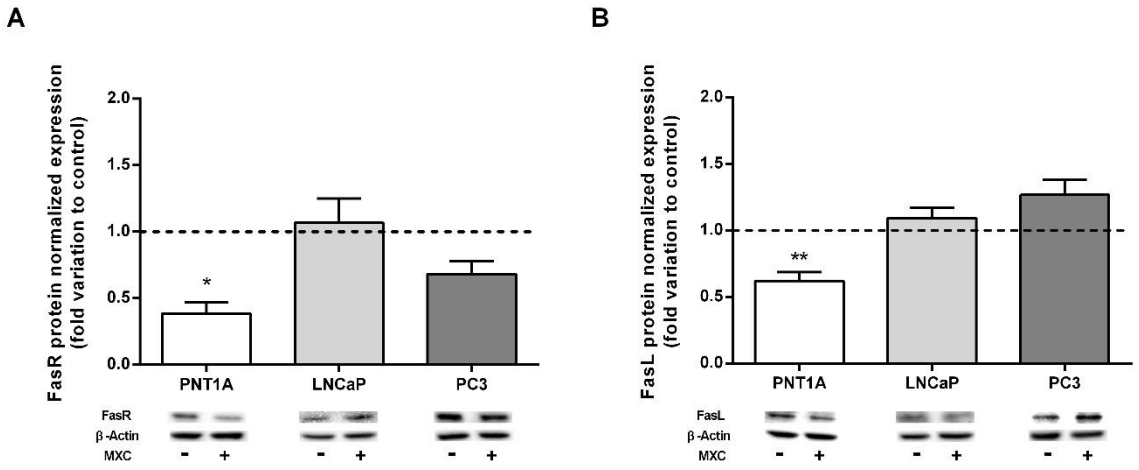

Figure 14. Expression of proteins involved in the extrinsic pathway of apoptosis, FasR (A) and FasL (B), in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after stimulation with 100 μM of MXC for 48 hours, obtained by Western blot analysis after normalization with ß-actin. Results are expressed as fold-variation comparatively to control (dashed line). Error bars indicate mean ± S.E.M (n=6). * p<0.05; ** p<0.01. Representative blots are shown below the respective graph.

Regarding FasL, PNT1A-treated cells demonstrated a significantly decreased expression of this apoptosis marker (approximately 38% of reduction, p=0.0089, Fig. 14B) when compared with control group. The expression of FasL in LNCaP- and PC3-treated cells was slightly increased, however, no significance was observed relatively to the untreated groups (p=0.3526 and p=0.1250, respectively, Fig. 14B).

Finally, the expression levels of the pro-apoptotic tumour suppressor p53 protein were also measured in non-neoplastic PNT1A cells and neoplastic LNCaP cells over MXC-treatment. The expression of p53 showed no alterations in MXC-treated PNT1A cells when compared with control group (p=0.2248, Fig. 15). Contrastingly, the expression of this marker was significantly increased in LNCaP cells stimulated with MXC (over than 3.0-fold variation, p=0.0047, Fig. 15) when compared with untreated group.

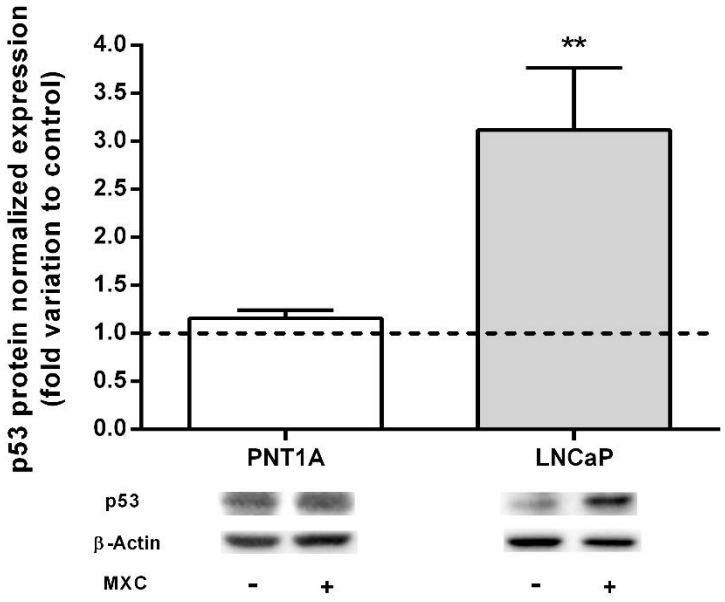

Figure 15. Expression of tumour suppressor p53 protein, in non-neoplastic PNT1A cells and neoplastic LNCaP human prostate cells after stimulation with 100 μM of MXC for 48 hours, obtained by Western blot analysis after normalization with β-actin. Results are expressed as fold-variation comparatively to control (dashed line). Error bars indicate mean ± S.E.M (n=6). ** p<0.01. Representative blots are shown below the respective graph.

3. Glycolytic metabolism of human prostate cells is stimulated by methoxychlor

The glucose consumption and lactate production by non-neoplastic, PNT1A, and neoplastic, LNCaP and PC3, human prostate cells were measured through biochemical spectrophotometric assays. In all cell line models of human prostate, a significant increase in glucose consumption was observed in MXC-treated cells when compared with the control non-treated cells (Fig. 16A). 100 μM of MXC for 48 hours of treatment augmented glucose consumption in PNT1A cells by approximately 4.4-fold variation relatively to control ($p<0.001$, Fig. 16A). Also, in LNCaP and PC3 cells a notorious increase in glucose consumption was observed after exposure to 100 μM MXC for 48 hours (approximately 3.83-fold and 2.64-fold variation relatively to control, respectively; $p<0.001$, Fig. 16A). Following the increase in glucose consumption also the lactate production was significantly augmented in the three human prostate cell lines (Fig. 16B). Lactate production was significantly increased in PNT1A cells after the treatment with the MXC by approximately 3.53-fold variation relatively to control group ($p<0.001$, Fig. 16B). Concerning LNCaP cells, a remarkable augment in lactate production was verified after stimulation with MXC (almost 9.5-fold variation relatively to control, $p<0.001$, Fig. 16B). PC3 cells also displayed a significantly increased lactate production after being exposed to MXC (nearly 2.75-fold variation relatively to control, $p<0.001$, Fig. 16B).

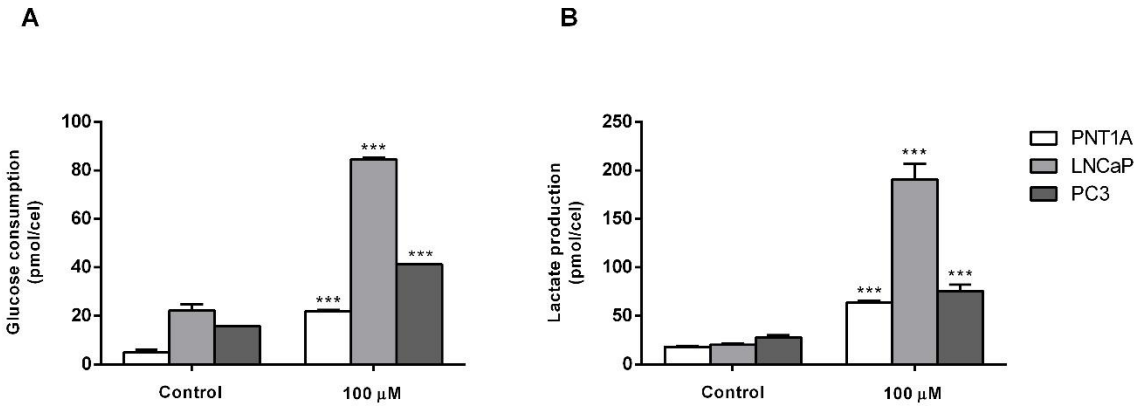

Figure 16. Glucose consumption (A) and lactate production (B) in non-neoplastic PNT1A epithelial cells and neoplastic LNCaP and PC3 human prostate cells after treatment with 100 μM of MXC for 48 hours, obtained by spectrophotometric assays. Errors bars indicate mean ± S.E.M (n=6). *** $p<0.001$ comparatively with the respective controls.

In accordance with the above-exposed results of glucose consumption, altered expression of GLUT1, GLUT2, and GLUT3 was observed in human prostate cell lines after treatment with MXC (Fig. 17). The expression of GLUT1 was significantly increased in both non-neoplastic PNT1A cells (2-fold variation relatively to control, p=0.0248, Fig. 17A) and neoplastic PC3 cells (approximately 1.3-fold variation relatively to control, p=0.0274, Fig. 17A) treated with the MXC. In LNCaP cells stimulated with MXC, no significant difference was observed in GLUT1 expression (p=0.9211, Fig. 17A) when compared to the control group. Concerning GLUT2 expression, a significant decrease was found in LNCaP cells exposed to the MXC (approximately 60% relatively to control, p=0.0453, Fig. 17B). No significant alterations were observed on GLUT2 expression in both PNT1A (p=0.4674, Fig. 17B) and PC3-treated cells (p=0.4622, Fig. 17B) when compared to non-treated groups. In what concerns GLUT3 expression, it was augmented in all human prostate cell lines after MXC treatment, though, significant differences were only observed in neoplastic LNCaP (~1.5-fold variation relatively to control, p=0.0478, Fig. 17C) and PC3 (~1.4-fold variation relatively to control, p=0.0271, Fig. 17C) cells. In the case of PNT1A-treated cells, no significant alterations were found in comparison with the control group (p=0.1724, Fig. 17C).

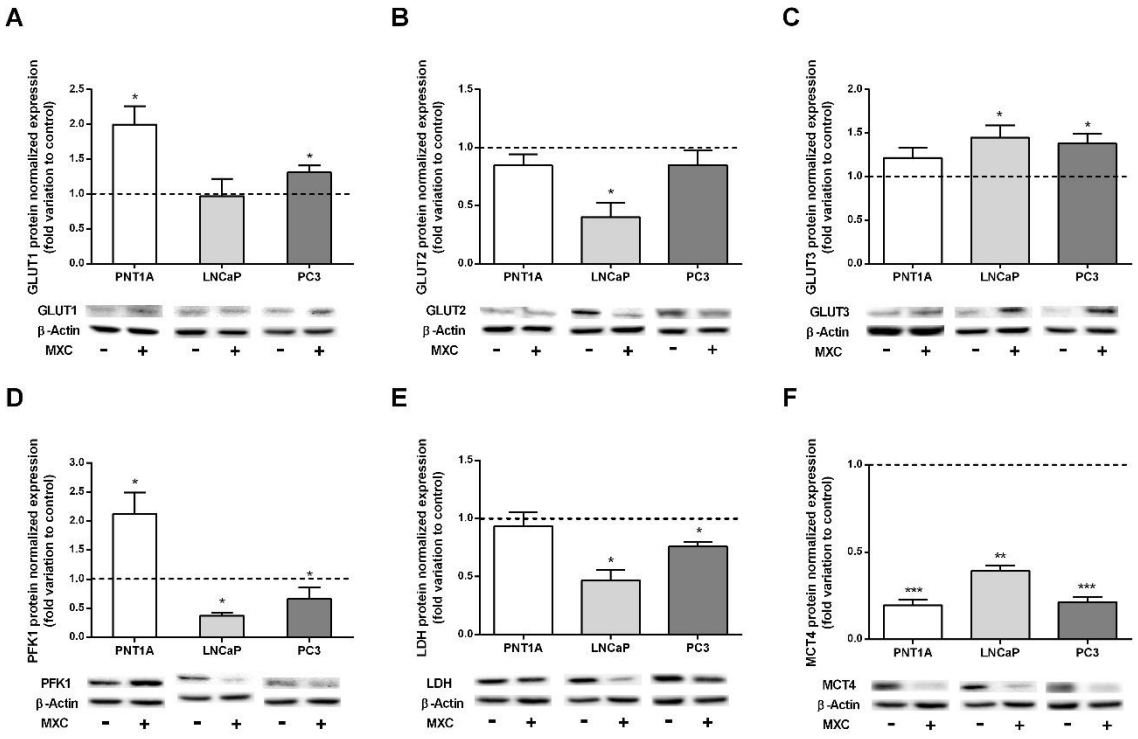

Figure 17. Expression of metabolism-associated proteins, glucose transporters GLUT1 (A), GLUT2 (B) and GLUT3 (C), glycolytic-associated enzymes, PFK1 (D) and LDH (E), and lactate exporter MCT4 (F) in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after stimulation with 100 μM of MXC for 48 hours, obtained by Western blot analysis after normalization with ß-actin. Results are expressed as fold-variation comparatively to control (dashed line). Error bars indicate mean ± S.E.M (n=6). * p<0.05; ** p<0.01; *** p<0.001. Representative blots are shown below the respective graph.

To evaluate the metabolization of the internalized glucose, the expression of PFK1 was analysed, which showed significant and distinct differences in response to MXC in the cell line models of human prostate. In PNT1A cells stimulated with 100 μM of MXC for 48 hours, a significant increase in the expression of this enzyme was observed (over than 2.0-fold variation relatively to control, p=0.0415, Fig. 17D). On the other hand, the expression of PFK1 in MXC-treated LNCaP and PC3 cells was significantly decreased compared with the respective control groups (~ 63%, p=0.0184 and ~ 34%, p=0.0331 reduction, respectively) (Fig. 17D).

The final product of glycolysis pyruvate can be converted to lactate by the activity of LDH. The expression of this enzyme was significantly decreased in both LNCaP- (approximately 53% relatively to control, p=0.0121, Fig. 17E) and PC3-treated cells (approximately 25% comparatively to control, p=0.0203, Fig. 17E). In the case of PNT1A cells treated with MXC, no significant alterations (p=0.7082, Fig. 17E) were obtained when compared with the non-treated group. Despite the diminished LDH expression observed in the neoplastic LNCaP and PC3 cells, the activity of this enzyme was clearly increased (Fig. 18). LNCaP and PC3 cells treated with 100 μM MXC showed nearly 1.85-fold (p=0.0201) and 2.3-fold (p=0.0030) increased activity of LDH, respectively (Fig. 18). PNT1A-treated cells also displayed increased LDH activity relative to the control, though not statistically significant (p=0.0768, Fig. 18).

Lastly, the expression of MCT4, the most important exporter of lactate to the extracellular space, in non-neoplastic and neoplastic human prostate cell lines also was evaluated. MCT4 expression was significantly decreased in all prostate cell lines (Fig. 17F); PNT1A (approximately 80% reduction, p<0.001), LNCaP (nearly 61% reduction, p<0.001) and PC3 cells (about 79% reduction, p<0.001) displayed a marked decreased of MCT4 expression after stimulation with MXC in comparison with the respective control groups (Fig. 17F).

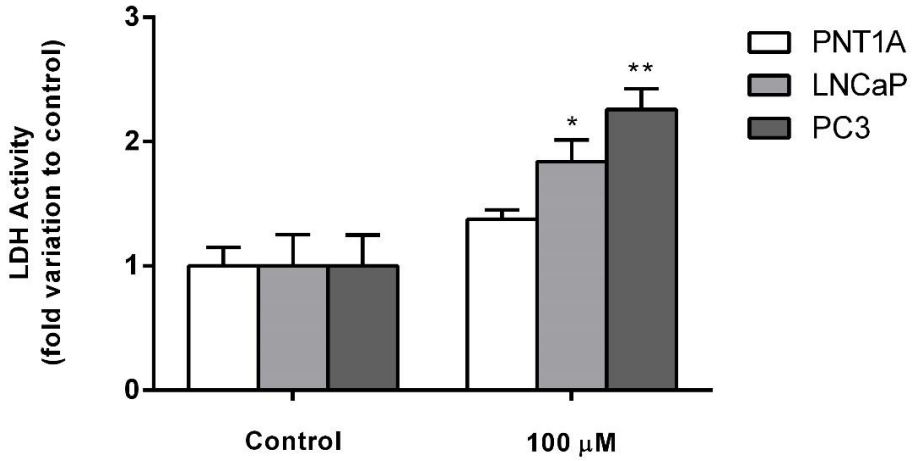

Figure 18. LDH enzymatic activity in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after treatment with 100 μM of MXC for 48 hours, determined by spectrophotometric assay. Results are expressed as fold-variation comparatively to control. Error bars indicate mean ± S.E.M (n=6). * p<0.05; ** p<0.01.

V. DISCUSSION AND CONCLUSION

The present dissertation investigated the effect of MXC, an endocrine-disrupting chemical, in the proliferation, apoptosis and glycolytic metabolism of non-neoplastic, PNT1A, and neoplastic, LNCaP and PC3, human prostate cell lines. PNT1A cells are immortalized cells representative of a non-neoplastic prostatic epithelium, while LNCaP and PC3 cells represent different stages of metastatic prostate cancer, androgen-dependent and androgen-independent, respectively. These cell line models have been widely used to study the behaviour of prostate cancer cells, including the evaluation of cytotoxic effects by different chemicals, the resistance to apoptosis and the alterations in glycolytic metabolism.

Firstly, the analysis of the results obtained in the MTT proliferation assays allowed to conclude that, generally, all the cell lines under study showed decreased proliferation in response to MXC-treatment, in a concentration and time-dependent manner (Fig. 11). This behaviour follows the observations in the literature in similar study cases using other EDCs with estrogen-like properties (255-258). However, at least for our knowledge, this is the first report evaluating the effects of MXC in prostate cancer cells.

Nevertheless, there were notorious differences on the effect of MXC influencing the viability of neoplastic and non-neoplastic prostate cells. LNCaP cells were the most affected by MXC showing the more pronounced reduction in cell viability, followed by PC3 cells and, lastly, by the non-neoplastic PNT1A cells (Fig. 11). These differences might be explained by the cell division and metabolic activity rates, that normally are higher in cancer cells, which may lead to an increased effect of MXC diminishing cell viability (259, 260).

After integrating all the information on the effects of MXC on cell viability, the concentration of 100 μM of MXC and the exposure time for 48 hours were selected for analysis of apoptosis and metabolism of non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 cells.

Over the last decades, fundamental research has produced enormous advances in cancer biology. Among these advances, one of the most important was the discovery of genes that control apoptosis and its effects on the establishment of the malignant phenotype. It is well known that some oncogenic mutations disrupt apoptosis, leading to tumour initiation and progression. On the other hand, evidence indicates that other changes in oncogenes can promote apoptosis and produce a selective pressure to override apoptosis in several stages of carcinogenesis (261).

The caspases enzymes are homologous cysteine-dependent cell death proteases and the main executioners of apoptosis (262). They can be activated by internal or external stimuli or by several receptors at cell membrane. Independently of the stimuli, the effectiveness of apoptosis relies on the activation of caspase-3, which is recognized an end point of apoptosis (263).

Other important regulators of apoptosis are the Bcl-2 protein family members. Moreover, the ratio of proapoptotic Bax/antiapoptotic Bcl-2 proteins has been used as an indicator of the cell propensity to apoptosis, and as a useful prognostic marker in cancer (264, 265). A

higher value of this ratio, normally, represents enhanced activity of some members of caspase family, which leads to an increased apoptosis (266).

p53 is a tumour suppressor protein that acts as a transcription factor regulating the expression of downstream genes that control cell cycle and apoptosis, which includes Bax and Bcl-2 (267, 268).

The decreased viability of PNT1A-, LNCaP- and PC3-treated cells determined by the MTT assay, was followed by the altered expression and activity of some regulators of apoptosis, associated both with the extrinsic and intrinsic pathways; generally confirming the results obtained in the MTT assays (Fig. 13, 14 and 15).

In the case of PNT1A cells, despite the decrease in cell viability in response to MXC treatment, the activity of caspase 3, was significantly decreased (Fig. 12). Concerning the expression of apoptosis regulators Bax and Bcl-2 expression, both were significantly decreased in PNT1A-treated cells (Fig. 13A and B). Thus, no significant alterations were observed in the Bax/Bcl-2 ratio, as well as in the expression of caspase-9, the initiator caspase associated with the intrinsic pathway of apoptosis (Fig. 13C and D). We also evaluated the expression of modulators of the extrinsic pathway of apoptosis, namely the FasR and FasL (Fig. 14). Both FasL and FasR expression levels were significantly reduced in the MXC-treated PNT1A cells, which indicates less activity of the extrinsic pathway and might explain the reduced activity of caspase-3. Despite increased expression of the apoptosis inducer and cell cycle regulator p53 has been described in PNT1A cells treated another EDC with estrogen properties, the nonylphenol (269), no significant differences were perceived in response to MXC (Fig. 15). Altogether, the results gathered in the apoptosis-related proteins indicate lower apoptotic rate in MXC-treated PNT1A cells.

Concerning LNCaP cells, and in agreement with the decreased cell viability verified in the MTT assay, the activity of caspase-3 was significantly increased in response to MXC (Fig. 12), which is indicative of augmented apoptosis. However, the analysis of the apoptosis markers of the intrinsic pathway showed no alterations in the expression levels of Bax and Bcl-2; thus, Bax/Bcl-2 ratio also was unaltered (Fig. 13A, B and C). Moreover, the expression of caspase-9 is decreased in MXC-treated cells (Fig. 13D). In the case of extrinsic pathway, the expression of both FasR and FasL was also unaltered in the LNCaP-treated cells (Fig. 14). Lastly, the most notorious alteration in these cell line after stimulation with MXC was the remarkable increase in p53 expression (Fig. 15). A possible explanation for the increased activity of caspase 3, despite the reduced activity of caspase 9, and the unchanged expression levels of Bax and Bcl-2 expression, is the fact that there are several other pro-apoptotic and anti-apoptotic proteins that are controlled by p53 (270, 271). p53 is known to regulate other pro-apoptotic proteins such as PUMA and Noxa that bind Bcl-2 and active proapoptotic Bax, which might be underpinning the increased caspase-3 activity in LNCaP-treated cells (270, 271). Also, it has been shown that other cell death receptors, namely, the death receptor 5 (DR5) also known as TNF-related apoptosis-inducing ligand (TRAIL) receptor 2, are controlled by p53 (272-274). Since FasR showed no alteration in MXC-treated LNCaP

cells, it is possible to consider that p53 can be activating another cell-death receptor responsible for the observed increase in the activity of caspase-3. This assumption would be confirmed by evaluating the expression of caspase-8, which is a target of cell-death receptors and its activation culminates with the activation of caspase-3.

Finally, regarding PC3-treated cells, the diminished cell viability obtained in MTT assay was followed by the significant increase in the activity of caspase-3 (Fig. 12). Moreover, the Bax/Bcl-2 ratio was clearly increased (Fig. 13C), as well as, the expression of caspase-9 (Fig. 13D). Concerning the extrinsic pathway of apoptosis, no significant differences were observed both in FasR and FasL expression levels in treated cells (Fig. 14). Integrating the obtained results, we can conclude that MXC treatment stimulated the intrinsic pathway of apoptosis in PC3 cells, leading to increased activity of caspase-3, and overall supporting increased apoptosis in the presence of this EDC. These findings are also in concordance with the available literature. PC3 cells treated with diethylstilbestrol, another synthetic EDC that mimics estrogens, showed augmented apoptosis (257).

One of the emerging hallmarks of cancer is the reprogramming of cell metabolism, which has been recognized, in the last years, as an interesting point for development of novel therapeutic approaches for cancer treatment (275, 276). It is well-known that to satisfy their energy necessities, cancer cells use an accelerated glycolysis rate, which allows maintaining a high rate of proliferation and an increased synthesis of macromolecules (277, 278). In order to achieve energy in a faster way, cancer cells prioritize the anaerobic pathway, even in normal oxygen conditions, which will result in an increased production of lactate, a metabolite that acidifies the tumour microenvironment enhancing the migration and invasion of cancer cells (111, 279).

Multiple molecular mechanisms converge to alter cell metabolism and provide support for the energetic needs of cancer dividing cells. In consequence of the acquisition of gain- or loss-of-function mutations in oncogenes and tumour suppressor genes, respectively, several signalling pathways are overactivated in cancer cells. This includes the PI3K, HIF, tumour suppressor p53, MYC and AMPK pathways, which have been shown to drive the metabolic alterations associated to the metabolic reprogramming of cancer cells (129). In hormone-dependent cancers, such as breast and prostate cancer, the steroid hormones have also been identified as important modulators of cancer cells metabolism (251, 280-282).

Recent findings of our research group and others have demonstrated the distinct metabolic profile of neoplastic LNCaP and PC3 cells relatively to the non-neoplastic PNT1A cells, as well as the ability of androgens, the master stimulators of prostate cancer, modulating glucose handling and lactate production (35, 251, 283). With the objective of evaluating the likely potential of MXC causing development and progression of prostate cancer, the glycolytic metabolism of PNT1A, LNCaP and PC3 cells was studied.

Firstly, a significant increase in glucose consumption was observed both in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 cells treated with 100 μM MXC for 48 hours (Fig. 16A), which was accompanied by the increased expression of GLUTs (Fig. 17). In

PNT1A-treated cells, GLUT1 expression levels were significantly increased, whereas LNCaP cells displayed increased expression of GLUT3 in response to MXC. PC3-treated cells presented a notorious and significant increased expression of both glucose transporters GLUT1 and GLUT3, which supports the increase in glucose consumption. On other hand, the expression of GLUT2 showed a tendency to be decreased in all cell lines, but only presented a significant reduction in LNCaP-treated cells. GLUT2 is a GLUT family member more associated with highly glycolytic cells and glucose uptake by liver, intestine and kidney cells (284, 285). In the case of prostate cells, GLUT1 and GLUT3 are the most well-characterized transporters and have been considered as the responsible for glucose uptake in cancer cells (286, 287). Therefore, the higher expression of GLUT1 and GLUT3 might imply a higher glucose intake and explains the increased glucose consumption seen in prostate cells even when GLUT2 expression is diminished.

Following the analysis of glycolytic process, the expression of PFK1, an important enzyme that catalyses a limiting step of glycolysis, the conversion of F-6-P into F-1,6-P, was evaluated. A distinct response was observed concerning the expression of PFK1 in MXC-treated PNT1A, LNCaP and PC3 cells (Fig. 17D). In the case of PNT1A cells, PFK1 expression was increased in response to MXC, which is in concordance with the observed augmented glucose consumption and lactate production (Fig. 16). The augmented expression of PFK1 would signify a cell increased capacity to produce pyruvate via the glycolytic pathway and its conversion to lactate, which is demonstrative of the stimulation of this energy route. However, an opposite effect was observed in LNCaP- and PC3-stimulated cells, which displayed diminished expression of PFK1, despite the augmented glucose consumption and lactate production (Fig. 16).

After the limiting step under the responsibility of PFK1, the glycolytic metabolism continues with a series of reactions that will culminate with the production of pyruvate, which is then transported to the mitochondria or converted into lactate. The latter case, though less efficient energetically is faster in the production of ATP and is the preferred metabolic route of rapidly-dividing cells including cancer cells. The activity of LDH ensures the conversion of pyruvate into lactate, and the produced lactate is then exported into the extracellular space by the MCTs, namely by MCT4 (Fig. 8) (288, 289).

The results obtained in response to the administration of MXC showed that exposure to this EDC, increased the activity of LDH in both neoplastic LNCaP and PC3 cells (Fig. 18), despite the fact that its expression levels were diminished. As discussed-above, the increased activity of LDH is indicative that MXC stimulates the glycolytic pathway in prostate cancer cells, and supports the increased lactate export observed in MXC-treated cells. However, PFK1 expression was diminished in LNCaP and PC3 cells treated with MXC indicating that less pyruvate might being produced. A possibility to explain these findings is that an increased activity of the LDH enzyme results in a higher lactate turnover despite the lower pyruvate production, and this lactate overproduction via pyruvate is a typical event in neoplastic cells. Also, it cannot be excluded from the discussion that additional pyruvate is being generated by

other metabolic routes, namely, glutaminolysis. This metabolic pathway encompasses cleavage of glutamine into glutamate, resulting in the production of pyruvate via citric acid cycle; pyruvate can leave mitochondria and generate lactate (290). Moreover, the activity of the enzyme alanine transaminase (ALT), which catalyses the reversible reaction that converts alanine into pyruvate could also contribute to the production of pyruvate, which in turn by the action of LDH can be converted to lactate. Finally, we must consider the hypothesis that despite PFK1 expression is diminished, its activity might be increased, and this can also explain the increased production of lactate. In the case of the non-neoplastic PNT1A cells, the increased lactate production upon MXC treatment is supported by the expect enhanced pyruvate production in consequence of increased expression levels of PFK1 and, though not followed by the altered expression or activity of LDH.

Concerning MCT4, its expression levels were clearly diminished after MXC-stimulation of all cell lines, non-neoplastic and neoplastic (Fig. 17F). As mentioned earlier, MXC stimulated the glycolytic pathway, as indicated by the enhanced glucose consumption and lactate production, which was supported by the increased expression of GLUTs and LDH activity. The down-regulated expression of MCT4, not supportive of the enhanced glycolytic metabolism and augmented lactate production, lead us to consider the possibility that other lactate exporter MCT family member would be involved, specifically MCT1. This is further sustained by the reports showing that MCT1 is expressed in both non-neoplastic and neoplastic prostate cell lines, as well as its capacities in the import and export of lactate in cancer cells (291, 292). The export of lactate by cancer cells is thought to be essentially mediated by both MCT1 and MCT4 since these are MCTs usually upregulated in cancers. However, in MCF7 breast cancer cells, MCT1 was shown as the main transporter involved in lactate handling, since its inhibition induced cell death through disruption of lactate export, glycolysis and glutathione synthesis (293). Moreover, MCT1 expression was shown to be elevated in glycolytic tumours, and high MCT1 expression predicts poor prognosis in breast and lung cancer patients (294, 295).

In conclusion, the results obtained in the present dissertation showed that MXC diminished the viability of both non-neoplastic and neoplastic prostate, but with a distinct response in the apoptotic cell death. The rate of apoptosis was augmented in LNCaP and PC3 cells after treatment with MXC whereas in PNT1A cells apoptosis was diminished. The decrease in apoptosis is a feature that has been linked to malignant transformation (103), which indicates that this EDC can modulate the phenotype of human prostate epithelial cells towards carcinogenesis. Moreover, it was shown that MXC disrupts the metabolism of PNT1A, LNCaP and PC3 cells stimulating the glycolytic pathway. The increase in the glycolytic flux, resulting in a higher production of lactate, alters tumour microenvironment and has been recognized as a critical aspect in initiation and progression of cancers (296). Therefore, it is liable to assume that the enhanced glycolytic metabolism observed in PNT1A cells after MXC treatment, may in some way, stimulate the malignant transformation of prostate cells. It is also known that lactate production contributes to acidification of the tumour

microenvironment, a feature that has been shown to allow the migration of cancer cells, favoring invasion and metastization (296). In addition, the lactate in the tumour microenvironment also has been shown to play a relevant role suppressing the immune system, which favors tumour progression. Following this rationale, the observed stimulatory effects of MXC over the glycolytic pathway of neoplastic prostate cells indicate that this EDC could prone the progression of prostate cancer. The increased apoptosis obtained might be a selective pressure to override apoptosis during prostate carcinogenesis.

The findings of this dissertation corroborate the existing evidence indicating the harmful effects of EDCs in general and MXC in particular, as well as, their actions as carcinogens. Also, the results obtained alert for the concern of using these chemicals and the need of defining the time points, exposure routes and at which concentrations safety is guaranteed. Moreover, apoptosis and metabolic alterations are important biological processes at the interplay of other human diseases, and the results obtained herein also highlight the impact of MXC and its side effects for example in diabetes, obesity, and infertility.

VI. FUTURE PERSPECTIVES

The present dissertation demonstrated that MXC diminished the viability of both non-neoplastic and neoplastic human prostate cells whereas stimulating its glycolytic metabolism. The metabolic alterations observed were sustained by the altered expression of GLUTs and LDH activity but in the case of PFK1 expression the results were not entirely coherent with the enhanced glycolytic flux. So, it is pertinent to confirm if PFK1 activity is augmented despite its diminished expression. Another interesting approach would be the investigation of the alternative sources of pyruvate that could compensate the diminished expression of PFK1, such as glutaminolysis and the alanine cycle. For example, analyzing the activity of ALT could be indicative that other metabolic pathways are generating pyruvate and contributing to the enhanced glycolytic profile.

Moreover, it was shown that MXC suppressed apoptosis in the non-neoplastic prostate cells, which can be a relevant step towards carcinogenesis. This decrease in apoptosis occurred through the reduction of FasL and FasR, modulators of the extrinsic pathway of apoptosis. However, it would be interesting to confirm whether the expression of caspase-8 is also reduced and if other receptors besides FasR, for example DR5, could be contributing to the suppressed apoptosis in PNT1A cells.

In a near future, it would also be interesting to study the effect of MXC in non-neoplastic human prostate epithelial cell lines over longer periods of exposure and at lower concentrations, evaluating their metabolic profiles, rate of proliferation and apoptosis, as well as, the epithelial-mesenchymal transition. This complementary strategy to the present study, importantly, will assess the effects of this compound in a long-term exposure, which is an experimental design closer to the reality since exposure to this type of chemicals usually occurs on a constant and prolonged base.

Another interesting approach would be the study of the *in vivo* effects of MXC. After the study performed that contributed to disclose the effects of MXC *in vitro* using human prostate cell lines, it would be determinant to confirm if these effects are maintained or can be potentiated in *in vivo* models since MXC and other EDCs are partially metabolized in the liver before they can reach other cells. In the case of MXC, it is known that liver metabolization produces other metabolites with estrogen-like properties also, and in some cases with higher affinity for ERs than the MXC itself. Thus, it is also pertinent to evaluate whether these metabolites can synergize with MXC and enhance the carcinogenic effects of this compound.

Despite the conclusions that were drawn, the results obtained in the present thesis opened new perspectives of research to further explore the association of MXC with the development and progression of prostate cancer.

VII. REFERENCES

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