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Endocrine disruptors, obesity, and prostate cancer: cell fate analysis

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Dedicatória

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Resumo

O cancro da próstata (PCa) é um dos cancros mais comuns nos homens e a sua progressão e desenvolvimento estão intimamente relacionados com a ação dos androgénios. A obesidade, uma epidemia mundial do século XXI, tem também sido associada à agressividade e pior prognóstico do PCa. Por outro lado, os fatores extrínsecos demonstraram contribuir para o risco de desenvolvimento do PCa em mais de 99,9%, o que estimula o interesse em explorar o efeito de fatores ambientais no aparecimento e progressão do cancro. É o caso dos disruptores endócrinos, compostos que têm efeitos que mimetizam a ação das hormonas endógenas, interferindo na sua síntese, secreção e mecanismo de ação, e que têm sido associados ao desenvolvimento de alguns cancros. Alguns desreguladores endócrinos são designados obesogénios, uma vez que induzem desregulação hormonal interferindo com as vias bioquímicas que controlam o apetite e/ou toda a homeostase metabólica e processamento de lípidos, promovendo assim a obesidade. O tributilestanho (TBT) é um desregulador endócrino e obesogénio utilizado como biocida, o qual tem também atividade androgénica. Outros autores demonstraram que a desregulação induzida pelo TBT pode aumentar a atividade proliferativa das células de PCa, no entanto, sem relação com o contexto de obesidade e disponibilidade de lípidos. Neste estudo, hipotetizamos que o TBT pode afetar diferencialmente o destino das células PCa em condições de obesidade e não-obesidade, sendo avaliado o impacto na viabilidade, proliferação, migração e invasão das células da próstata na presença e ausência de colesterol LDL (*low-density lipoprotein*). Linhas celulares prostáticas, não neoplásicas (PNT1A) e neoplásicas (LNCaP e PC3) foram tratadas com diferentes concentrações de TBT durante 48 horas, e a viabilidade celular avaliada pelos ensaios MTT e SRB. Nas células LNCaP tratadas com 100 nM TBT, a proliferação celular foi avaliada pela marcação do Ki-67 por imunocitoquímica de fluorescência. A expressão das proteínas Akt, p-Akt, MAPK, p-MAPK e c-Myc foi avaliada por *Western blot* (WB). A migração e invasão celular foram avaliadas por ensaios com matrigel, e a expressão da proteína E-caderina foi analisada por WB. Estes ensaios foram repetidos para uma concentração de TBT 10 nM em meio enriquecido com colesterol LDL (100 μg / ml) e na presença de 5α-dihidrotestosterona (DHT, 10 nM). Os resultados obtidos demonstraram que o TBT (100 nM) aumenta a viabilidade das células LNCaP. Para além disso, as células LNCaP tratadas com TBT, apresentaram uma maior capacidade proliferativa, como indicado pelo aumento da marcação do Ki67, bem como da expressão da proteína c-Myc. O TBT também promoveu a migração e invasão celular, o que foi observado nos ensaios de matrigel e suportado pela diminuição da expressão da E-caderina. Finalmente, as células LNCaP tratadas com uma concentração mais baixa

de TBT (10 nM) na presença de LDL, demonstraram igualmente maior taxa de proliferação e capacidade de migração, o que foi ainda favorecido pela presença de DHT. De um modo geral, podemos concluir que o TBT tem a capacidade de alterar o comportamento das células de PCa, podendo contribuir para a progressão tumoral. De ressaltar que estes efeitos foram mantidos, mesmo em concentrações mais baixas de TBT, e parecem ser mais impactantes em condições de obesidade, o que alerta para o maior perigo de uma eventual exposição em doentes de PCa obesos.

Palavras-chave

Cancro da próstata; obesogene; TBT; proliferação; migração; invasão.

Resumo alargado

O cancro da próstata (PCa) é um dos cancros mais frequente nos homens e representa a quinta maior causa de morte por cancro na população masculina. Numa fase inicial da doença, as células tumorais são sensíveis aos androgénios, que estimulam a sua sobrevivência e proliferação. Assim sendo, as terapias de privação de androgénios são frequentemente utilizadas no tratamento do PCa. No entanto, na maioria dos casos, as células tumorais adaptam-se e adquirem resistência à terapia, conseguindo sobreviver e proliferar mesmo na ausência dos androgénios. Esta fase mais avançada do tumor é designada por cancro da próstata resistente à castração (CRPC, "castrate-resistant prostate cancer"), no qual as células adquiriram a capacidade de metastatizar para outros órgãos. De entre os fatores de risco que têm sido associados ao desenvolvimento do PCa destacam-se a idade, o histórico familiar, a etnia e os níveis de testosterona. No entanto, a obesidade, uma patologia crónica e complexa, parece estar fortemente associada com a agressividade e mau prognóstico do PCa. Para além disso, um estudo recente demonstrou que os fatores extrínsecos contribuem em cerca de 99,9% para o desenvolvimento do PCa. Nestes fatores extrínsecos estão incluídos o estilo de vida, fatores dietéticos associados ao desenvolvimento de obesidade, o consumo de álcool, a exposição a radiações ultravioletas ou mesmo a exposição a fatores ambientais. Um exemplo de fatores ambientais que podem estar relacionados com o desenvolvimento tumoral são os disruptores endócrinos, compostos que têm efeitos que mimetizam a ação das hormonas endógenas, interferindo na sua síntese, secreção e mecanismo de ação. Alguns desreguladores endócrinos são designados obesogénios, uma vez que têm a capacidade de aumentar o número de adipócitos e/ou armazenamento de tóxicos nos mesmos, alterar a quantidade de calorias armazenadas e/ou alterar os mecanismos moleculares através dos quais são regulados a saciedade e o apetite. Desta forma, para além de exacerbarem os efeitos dos hábitos diários, os obesogénicos induzem desregulações endócrinas que, por conseguinte, levam a severas disfunções metabólicas. De entre as várias substâncias que predispõem os indivíduos para o aumento de peso, destaca-se o tributilestanho (TBT), o obesogénio modelo, considerando mesmo um dos compostos mais tóxicos alguma vez introduzidos de forma deliberada no ambiente. Este composto era inicialmente utilizado apenas como algicida e moluscicida na indústria da navegação, no entanto chega hoje à população não só através da cadeia alimentar, mas também pelo contacto com o pó doméstico e produtos de preservação de madeiras. Diversos efeitos biológicos adversos em diferentes espécies têm vindo a ser atribuídos à contaminação por TBT, nomeadamente

ao nível do sistema reprodutor. A nível mecanístico parece atuar através da ativação do RXR ("Retinoid X Receptor") e PPARγ ("Peroxisome proliferator-activated receptor gama"), promovendo assim a adipogénese e o armazenamento dos lípidos. O TBT tem também como característica atividade androgénica, e foi previamente descrito o seu efeito no aumento da atividade proliferativa das células de PCa, no entanto, sem relação com o contexto de obesidade e disponibilidade de lípidos. Neste estudo, hipotetizamos que o TBT pode afetar diferencialmente o destino das células PCa em condições de obesidade e não-obesidade, sendo avaliado o impacto em diferentes marcos associados ao desenvolvimento tumoral, como a viabilidade, proliferação, migração e invasão das células da próstata na presença e ausência de colesterol LDL (low-density lipoprotein) e ou 5α-di-hidrostestosterona. Deste modo, diferentes linhas celulares prostáticas, não neoplásicas (PNT1A) e neoplásicas (LNCaP e PC3) foram tratadas com várias concentrações de TBT durante 48 horas, e a viabilidade celular avaliada pelos ensaios MTT e SRB. Nas células LNCaP tratadas com 100 nM TBT, a proliferação celular foi avaliada pela marcação do Ki-67 por imunocitoquímica de fluorescência. A expressão das proteínas Akt, p-Akt, MAPK, p-MAPK e c-Myc foi avaliada por Western blot (WB). A migração e invasão celular foram avaliadas por ensaios com matrigel, e a expressão da proteína E-caderina foi analisada por WB. Estes ensaios foram repetidos para uma concentração de TBT 10 nM em meio enriquecido com colesterol LDL (100 μg / ml) e na presença de 5α-dihidrotestosterona (DHT, 10 nM). Os resultados obtidos nesta dissertação demonstraram que o TBT em diversas concentrações aumenta a viabilidade das células LNCaP. Para além disso, as células LNCaP tratadas com TBT (100 nM) apresentaram uma maior capacidade proliferativa, podendo ser verificado pelo aumento da marcação do Ki67, bem como da expressão da proteína c-Myc. O tratamento com TBT também induziu um aumento na migração e invasão celular como observado nos ensaios de matrigel e suportado pela diminuição da expressão da E-caderina. Para além disso, o tratamento das células LNCaP com uma concentração mais baixa de TBT (10 nM) na presença de LDL, demonstraram igualmente maior taxa de proliferação e capacidade de migração, o que foi ainda favorecido pela presença de DHT. De um modo geral, os resultados obtidos nesta dissertação permitem-nos concluir que o TBT tem a capacidade de alterar o comportamento das células de PCa, aumentando a sua sobrevivência, capacidade proliferativa, de migração e invasão, podendo deste modo contribuir para a progressão tumoral. Estes efeitos foram mantidos, mesmo em concentrações mais baixas de TBT. Assim este composto poderá ser considerado um agente carcinogéneo no desenvolvimento do PCa. De salientar que esta influência no comportamento das células do PCa parece ser mais impactante em

condições de obesidade, o que alerta para o maior perigo de uma eventual exposição em doentes de PCa obesos.

Abstract

Prostate cancer (PCa) is one of the most common cancers in men and its progression and development are intimately related to androgens actions. Obesity, a worldwide epidemic of the 21st century has been associated with the aggressiveness and worse prognosis of PCa. On the other hand, the extrinsic factors have been shown to contribute to the risk of PCa development in more than 99,9 %, which stimulates the interest in exploiting the effect of environmental factors on cancer onset and progression. It is the case of endocrine disruptors, a group of compounds that interfere with the synthesis, secretion, and mechanism of action of endogenous hormones, being also implicated in the carcinogenesis of several tissues. Some endocrine disruptors are also called obesogens, since can induce a hormone dysregulation, interfering with the biochemical pathways that control appetite and/or the entire metabolic homeostasis and lipid handling, thus promoting obesity. Tributyltin (TBT) is an endocrine disruptor and an obesogen used as a biocide, which has also androgenic activity. It was demonstrated that the deregulation induced by TBT can increase the proliferative activity of PCa cells. However, no relationship was established in the context of obesity and lipid availability. This study hypothesizes that TBT may differentially affect PCa cells fate dependently on obesity or non-obesity conditions. The effect of TBT on prostate cells' viability, proliferation, migration, and invasion in the presence or absence of low-density lipoprotein (LDL) cholesterol was evaluated. Non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) prostate cell lines were treated with different concentrations of TBT for 48 hours, and cell viability assessed by the MTT and SRB assays. In LNCaP cells treated with 100 nM TBT, cell proliferation was accessed by the Ki-67 fluorescence immunocytochemistry. The expression of Akt, p-Akt, MAPK, p-MAPK and c-Myc proteins was evaluated by Western blot (WB). Cell migration and invasion were assessed by Matrigel assays, and E-cadherin protein expression was analysed by WB. Experiments were repeated with a lower concentration of TBT (10 nM) in a medium enrich with LDL-cholesterol (100 µg / ml) and 5α–dyhidrotestosterone (DHT, 10 nM). Our results demonstrated that TBT (100 nM) induces LNCaP cell viability. Also, a higher proliferative capacity was observed in LNCaP cells treated with TBT, as indicated by the increase in Ki67 staining, as well as in the expression of c-Myc protein. TBT also promoted LNCaP cells' migration and invasion, which was observed by the matrigel assays and supported by the decreased expression of E-cadherin. Finally, LNCaP cells exposed to a lower concentration of TBT (10 nM) in the of LDL showed a higher proliferation and migration capacity, which was favored by DHT. Overall, TBT has the capacity to disrupt the behavior of PCa cells, likely contributing to tumor's progression. Noteworthy, these effects were maintained even in

lower TBT concentrations, and seem to be more impactful in obesity conditions, which highlights the greater danger of a possible exposure in obese PCa patients.

Keywords

Prostate cancer; obesogen; TBT; proliferation; migration; invasion.

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List of Abbreviations

2,4-D	2,4-dichlorophenoxyacetic
AFS	Anterior Fibromuscular Stroma
AngII	Angiotensin II
AR	Androgen Receptor
BCA	Bicinchoninic acid
BMI	Body Mass Index
BPA	Bisphenol A
BPH	Benign Prostatic Hyperplasia
BSA	Bovine Serum Albumin
CRPC	Castrate-Resistant PCa
CZ	Central Zone
DHT	Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
EDCs	Endocrine Disrupting Chemicals
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EMT	Epithelial Cells To Mesenchymal Cells
ERs	Estrogen Receptors
FBS	Fetal Bovine Serum
HED	High-Energy Diets
IGF-1	Insuline-Like Growth Factor 1
IL-6	Interleukin-6
KGF	Keratinocyte Growth Factor
LDL	Low-Density Lipoproteins
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattracting Protein
MIC-1	Macrophage-Inhibiting Cytokine 1
MTG	Matrigel
MTT	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NK	Natural Killer Lymphocytes
NR	Nuclear Receptors
PAP	Prostatic Acid Phosphatase
PBDEs	Polybrominated Diphenyl Ethers
PBS	Phosphate Buffer Saline

PBS-T	Phosphate Buffer Saline Tween-20
PCa	Prostate Cancer
PCBs	Polychlorinated Biphenyls
PFOA	Perfluorooctanoic Acid
PFA	Paraformaldehyde
PI3K	Phosphoinositide-3-Kinase Pathway
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic Intraepithelial Neoplasia
PPAR	Peroxisome proliferator-activated receptor gama
PSA	Prostate Specific Antigen
PTEN	Phosphatase And Tensin Homolog
PVC	Polyvinyl Chloride
PVDF	Polyvinylidene Difluoride
PZ	Peripheral Zone
RIPA	Radioimmunoprecipitation Assay
RXRs	Retinoid X Receptors
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel
S.E.M	Standard Error Of The Mean
SRB	Sulforhodamine B Assay
TBT	Tributyltin
TGF-β	Transforming Growth Factor-β
TNFα	Tumor Necrosis Factor
TPT	Triphenyltin
TZ	Transition Zone
WB	Western Blot

Chapter 1

Introduction

1.1 Brief overview of anatomy and physiology of prostate

Prostate gland is the largest accessory gland of the male reproductive system [1]. 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 [2, 3]. Anatomically is constituted of a base, an apex and anterior, posterior, and inferior lateral surfaces. The prostatic plexus is responsible for the nerve supply and the artery supply and is maintained by the branches of the internal iliac artery [3].

The human prostate is composed of glandular or epithelial and stromal components which are tightly fused within a pseudo capsule. The prostate capsule is lined externally by a layer of collagen and internally by smooth muscle [3]. The gland is divided in four zones with different sizes (Figure 1), which have specific particularities in what concerns to the embryogenic origin, histology, anatomic landmarks, biological functions, and susceptibility to pathologic disorders. The peripheral zone (PZ), that compound about 70% of the glandular tissue and is the most susceptible zone to develop carcinoma, chronic prostatitis, and post inflammatory atrophy development. The transition zone (TZ) that surrounds proximal prostatic urethra, involves approximately 5% of the glandular tissue and with aging usually occurs hypertrophy development. Both TZ and PZ seem to have the same embryological origin, the urogenital sinus, but differ in the incidence of prostate cancer (PCa), with 70% arising in PZ and 25% in the TZ. These zones have differences in the stromal component, being the TZ stroma more fibromuscular, and therefore more susceptible to develop benign prostatic hyperplasia (BPH) [3]. The area that surrounds the ejaculatory ducts is called the central zone (CZ), derives from the Wolffian duct, contains 25% of glandular tissue and has a low incidence of PCa. Finally, the last zone does not contain glandular tissue, is located anteriorly and is called anterior fibromuscular stroma (AFS) [4-6].

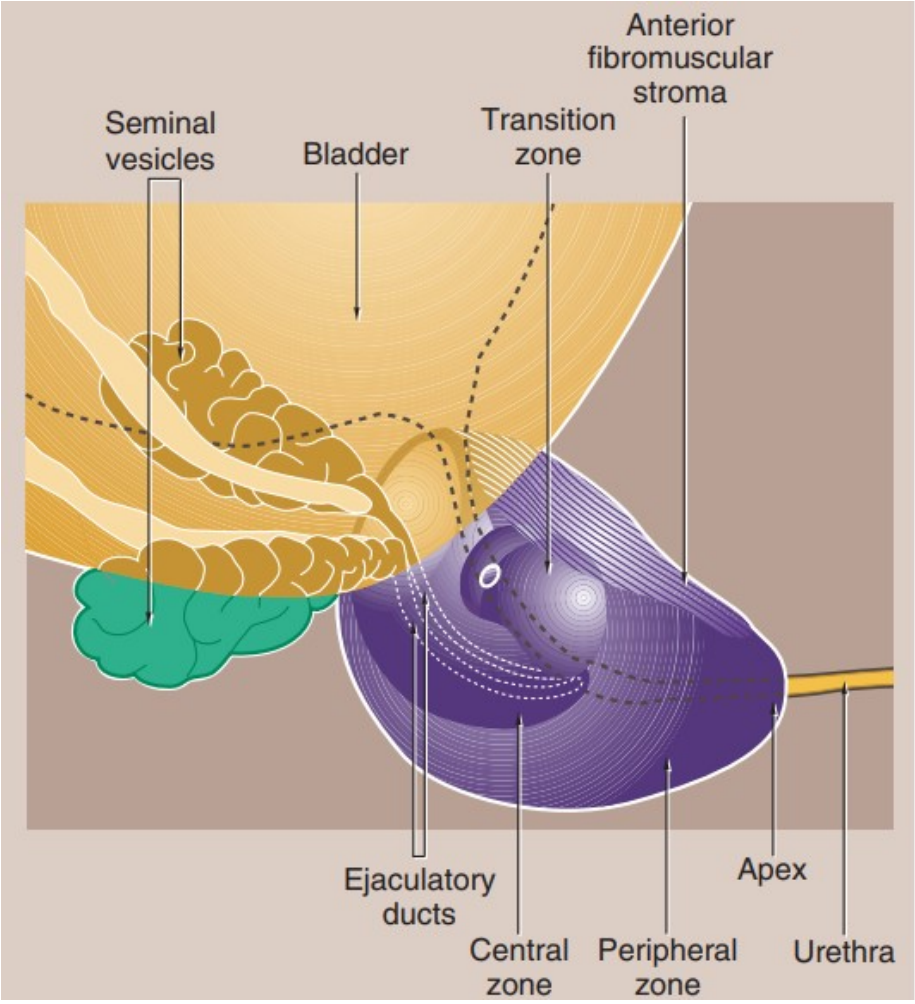

Figure 1. Anatomy of the human prostate. The prostate is divided in four zones, peripheral zone that consists in about 70% of prostate, transition zone that surrounds proximal prostatic urethra, central zone that surrounds the ejaculatory duct and the anterior fibromuscular stroma (AFS), which allows the connection between anterior and apical surfaces (Adapted [8])

The glandular prostatic epithelium is constituted by the secretory epithelial cells, basal cells, stem cells and neuroendocrine cells [7]. The secretory luminal layer comprises of columnar epithelial cells responsible for the production of prostatic secretions [8], including the prostate specific antigen (PSA), routinely used as PCa indicator [9]. Basal cells form a layer between the secretory cells and the basement membrane [8]. 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 [10]. Finally, neuroendocrine cells are responsible for secretion of neurosecretory products promoting prostate growth [11, 12].

Prostate is responsible for the production of the prostatic fluid that contributes for about 30% of the ejaculation volume [7, 9]. The major functions of the prostatic secretions are related with semen gelation, coagulation and liquefaction [13] and guarantee sperm motility, due to the production of secretory proteins that involve the coating and

uncoating of spermatozoa [13]. The environment of the vas deferens and the female vagina is acidic, so the milky secretions, with basic pH, from the prostate will neutralize the acidic pH. In addition, there are also other factors produced by epithelial cells that will be useful in sperm, such as citric acid and proteolytic enzymes [14].

1.2 Prostate cancer: etiology and development of prostate cancer

1.2.1 Epidemiology

Prostate cancer has been considered in the last decade as the second most diagnosed cancer in the men and it is estimated 15% of the diagnosis of male cancer and 8% of all cancer cases [15]. It is expected a 1.05% increase in mortality and an estimate of 2 293 818 new cases in 2040 [16]. The highest incidence rates of PCa 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 [15]. Developing countries have more capacity to apply early detection methods, including PSA test and biopsies, which can partly explain the highest incidence rate.

In Portugal, PCa is the most common cancer in men, with 5433 new cases each year in a total of 22.4% of the overall cancer cases [17]. It is predicted that in 2020 there will be 8600 new cases and 1700 deaths from PCa [17]. Studies have shown that there is an increase of the incidence in Portugal, but the mortality that is associated with this cancer appears to be steadily decreasing over time (Figure 2) [18].

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 (Adapted [18]).

1.2.2 Risk factors for prostate cancer

The causes and risk factors for PCa have been described, but the specific causes are still poorly understood. The greatest factor that increases the probability to develop PCa is the age. It is known that there is a lower incidence of PCa in men under 50, which represents less than 0.1% of all PCa patients, and approximately 85% of cases with PCa are diagnosed above 65 years old [19]. The highest incidence of this cancer in men is between 55 and 74 years old [20]. Other risk factors for PCa are ethnicity and race. Oceania, followed by Northern America, Western Europe, Northern Europe, and the Caribbean have among the highest PCa incidence rates in the world (Figure 3) [21].

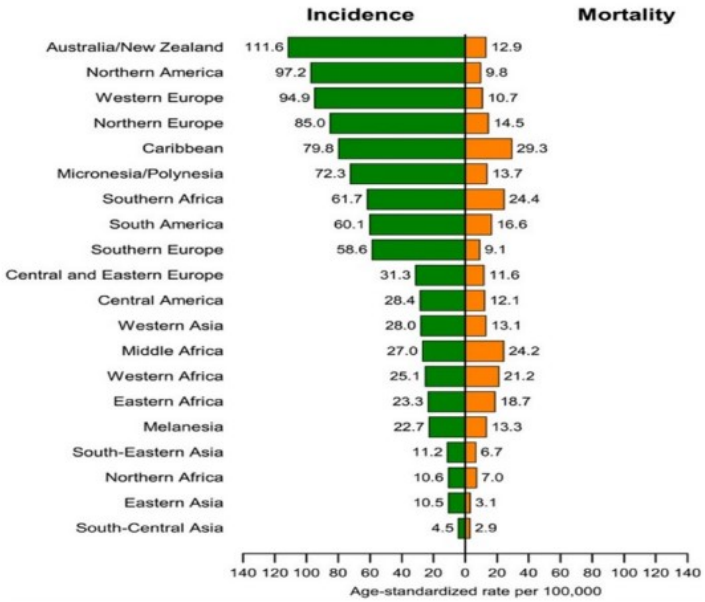

Figure 3. Differences in incidence and mortality of prostate cancer among geography. The difference between developed and less developed countries when comparing the incidence mortality rate (Adapated [21]).

Genetic background, as, family, and genetic history seem to be associated with PCa development. The risk to acquire increased with an affected first-degree relative. Furthermore, the risk to develop PCa increases, with multiple family members affected and with the degree of relatedness and is inversely related with the age at which the family members were diagnosed [22-24]. Several PCa susceptibility genes have been identified, such as RNASEL, ELAC2, MSR1, OGG1, CHEK2, BRCA2, PON1 and GDF15 [25].

There are also factors that damage the prostate epithelium, such as inflammation and infection triggered by infectious agents via urine and sexual activity, which probably leads to the prostate tumor-suppressor genes and potentiates cell hyperproliferation, stress oxidative and DNA damage[26]. This fact was indicated in tumor suppressor genes lose (tumor-suppressor phosphatase and tensin homolog (PTEN) and TP53) and in the increase of oncogenes activation, as Myc [27]. Apoptosis is interrupted and proliferation increases, affecting cells metabolism and thus contributing to cancer development [28-31] .

Interestingly, extrinsic factors seem to contribute to the risk of PCa development in more than 99.9 % [32]. Chemical agents, lifestyle, type of diet, seem to be associated PCa development [32-34]. The average fat intake can be correlated with the incidence and mortality of PCa [35, 36]. Obesity seem to me correlated with progression and aggressiveness of PCa [37]. On the other hand, vitamin E, selenium and lycopene seem

to be a protective role of PCa risk [38, 39]. Alcohol consumption is another risk factor for PCa, but seem to be dose dependent[40].

1.2.3 Diagnosis and therapeutic options for prostate cancer

The detection of PCa as early as possible is crucial, since this disease is usually asymptomatic until the latest stages [41]. One of the first serum biomarker for PCa detection was the human prostatic acid phosphatase (PAP) but has poorly sensitivity. [41-43]. Actually, the most common methods used to diagnose PCa are the digital rectal exam, which is a physical exam, and a biochemical examination of the serum content of the biomarker PSA [44, 45]. The PSA test is not 100% reliable, as it is known that 15% of men who have a normal or low PSA level can have PCa [46, 47]. To be sure of the diagnosis, a biopsy must be performed to allow elimination of false positives and false negatives due to the PSA test [48]. Each year science evolves as new biochemical techniques are tested, but produced only preliminary results, such as the 4kscore blood test, that tests four biomarkers instead of one [49].

Relatively to the therapeutic options for PCa, there is a wide variety of possible therapies dependently of the stage of the disease. "Waiting Surveillance" is a monitoring strategy used when there is a small tumor, detected locally in the gland [50]. Radical prostatectomy, brachytherapy, external beam radiation therapy and androgen deprivation therapy are hypotheses for pre-metastatic stages [51]. In more advanced stages, with metastases, more drastic therapies for the human body will have to be used, such as chemotherapy, radiotherapy and hormonal therapy [52]. There are several therapies and methods available for PCa, however, and mainly in the most aggressive stages there are not efficient, so there is still a need to discover more innovative therapies.

1.2.4 Mechanism underlying the development and progression of prostate cancer

Proliferative inflammatory atrophy (PIA) is a condition that occurs when the prostate epithelium is damaged by inflammation, infection and / or carcinogens, and the morphological alterations in the prostatic epithelium can lead to histological lesions as the high-grade prostatic intraepithelial neoplasia (PIN) [26]. PIN is characterized by the beginning of the loss of distinct basal and secretory layers and the appearance of luminal epithelial cells dysplasia [53, 54]. Finally, it is known that one of the precursors of PCa and its invasive state is the high-grade PIN (Figure 4) [53].

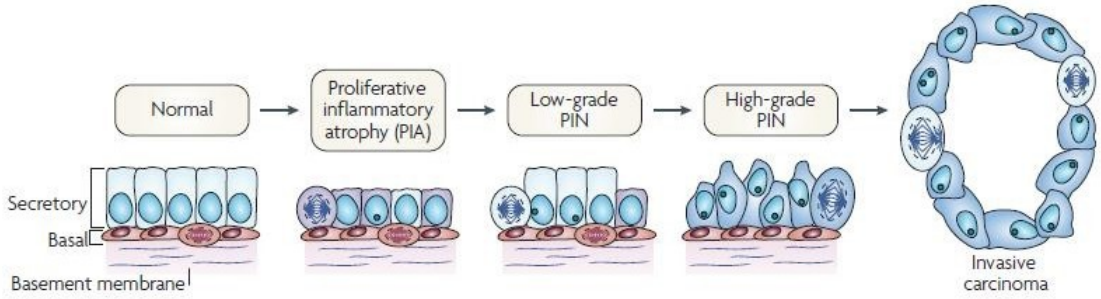

Figure 4. Cellular model of early prostate neoplasia progression. Initially, there is an infiltration of lymphocytes, macrophages, and neutrophils. Phagocytes will release reactive oxygen species that will cause DNA damage, cell damage and cell death, which leads to epithelial regeneration. The downregulation 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 [53]).

PCa is initially represented as a small foci of intraductal dysplasia, the prostate structures become less organized, the gland suffers an excessive enlargement and ultimately evolves to invasive stages [26, 55, 56]. At the first stages, PCa growth is dependent on the action of androgens. The androgens regulate the proportion of cells that proliferate and die, stimulating proliferation and inhibiting apoptosis [57]. The main androgen is testosterone, mainly secreted by the testis, that is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase in prostate, becoming the most active hormone, with a higher affinity for the androgen receptor (AR). The DHT- AR binding lead to the increase on PCa proliferation rates than cell death, which enables continuous proliferation and growth. Therefore, the main therapy for progressive PCa would be the androgen ablation, which leads to the regression of androgen-dependent tumors [58, 59]. However, in several cases, a selection of cell subpopulations still survives and adapt to the microenvironment, independently of the low circulating levels of androgens, which lead to the progression of the disease to the most lethal stage, the so

called castrate-resistant PCa (CRPC). At this point the androgen-ablation therapies become inefficient, and the tumor progresses and can metastasize to the lymph nodes, brain, bladder, and bone.

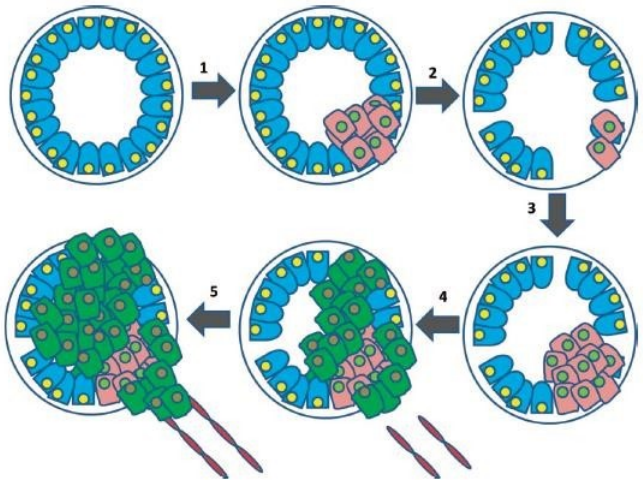

Figure 5. Malignant transformation of prostate and progression from androgen-dependent to androgren-independent prostate cancers. 1. Several carcinogenic alterations occur, and some prostate cells proliferate out of control. 2. Prostate cancer cells are initially androgen-dependent; thus, the androgen-deprivation 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 (Adapted [61]).

The mechanisms underlying the acquisition of androgen independence can involve different pathways. The first one is named Hypersensitive Pathway, in which an enhanced AR sensitivity to low androgens levels occurs, resultant from the amplification of the AR expression or from the increase of production of androgens on the local levels (Figure 5) [60, 61]. The promiscuous pathway is independent of androgens, and results from genetic changes, such as missense mutations of AR promoting a less specific binding to the ligand [62]. Therefore, mutated AR can become responsive to other non-androgenic steroid molecules or androgen antagonists [60]. The third pathway, the Outlaw Pathway, is dependent on the activation of AR with a ligand-independent mechanism [60], using growth factors overexpressed in some PCa, like insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF) or epidermal growth factor (EGF) [63]. The fourth pathway, called Bypass Pathway, is associated with the capacity of the cells to inhibit the apoptosis cascade, regardless of the activation of AR and the absence of androgens [61, 62]. The last pathway is known as Lurker Cell Pathway, and it is known that there is a group of stem cells from PCa or progenitor cells in the tumor

microenvironment, without tumor, that are carcinogenic and do not express ARs, surviving androgen depletion therapy [60].

1.2.5 Molecular mechanisms driving cancer cell proliferation, migration, and invasion

The normal prostate grow is regulated by the activation of AR. Upon the binding of DHT or similar steroid hormones, nuclear AR will dissociate from heat shock proteins and bind to specific promoters to stimulate transcription. As previously described, in PCa, the mutation and altered expression of AR and related proteins can influence cancer growth. The 5α-reductase enzyme that converts testosterone to dihydrotestosterone, can suffer an amino acid substitution that confers greater enzyme activity and consequently a slightly higher risk of PCa [64]. There are other survival and anti-apoptotic genes, growth factors and their receptors contributing to prostate tumor growth and progression. Loss of the PTEN and p53, or their mutations are linked to PCa progression, since a deregulation on cell cycle occurs [65]. One of the most characterized pathways of PCa is the phosphoinositide-3-kinase pathway (PI3K) which is activated by several tyrosine kinase receptors. The tumor suppressor PTEN, is regulator of PI3K, and when PTEN is suppressed the PI3K pathway is activated, triggering the cell proliferation by Akt activity [66]. The overactivation of PI3K signaling in PCa is accompanied by PTEN deletion [67-70]. P53 is a tumor-suppressor and proapoptotic factor, and deletions and mutations on the p53 gene have been detected in approximately 40 % of PCa cases [71]. p53 functions as a transcription factor regulating downstream genes important in cell cycle arrest and apoptosis, including, p21, Bax and Bcl-2 [72-74].

Ras family members can also drive PCa progression, activating the mitogen-activated protein kinase (MAPK) pathway, specifically protein kinases ERK1 and ERK2. Alterations on the MAPK pathway seem contribute to the severity of PCa [75-78]. Also, the increased expression of ERK was observed on 70% of prostate tumors with Gleason score of 8 to 10, evidencing the relation of this pathway with cancers with poor prognostics.

There are several reports indicating that the amplification of the c-Myc oncogene occurs in the earliest phases of PCa, as PIN lesions, but is also linked to the aggressiveness of the tumor [65].

The cancer aggressiveness is associated with the transition from epithelial cells to mesenchymal cells (EMT), making possible to cancer cells multiple properties like stems and migratory abilities. The alterations that the epithelial cells suffer during EMT are promoted by several molecular mechanisms that are strongly regulated and influenced by a cell-to-cell signaling network and environmental factors. EMT enables to cancer cells acquire more malignant characteristics, crucial to the development of metastatic capacity, such as loss of adhesion and alteration of cell morphology. These changes mainly result from the diminished expression of cytokeratins and E-cadherin (an intercellular adhesion molecule and key marker of the epithelial phenotype), and increased expression of E-cadherin repressors (*e.g.,* SNAIL), and N-cadherin and vimentin, both well-known markers of the mesenchymal phenotype (Figure 6) [79-81]. The EMT execution usually occurs in a hostile microenvironment (such as hypoxia, low pH and low glucose). Metabolic alterations are needed to favor the rapid tumor growth that support the execution of the EMT, helping cancer cells to ignore the limitation of nutrients and oxygen supply caused by the rapid primary growth of cancer and colonize secondary sites to ensure adequate support of energy and nutrients [82].

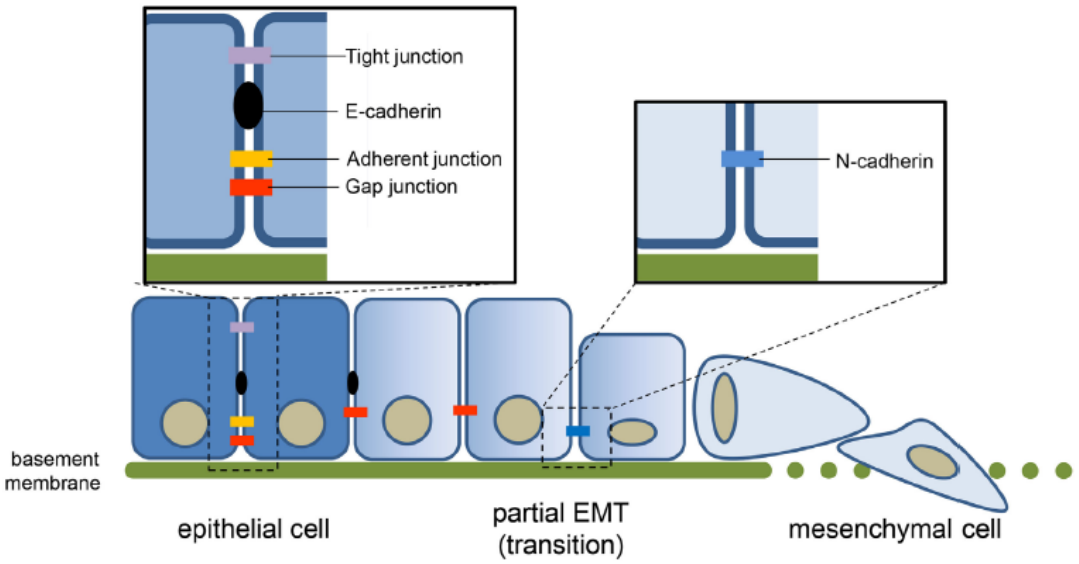

Figure 6. General features of epithelial-to-mesenchymal transition (EMT). When there is a transition from epithelial cells to the mesenchymal phenotype, several environmental or soluble factors are induced, their characteristic is lost which is the cell-cell contact and the cellular polarity, it makes the mesenchymal cells acquire competences to migratory and invasive competences. The EMT will be modulated by known markers, one of which is the loss of the epithelial marker E-cadherin, which is induced by the positive regulation of its transcriptional repressors (Snail1 / 2, Twist, ZED 1/2), and is one of the priming events. The concomitant acquisition of mesenchymal markers sustains and stabilizes the newly acquired phenotype (Adapted [65]).

1.3 Obesity, obesogens and prostate cancer

1.3.1 Obesity-driven prostate cancer

Obesity is defined as a disease resultant from excess body fat accumulation that will bring health consequences for humans. Overweight is classified by a body mass index (BMI) between 25-29,9 kg/m2 and obesity greater than 30 kg/m2 [83]. Obesity is mainly acquired in the presence of underlying disease or organic cause, but it can also be the primary cause, such as heredity, genetics, or endocrine deregulation. The excess fat has a negative impact on a person's health, and strong associations was related between obesity and metabolic syndrome, type 2 diabetes, cardiovascular disease, sleep-related breathing abnormalities, infertility, osteoarthritis, and liver and gallbladder disease [84]. Also a relationship between obesity and several types of cancer, as PCa, has been described [83]. Several human studies demonstrate that obesity is correlated with an increased risk of aggressive PCa development [85-87]. It seems to be also correlated with disease recurrence and PCa-specific mortality [88, 89]. In addition, *in vivo* models showed that high-fat diet support a link between obesity and PCa progression [15, 90, 91]. Despite these epidemiological, clinical and *in vivo* studies, the biological mechanisms linking obesity and PCa remain poorly understood [15].

The adipose tissue is formed by several types of cells, adipocytes, adipocyte progenitor cells, mesenchymal stem cells, endothelial cells and several resident and infiltrating immune cells [15]. Adipose cells produce and secrete leptin and adiponectin, known as adipokines, which regulate appetite, metabolism, and tissue expansion. When there is a greater amount of fat, serum leptin levels increase, but adiponectin may be reduced. Leptin helps to increase cell proliferation by activating JAK/STAT, ERK and PI3K/AKT/mTOR [92]. Prostate cells have receptors and are responsive to the actions of leptin and adiponectin present in the serum [92, 93].

In addition, adipose tissue of obese humans, has infiltration and accumulation of macrophages [20]. Other immune cells, especially lymphocytes produce pro-inflammatory cytokines and chemokines, as tumor necrosis factor (TNFα), interleukin (IL-6), IL-1b and monocyte chemoattracting protein (MCP-1) [94]. It was described that IL-6 is associated with the progression of PCa due to the high serum levels of IL-6 in patients who have advanced PCa, as CRPC. In addition, the periprostatic adipose tissue produces IL-6, that may be a local factor in prostate carcinogenesis. There are also high levels in circulation of macrophage-inhibiting cytokine 1 (MIC-1), also a member of the transforming growth factor-β (TGF-β) superfamily, which is related with a poor prognosis in PCa. Finally, there are other proinflammatory cytokines that are correlated with PCa and bone metastases, including IL-8 and TNFα (Figure 7).

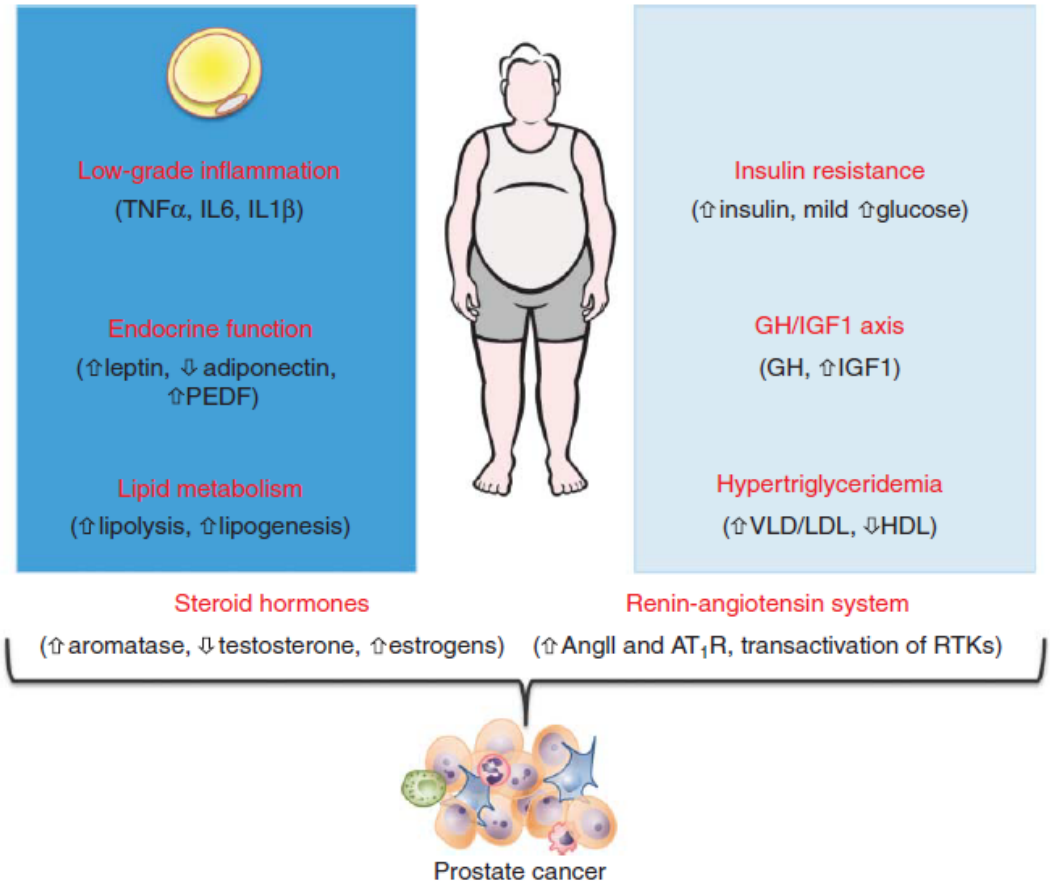

Figure 7. Linking obesity to prostate cancer. Changes in endocrine and metabolic function and the inflammatory milieu occur during the development of obesity. Changes associated with alterations in adipose tissue function are shown in the dark blue box and changes in the systemic metabolism and endocrine function are shown in the light blue box. Arrows indicate the direction of change in obese vs lean individuals. AngII, angiotensin II; AT11R, angiotensin II type 1 receptor; PEDF, pigment epithelium-derived factor; RTK, receptor tyrosine kinase (Adapted from [15]).

The synthesis and bioavailability of sex hormones are influenced by adiposity. Therefore, in obese men with PCa these alterations must be considered, since PCa is initially hormone dependent. The prostate is an organ that is regulated by androgens but also by estrogens. There are several reports describing that in adult men, total testosterone levels are inversely proportional to BMI and subcutaneous and visceral fat content.

Another important mechanism that can contribute to cancer progression is the availability of fatty acids, being involved in the bioenergetic requirements for: cell proliferation, energy storage depot and cellular membrane construction. Fatty acids can be derived from triglycerides contained in the low-density lipoproteins (LDL). Cholesterol is absorbed by the PCa cells in a process mediated by LDLr [95]. On the other hand, it was found that the inhibition of cholesterol biosynthesis with statins lead to the development of a compensatory mechanism by increasing the expression of LDLr in PCa

cells [96]. Cholesteryl ester induced the opposed scenario, significantly reducing the cell proliferation and invasion, and suppressing tumor growth [97]. Moreover, the aberrant accumulation of esterified cholesterol in lipid droplets was detected in high-grade PCa and metastases [97].

Another endocrine change in obesity is an increase in the production of estrogen since the adipose tissue also express the enzyme that metabolizes estrogen, the aromatase [15]. Those elevated levels of E2 create a hormonal imbalance and might influence PCa development and progression. In addition, the reduction of testosterone levels by the E2 negative feedback on the hypothalamus is accompanied by lower PSA levels, turning PCa less likely to be diagnosed [15, 98].

Future studies to understand the real connection between obesity and PCa are crucial to develop new therapeutic and preventive strategies.

1.3.2 Endocrine-disrupting chemicals (EDCS) and obesity: obesogens

The US Environmental Protection Agency has defined endocrine disrupting chemicals (EDCs) as "agents that interfere with the synthesis, secretion, transport, binding or even elimination of natural hormones in the body, which are responsible for maintaining homeostasis, reproduction, development and/or behavior" [99]. The two major categories of EDCs are natural and anthropogenic sources [100], including pesticides and herbicides, biocides, thermal stabilizers, chemical catalysts, plastic components, pharmaceutical products , ions (cadmium) and some Ultraviolet filters [101-103]. Exposure to EDCs may lead to changes in the neuroendocrine system, epigenetic alterations and/or direct effects on gene expression. The physiological responses and the mode of action depend on the class of the compounds because it is grouped into four categories: xenoestrogens, xenoandrogens, antiestrogens and antiandrogens. Xenoestrogens and xenoandrogens are chemical compounds that exhibit estrogenic and androgenic properties, respectively, and can interfere with the nuclear receptors [104-106]. Antiestrogens and antiandrogens have antagonistic actions, blocking or reducing the activity of estrogen receptors (ERs) and AR respectively, thus, it will disrupt hormonal actions and affect the various physiological responses in a wide range of tissues [107-110]. Mechanistically EDCs activate or block the nuclear receptors (NRs), regulating

specific receptor proteins that act as transcription factors or can also modulate gene expression. Epidemiological studies have given the information that there is an increase in the incidence of some diseases that are associated with EDCs, such as cancer of the prostate, breast, testis, also, diabetes, obesity and decreased fertility [111].

The term “obesogen” emerged in the recent years and refers to chemical compounds usually present in high-energy diets (HED) or in the surrounding environment, which are able to enhance adipogenesis by increasing the number of fat cells or the storage of fat-soluble toxicants into existing fat cells [112]. It has been described that exposure to obesogens may affect hormonal deregulation, interfering with the biochemical pathways that control appetite and/or whole metabolic homeostasis, and thus promote the development of obesity.

Specifically, obesogens interferes with receptors activated by the peroxisome proliferator (PPARα, PPARδ and PPARγ), steroid hormone receptors and with nuclear transcriptional regulators that have the function of controlling lipid flow and/or adipocyte proliferation / differentiation. PPARs will act by heterodimerization with retinoid X receptors (RXRs) and stimulate the breakdown of β oxidation of fatty acids. [113]. Several compounds have been suspected to present obesogenic activity (Table 1).

Table 1: Obesogens and their sources of exposure. Legend: 2,4-D - 2,4-dichlorophenoxyacetic; BPA – Bisphenol A; PBDEs - Polybrominated Diphenyl Ethers; PCBs - Polychlorinated biphenyls; PFOA - Perfluorooctanoic acid; PVC - Polyvinyl chloride; TBT – Tributyltin; TPT - Triphenyltin.

Obesogen	Main Sources	Function	Reference
Benzo[α]pyrene	Residential wood burnings, cigarette smoke, charbroiled food, coal tar and automobile fume emissions	Xenoestrogen	[114, 115]
BPA	Food and drink packaging plastics, medical devices, and thermal paper	Xenoestrogen	[116, 117]
Chlorpyrifos	Insecticides	Antiandrogenic	[118, 119]
Diazinon	Insecticides	Antiandrogenic	[120, 121]
Diethylstilbestrol	Cattle feed and medical treatments for breast and prostate cancers	Xenoestrogen	[117, 122]
Genistein	Soybeans and soy products, fava beans and coffee	Antiestrogen	[123, 124]
Lead	Water, artificial turf, and infant toys	Antiestrogen	[125, 126]
Nicotine	Tobacco, insecticides, and nightshade plants	Antiestrogen	[127, 128]
Parathion	Insecticides and acaricides	Antiandrogenic	[129, 130]
PBDEs	Flame retardant in building materials, electronics, furnishings, plasticizers and textiles	Antiandrogenic	[131, 132]
PCBs	Electric equipment, plasticizers, surface coatings, flame retardants, paints and carbonless copy paper	Antiandrogenic	[133, 134]
PFOA	Crawl and stain repellent on carpets, furniture, waterproof clothing, mattresses and microwavable food items	Xenoestrogen	[135, 136]
Phtalates	Plasticizers, PVC products, infant toys, detergents and personal care products	Xenoestrogen	[137, 138]
TBT	Antifouling paints, wood preservers and stabilizers of plastic products	Xenoandrogen	[117, 139]
TPT	Fungicide and miticide	Xenoandrogen	[117, 140]

1.3.3 The Obesogen Tributyltin: a relation with Prostate cancer?

Tributyltin (TBT) is an EDC with androgenic and obesogenic activity, commonly used in industry and it can be found in man by food sources, such as contaminated seafood and shellfish, in shipping applications or fungicides in paper mills and industrial water systems. As a consequence, severe restrictions have been adopted, including a global ban since 2008 [141]. However, present and future restrictions will not immediately remove TBT and their products from the marine environment, since these compounds are retained in the sediments [142]. In fact, TBT can remain in the ecosystem for tens of decades [143], and so, their influences on the microenvironment and human health is a matter to be aware of [144]. According with the regulatory scientific advisory panels it is tolerable a daily intake of 270 ng TBT/kg of body weight/day in relation to human health risk assessments [145]. However, other "sources" of TBT exposure comes from contaminated household dust (in an estimated range for total organotins between 0.3–28 μg/g) [146] and from wood preservatives [144].

The obesogenic properties of TBT are described in the activation of the "main regulator" of adipogenesis, PPARγ and its heterodimeric partner, 9-cis RXR receptor, promoting adipogenesis and alter lipid homeostasis [139, 147, 148]. Environmental exposure of relevant levels (nanomolar) of TBT, a PPARγ-dependent pathway, cause human mesenchymal stem cells and pre-adipocytes differentiation [149]. In adult rats exposed to TBT in the uterus, it was demonstrated an increase in lipid accumulation in adipose deposits, livers and testicles [150]. Also, in an epidemiological study, an increase in the weight index in human babies with greater prenatal exposure to TBT was described [150].

The androgenic properties of TBT started to be recognized after the observation that marine organisms exposed to TBT presented sexual abnormalities, known as imposex, which means the superimposition of male organs (penis and/or vas deferens) onto the female genital organs. The mechanism underling the TBT-induced masculinization, as well as the way as TBT affect androgen accumulation in other species including mammals has not been fully understood. Some authors have been suggested that inhibition of androgen excretion due to a decrease in sulfur conjugation of androgen is the major cause of the accumulation of androgens after TBT exposure in gastropod tissues, but the mechanism remains unexplained [151]. On the other hand, several reports have suggested that TBT inhibits the activity of cytochrome P450 aromatase, the enzyme

responsible for the conversion of androgen to estrogen, and consequently, an increase in the levels of unconverted androgens [117, 152-157].

In addition, it was demonstrated that TBT may induce an imbalance of ovarian hormones reducing the number of gonocytes and germ cells [152]. Other data showed that TBT can cause changes in the testis and ovaries weight, with a reduction in serum progesterone levels and consequently a reduction in the pregnancy rate [154, 158].

TBT has also been associated with the suppression of immunity system cells, as the natural killer lymphocytes (NK) making the humans susceptible to viral infections and eventually to tumor development [159].

Nowadays, with the increase of epidemiological studies, there is more evidence, that several EDCs, some of them obesogens may influence the development or progression of PCa [160-162]. A link has been found between PCa rates and pesticides[163]. A chronic or intermittent exposures to these chemicals that contribute to higher rates of this disease [164]. Specifically, the obesogen TBT, which has been described as an EDC with androgenic and obesogenic properties seems to be related with PCa development. In an androgen-responsive PCa cell line, the exposure to TBT and triphenyltin (TPT) increased the proliferation of cells, as well as, the AR dependent transcription, such as regulation of transcription factors such as GATA2, PDEF, ETV1, CREB3L4, HOXB13 and NKX3.1, mainly in the 100 nM concentration [165].

Despite the reports demonstrating a relationship between obesogens with adipogenesis alteration of lipid homeostasis, deregulation of sex steroids and influence of progression the cancer, the relationship between TBT and PCa on proliferation, migration and apoptosis remains unexplored.

Chapter 2
Aims

Over the years, it has been observed that the development of PCa is related with the lifestyle and dietary habits, being more common in Western societies. Obesity and hypercholesterolemia are among the factors that have been linked to the aggressiveness of PCa. Interestingly, it was also demonstrated that the extrinsic factors contribute to the risk of developing PCa approximately in 99.9%. Indeed, several extrinsic agents have been speculated to contribute to PCa progression, which includes chemical agents as the EDCs. Moreover, some EDCs have obesogenic activity, contributing for adipogenesis and development of obesity. Therefore, to study the impact of obesogens in the development of PCa emerges as an exciting field of work. TBT is the gold standard to study the effects of obesogens, and it is particularly relevant in the case of PCa as it acts as a main regulator of adipogenesis and also has androgenic activity.

Although it is known that TBT has the potential to affect human health, its action contributing to the malignant transformation of prostate cells remains to be clarified. Herein, we hypothesized that TBT can differentially affect PCa cell fate dependently on the obesity or non-obesity conditions. Therefore, the present dissertation aims to:

- Study the effect of the obesogen TBT on the viability, proliferation and migration of cell line models mimicking different stages of PCa;
- Analyse the effects of TBT in PCa cells in conditions of high LDL-cholesterol availability (obesity-like).

Chapter 3
Material and Methods

3.1. 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). LNCaP is an androgen-responsive cell line derived from a lymph node metastasis of prostate cancer [166]. PC3 cells were derived from bone metastasis and are non-responsive to androgens [167]. 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 PCa respectively [29, 168].

3.2. Cell culture and treatments

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. For cell viability, proliferation and western blot experiments, cells were seeded and at 60% of confluence, the medium was changed for RPMI medium without phenol containing 5% charcoal-stripped FBS (Sigma-Aldrich) during 24 hours. Cells were stimulated with 100 nM of TBT for 24 or 48 hours. Additionally, the experience was repeated using 1% lipid-depleted FBS (Biowest, Riverside, MO, USA) and exposed to TBT (10 nM), LDL (100 μg / mL) and/or 5α-dihydrotestosterone (DHT, 10 nM) for 24 or 48 hours. After treatment, cells were trypsinized and harvested for posterior analysis.
The TBT stock solution (T50202, 100g, Sigma-Aldrich) was dissolved in ethanol, resulting in a 10 mM TBT solution. All TBT working solutions were freshly prepared from the stock solution in medium.

3.3. Cell viability assay

PNT1A (7500 cells/well), LNCaP (15000 cells/well) and PC3 (4000 cells/well) cells were grown in 96-well plates with 100 μL of culture medium and cell viability was assessed by the 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay at 24 hours and 48 hours after treatment with TBT (0 nM, 1 nM, 10 nM, 100 nM) , and TBT (10 nM) plus LDL (100 μg/ml) or DHT (10 nM). The MTT stock solution (5 mg/ml) (Sigma-Aldrich) was prepared dissolving the powder in phosphate buffer saline (PBS) at

pH 7.4 and filtered. After TBT treatment, 10 µL of MTT stock solution and 90 µL phenol red-free RPMI 1640 medium were added and cells were incubated for 4 hours at 37°C, away from light. At the end, the solution with MTT was removed and 100 µl of dimethyl sulfoxide (DMSO) was added in each well, the plates were shaken to dissolve the formed formazan crystals, and the absorbance was measured spectrophotometrically using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA) at 570 nm, being the value of absorbance directly proportional to the number of viable cells.

3.4. Sulforhodamine B assay

PNT1A (7500 cells/well), LNCaP (25000 cells/well) and PC3 (5000 cells/well) cells were grown in 96-well plates with 100 µL of culture medium and cell mass was assessed by the Sulforhodamine B assay (SRB) assay at 24 hours and 48 hours after treatment with TBT (0 nM, 1 nM, 10 nM, 100 nM). The SRB (S1402, Sigma-Aldrich) is a bright pink aminoxanthene dye that binds to basic amino acids of cellular proteins. The quantity of SRB that binds to the basic amino-acid residues, which is obtained in absorbance values, is directly proportional to the cell mass, functioning as an indicator of proliferation [169]. After TBT treatments, the medium was removed, and cells were fixed in 200 µL of phenol red-free RPMI 1640 medium and 50 µL of cold 60% TCA solution. Then, the medium with TCA was removed, and cells were washed with distilled water and dried at room temperature. 100 µL of SRB (0.05%) was added to each well and the plate was incubated for 1 hour at 37°C, away from light. Unbound dye was removed with 1% acetic acid. Dye bound was extracted with, 200 µL of 10 mM Tris base solution at pH 10. The plate was shaken for 10 minutes, and the absorbance was measured spectrophotometrically using the xMarkTM Spectrophotometer (Bio-Rad, Hercules, CA, USA) at 540 nm. The value of absorbance obtained is directly proportional to the cell mass.

3.5. Ki-67 fluorescent immunocytochemistry

LNCaP cells (1.5 x10^5/well) were fixed with 4% paraformaldehyde (PFA) and permeabilized with 1% Triton X-100 for 5 minutes at room temperature. Unspecific staining was blocked by incubation with PBS containing 0.1% (w/v) Tween-20 (PBS-T) and 20% FBS for 1 hour. Posteriorly, cells were incubated with rabbit anti-Ki-67 primary

antibody (1:50, ab16667, Abcam, Cambridge, United Kingdom) for 1 hour at room temperature. Alexa Fluor 546 goat anti-rabbit IgG (1:1000, Invitrogen, Carlsbad, CA, USA) was used as secondary antibody, and the cells were incubated for 1 hour at room temperature. Cells were washed and incubated for 10 min at room temperature in Hoechst 33342 (5 μg/mL, H3570, Invitrogen, Carlsbad, CA, USA) Lamellae were washed with PBS-T and mounted in Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Specificity of the staining was assessed by the omission of primary antibody. Images were acquired using the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany). Proliferation was determined by the percentage of Ki-67-positive cells out of the total number of Hoechst-stained nuclei in 10 randomly selected ×40 magnification fields in each lamella.

3.6. Migration and Invasion assay

LNCaP cells (3.0 x10^5 cells/transwell) were plated on the upper chamber of Matrigel (MTG)-coated chambers (BD Biosciences) and non-coated chambers (35224, SPL, Life Sciences, Naechon-Myeon Pocheon, South Korea) for migration and invasion, respectively, and incubated with serum-free media in the presence or absence of TBT (10 and 100 nM) LDL (100 μg/ml) and DHT (10 nM). The lower chambers contained media with 10% FBS, as a chemoattractant. Cells were incubated at 37°C and 5%CO_2 for 24 hours. Then, cells on the lower surface of the transwell were fixed, washed, and stained with Hoechst 33342 (5 μg/mL, H3570, Invitrogen, Carlsbad, CA, USA). Migration and invasion were determined by the total number of Hoechst-stained nuclei in 5 randomly selected ×40 magnification fields per transwell.

3.7. Total protein extraction

Total protein was isolated from PNT1A, LNCaP and PC3 cells using radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA, 1% protease inhibitor cocktail, 10% phosphatase inhibitor cocktail). According to the amount of pellet obtained in the eppendorf, the appropriate amount of RIPA was placed in each one. Then, the samples were placed on ice for 20 minutes and vortexed every 5 minutes. After 20 minutes, the samples were centrifuged at 14000 rpm for 20 minutes at 4°C. At the

end, the supernatant was placed on new identified eppendorfs. The total protein concentration was determined by the Bicinchoninic acid (BCA) assay (Thermo Fisher, Rockford, USA) [170]. This assay was carried out with a kit that has two reagents, with reagent A being a carbonate buffer containing BCA reagent and reagent B a cupric sulfate solution. Both reagents were mixed in a proportion of 50 parts of reagent A to 1 part of reagent B, to obtain the working reagent. The working reagent (80 µL) was added to a 96 well- plate containing the sample (1 µL) and water (19 µL). A standard curve was performed using several concentrations of serum bovine albumin (BSA) and 1 µl of RIPA buffer. The plate was then incubated in the dark at 37 ° C for 30 minutes and the absorbance was measured by the spectrophotometer xMark ™ Microplate Absorbance Spectrophotometer at 562nm. The total protein concentration of the samples was calculated by the Lambert-beer law using the standard curve.

3.8. Western Blot

Total proteins (25 µg) from the 3 cell lines used were denatured at 100ºC for 5 minutes and later loaded and resolved in a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) by electrophoresis in at 180 V for 1 hour.

Protein transfer was carried out to a polyvinylidene difluoride (PVDF) membrane for 1 hour and 30 minutes or 2 hours at 4ºC and, depending on the use of 2 or 4 membranes, the amperage varied between 750 mA or 1 A. Then, membranes were placed in a blocking solution containing 5% of skimmed dried milk with washing buffer 1x, for 1 hour. Subsequently, membranes were incubated overnight at 4˚C with rabbit anti-Akt (1:500, #9272; Cell Signaling Technology, Danvers, Massachusetts, EUA), rabbit anti- Phospho-Akt (1:500, #9271; Cell Signaling Technology, Danvers, Massachusetts, EUA), rabbit anti-MAPK (1:500, #9102; Cell Signaling Technology, Danvers, Massachusetts, EUA), rabbit anti- Phospho-MAPK (1:500, #9101; Cell Signaling Technology, Danvers, Massachusetts, EUA), rabbit anti- C-Myc (1:500, #13748; Cell Signaling Technology, Danvers, Massachusetts, EUA) or mouse anti-E-cadherin (1:1000, sc-8426, Santa Cruz Biotechnology, Heidelber, Germany) primary antibodies. A mouse anti-β-actin monoclonal antibody (1:10000, A1978) was used for protein loading control in all Western Blot (WB) analysis. Goat anti-rabbit IgG HRP-linked (1:10000, #7074, Cell Signaling Technology, Danvers, Massachusetts, EUA) or m-IgGκ BP-HRP (1:20000, sc-516102; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies. The membranes were incubated with ECL substrate (Bio-Rad, Hercules, CA,

USA) for 5 minutes and scanned with the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). The band densities were obtained according to standard methods using the Image Lab 5.1 software (Bio-Rad, Hercules, CA, USA) and normalized by division with the respective β-actin band density.

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). $P<0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean (S.E.M).

Chapter 4
Results

4.1. TBT increased PCa cells viability, proliferation, migration, and invasion

The effect of TBT on the viability of non-neoplastic and neoplastic prostate cell lines was investigated by MTT and SRB methods. 10 nM and 100 nM TBT significantly decreased the viability of PNT1A cells for 48 hours (respectively 85.30 ± 1.50 and 77.33 ± 1.38-fold variation to TBT-untreated group, Figure 8 A), and no effect was observed with other TBT concentrations. In the androgen-sensitive PCa cells, LNCaP, there was an increase in cell viability after treatment with 100 nM TBT for 48 hours (133.8 ± 5.26-fold variation to TBT-untreated group, Figure 8 B). Moreover, the viability of the CRPC PC3 cells was significantly increased after 1 nM, 10 nM and 100 nM TBT treatment for 24 hours (respectively 118.3 ± 5.17, 120.4 ± 5.60 and 119.0 ± 5.25-fold variation to TBT-untreated group, Figure 8 C). Considering the SRB assay, a decrease in PNT1A cells viability was observed after stimulation with 100 nM TBT for 24 hours (81.18 ± 2.79-fold variation to TBT-untreated group, Figure 8 D). In addition, an increase in LNCaP cell viability was observed after treatment with 1 nM, 10 nM and 100 nM TBT for 48 hours (respectively 111.4 ± 2.95, 111.6 ± 2.01 and 109.8 ± 1.19-fold variation to TBT-untreated group, Figure 8 E). TBT (1 nM) significantly enhanced PC3 cell viability after 24 hours and 48 hours of treatment (respectively 119.5 ± 5.21 and 116.8 ± 0.85-fold variation to TBT-untreated group, Figure 8 F).

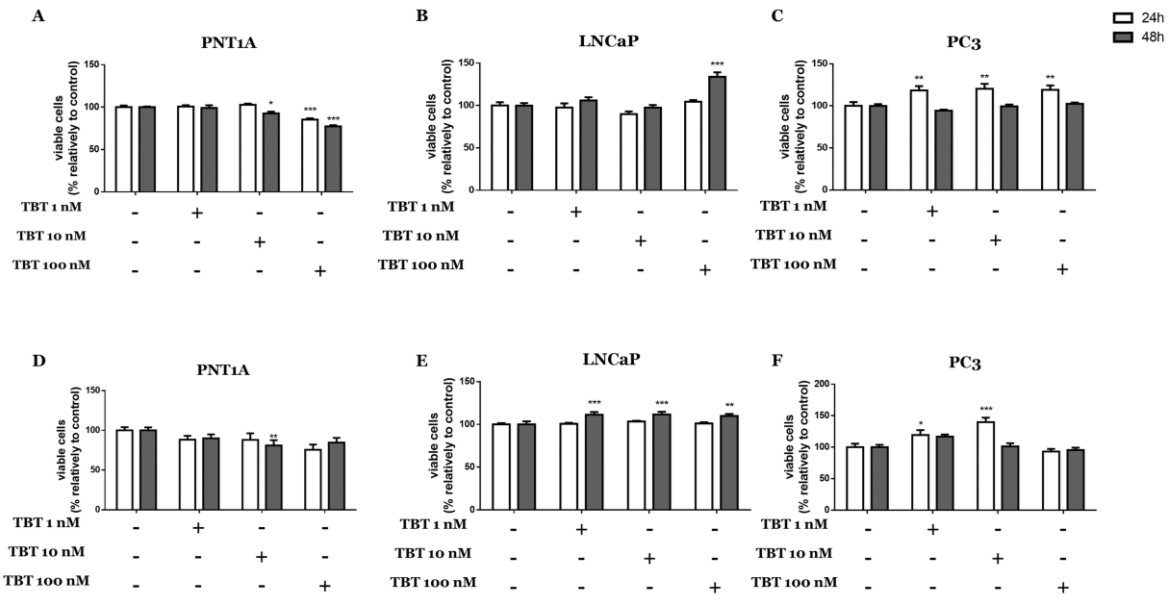

Figure 8. Effect of TBT on non-neoplastic, PNT1A, and neoplastic, LNCaP and PC3 cells viability after 24 and 48 hours of treatment. Percentage of viable cells was evaluated by the MTT (A, B and C) and SRB (D, E and F) assays. Results are expressed as % of control group. Error bars indicate mean ± S.E.M (n≥6). * p<0.05; ** p<0.01; *** p<0.001 when compared with the control group.

The immunofluorescent labeling of the nuclear proliferation marker Ki-67 was used to confirm the effect of 100 nM TBT on LNCaP cells proliferation. Indeed, cell proliferation was significantly augmented in the TBT treated cell line comparatively with the control (1.63 ± 0.14-fold variation to TBT-untreated group, Figure 9 A and 9 B).

The protein expression of key molecular targets involved in PCa cells proliferation/survival pathways in response to 100 nM TBT was evaluated by WB. TBT induced a significant increase in AR and c-Myc protein expression in LNCaP treated cells when compared to untreated cells (respectively 1.65 ± 0.23 and 1.18 ± 0.04-fold variation to TBT-untreated group, Figure 9 C). Akt and MAPK expression was significantly lower in LNCaP cells treated with TBT (respectively 0.54 ± 0.03 and 0.53 ± 0.08-fold variation to TBT-untreated group, Figure 9 C), whereas the expression of their phosphorylated forms, respectively p-Akt and p-MAPK, did not present significant differences.

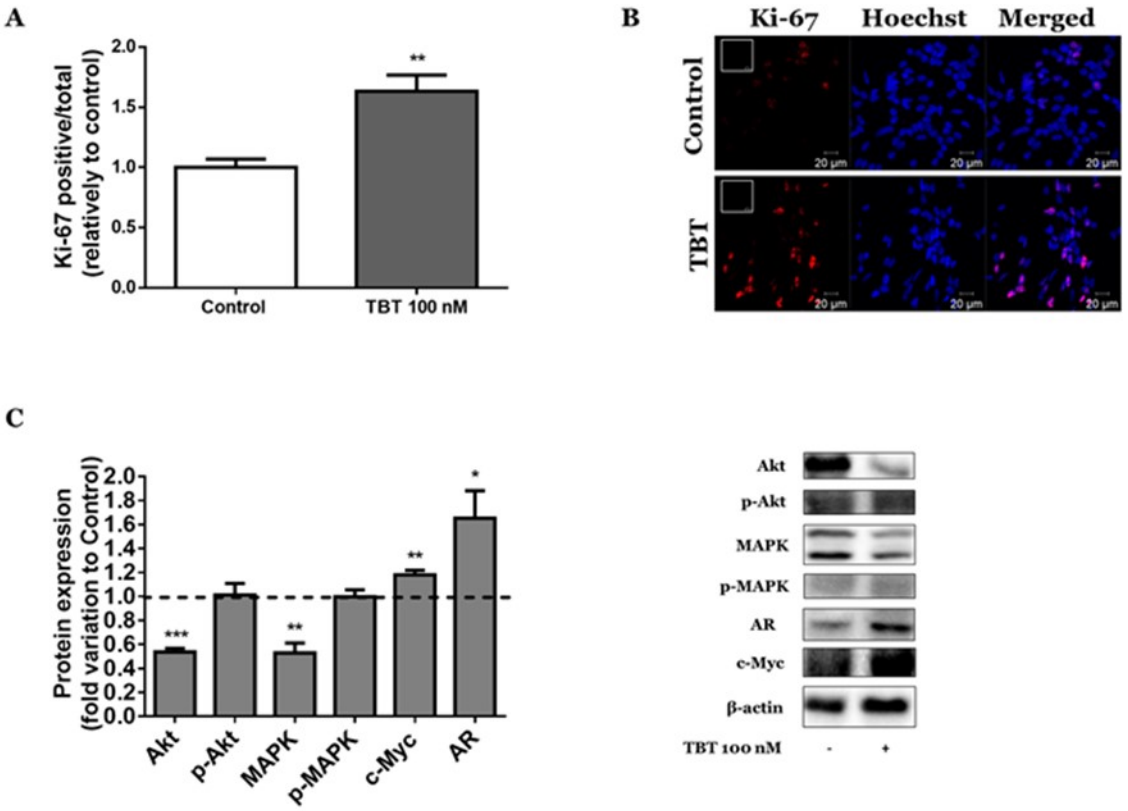

Figure 9. Effect of 100 nM TBT on the expression of proliferation/ survival key regulators (Akt, p-Akt, MAPK, p-MAPK and c-Myc) and proliferation in LNCaP cells after 48 hours of treatment. (A) Representative confocal microscopy images showing Ki-67 labelling in the different groups. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue) and Ki-67 positive staining appears as red. Negative controls for Ki-67 were obtained by omission of the primary antibody and are provided as insert panels (-). (B) Proliferation of LNCaP cells was determined by the immunofluorescence analysis of Ki67. Data are expressed as the mean of Ki67-positive cells relatively to the total cell number. (C) Protein expression was analysed by WB after normalization with β-actin. All results are expressed as fold-variation relative to the control group (dashed line). Representative immunoblots are shown in the right panel. 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.

Transwell assays were performed to assess cell migration and invasion of LNCaP cells stimulated or non-stimulated with 100 nM TBT. TBT treatment significantly increased LNCaP cell migration (1.22 ± 0.09-fold variation to TBT-untreated group, Figure 10 A) and cell invasion (1.32 ± 0.04-fold variation to TBT-untreated group, Figure 10 B). The expression of the adhesion protein, E-cadherin, that is usually lost with the EMT transition, increasing the invasion capacity of the cells, was studied by western blot. E-cadherin expression was significantly decreased in LNCaP cells treated with TBT when compared with the control group (respectively, 0.62 ± 0.09-fold variation to control, Figure 10 C).

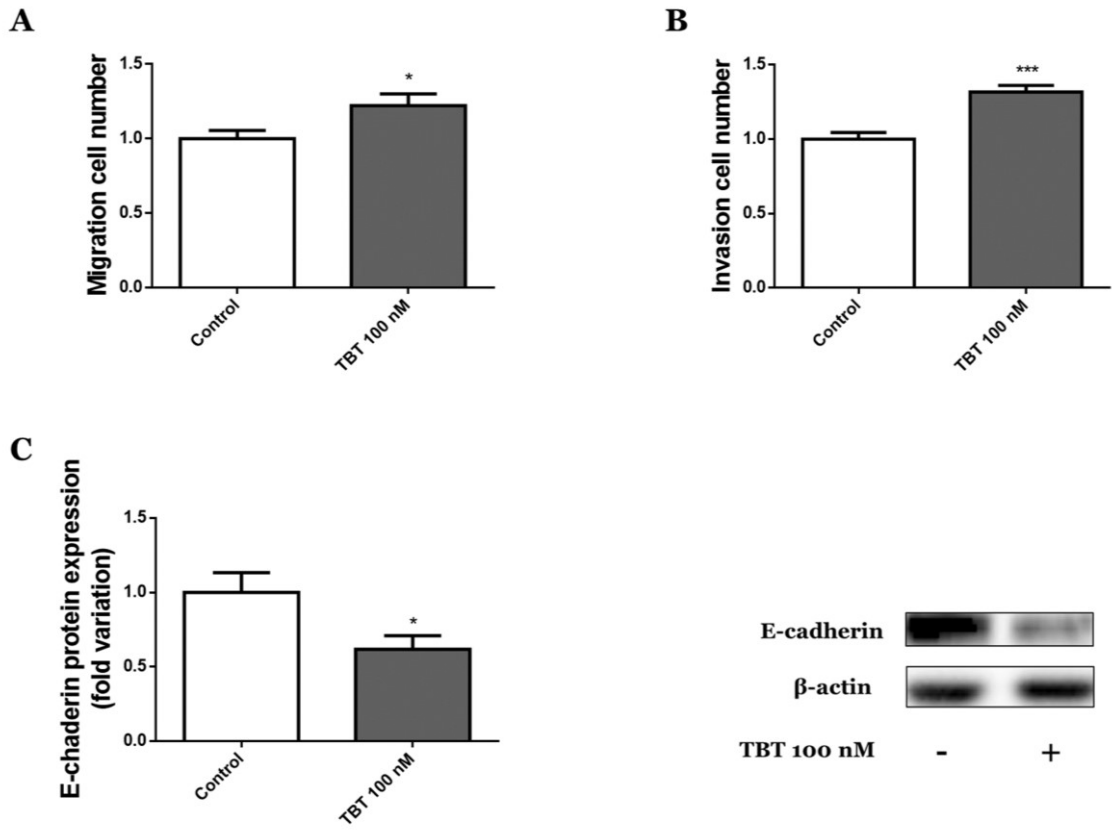

Figure 10. Effect of 100 nM TBT on migration (A), invasion (B) and on the expression of E-cadherin (C) in LNCaP cells after 48 hours of treatment. (A) Migration and (B) invasion of LNCaP cells were determined by trans-wells assays in uncoated chambers or in Matrigel coated chambers, respectively. The upper chamber contained serum free medium and LNCaP cells in the presence or absence of TBT. Lipid depleted medium in the lower chamber was used as chemoattractant. Data are expressed as the mean number of migrating cells per 20x magnification field (10 fields were assessed for each experimental condition). (C) Protein expression was analysed by WB after normalization with β-actin. All results are expressed as fold-variation relative to the control group. Results are expressed as % of control group. Error bars indicate mean ± S.E.M (n=6). * $p < 0.05$; *** $p < 0.001$ when compared with the control group.

4.2. TBT increased LNCaP cells viability, proliferation, and migration in conditions of high LDL-cholesterol availability

The effect of TBT at a concentration previously found in man, 10 nM, with or without LDL and DHT supplementation, non-neoplastic and neoplastic prostate cells viability was investigated by MTT. The stimulation with TBT promoted an increase in LNCaP and PC3 cells viability after 48 hours of treatment (respectively 134.9 ± 1.01 and 106.7 ± 10.00-fold variation to control, Figure 11 B and C). LDL-stimulation significantly augmented the viability of PNT1A and PC3 cells after 48 hours of treatment (respectively 130.7 ± 3.49 and 212.6 ± 12.91-fold variation to control, Figure 11 A and C). There was also a significant increase in all prostate cells evaluated, PNT1A, LNCaP and PC3, when treated with TBT in LDL positive conditions comparatively with the control non-treated cells (respectively 124.6 ± 3.60, 141.1 ± 6.49 and 208.2 ± 8.25-fold variation to control, Figure 11 A, B and C). Moreover, the viability of PNT1A, LNCaP and PC3 prostate cells was significantly increased when TBT was combined with LDL and DHT treatment when compared with the respective untreated cells (respectively 126.5 ± 5.69, 184.3 ± 4.42 and 193.1 ± 5.84-fold variation to control, Figure 11 A, B and C). The viability of LNCaP cells treated with TBT and in DHT and LDL positive conditions is significantly higher than the group treated only with TBT or with TBT and LDL (respectively 134.9 ± 1.01, 141.1 ± 6.49 and 184.3 ± 4.42-fold variation to control, Figure 11 B).

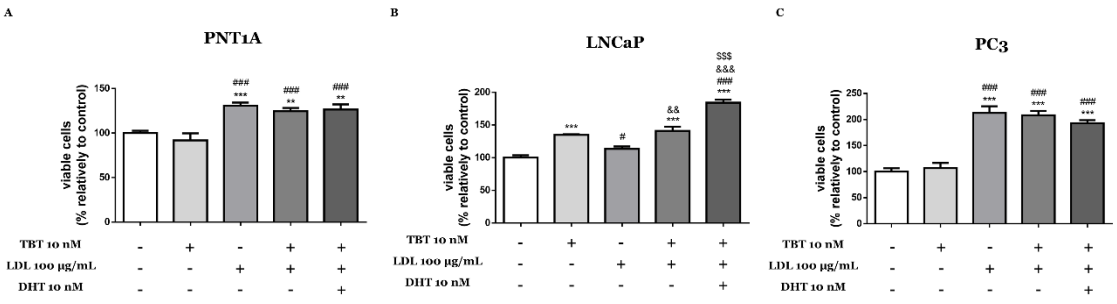

Figure 11. Effect of TBT (10 nM), LDL (100 µg/ml) and DHT (10 nM) on non-neoplastic, PNT1A (A) and neoplastic, LNCaP (B) and PC3 (C) cells viability. Cell viability was determined by the MTT assay after 48 hours of treatment. All results are expressed as fold-variation relative to the control group. Results are expressed as % of control group. Error bars indicate mean ± S.E.M (n=6). ** p< 0.01, *** p< 0.001, when compared with the control group; # p< 0.05; ### p< 0.001 when compared with the TBT (10 nM); && p< 0.01 &&& p< 0.001 when compared with the LDL (100 µg/ml); $$$ p< 0.001 when compared with the LDL (100 µg/mL) + TBT (10 nM).

The immunofluorescent labelling of Ki-67 and the expression of proteins involved in proliferation/ survival signaling pathways was analysed in LNCaP cells stimulated with TBT (10 nM) in LDL-cholesterol supplemented conditions for 48 hours as above described. Cell proliferation was significantly increased in the 10 nM TBT treatment, in the presence and absence of LDL and/or DHT, presented higher proliferation index than non-treated cells (respectively 1.18 ± 0.05, 1.54 ± 0.06 and 1.74 ± 0.07-fold variation to control, Figure 12 A and 12 B). An increase on cell proliferation of the group treated with TBT in LDL positive conditions was observed, an effect that was intensified in LDL and DHT combined conditions (Figure 12 B and 12 C). Concerning MAPK expression, there was a significant decrease of this protein expression in LNCaP cells treated with TBT and with LDL and DHT supplementation when compared to the control group (0.67 ± 0.007-fold variation to control, Figure 12 C). There was no significant difference on the expression of Akt, p-Akt, p-MAPK and c-Myc proteins between the experimental groups evaluated (Figure 12 C).

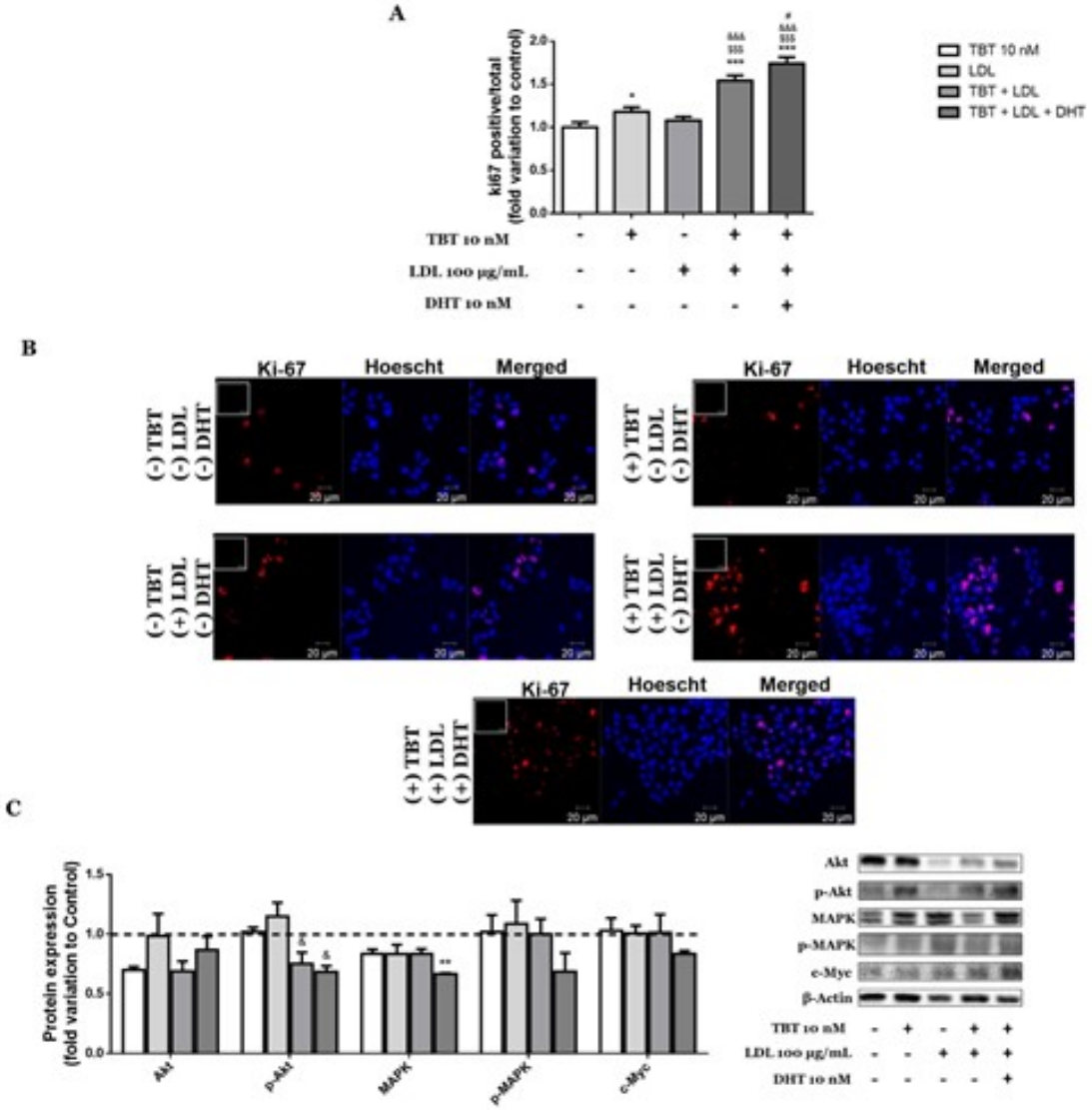

Figure 12. Effect of TBT (10 nM), LDL (100 µg/ml) and DHT (10 nM) on the expression of proliferation/ survival key regulators (Akt, p-Akt, MAPK, p-MAPK and c-Myc) and proliferation in LNCaP cells after 48 hours of treatment. (A) Representative confocal microscopy images showing Ki-67 labelling in the different groups. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue) and Ki67 positive staining appears as red. Negative controls for Ki-67 were obtained by omission of the primary antibody and are provided as insert panels (-). (B) Proliferation of LNCaP cells was determined by the immunofluorescence analysis of Ki-67. Data are expressed as the mean of Ki67-positive cells relatively to the total cell number. (C) Protein expression was analysed by WB after normalization with β-actin. All results are expressed as fold-variation relative to the control group (dashed line) Representative immunoblots are shown in the right panel. 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 when compared with the control TBT (10 nM); & p< 0.05; &&& p< 0.001 when compared with the LDL (100 µg/ml); $$$ p< 0.001 when compared with the LDL (100 µg/ml) + TBT (10 nM).

Transwell assays were used to assess cell migration and invasion in cells treated with TBT (10nM), LDL and/or DHT versus non-supplemented conditions. Treatment with TBT in the presence or absence of LDL significantly increased LNCaP cells migration when compared with the control group (1.42 ± 0.08, and 1.49 ± 0.05-fold variation to control, Figure 12A). LNCaP cells stimulated with TBT, LDL and DHT significantly enhanced cell migration when compared with groups treated only with TBT or with TBT plus LDL (respectively 1.42 ± 0.08, 1.49 ± 0.05 and 2.14 ± 0.10-fold variation to control, Figure 12 A). Relatively to invasion assay, there was a significant increase in LDL treated cells invasion compared with the control group (4.10 ± 0.47-fold variation to control, Figure 12 B), an effect reversed in the presence of TBT (2.72 ± 0.35-fold variation to control, Figure 12 B). Concerning E-cadherin, a significantly decreased was verified in the groups with LDL comparatively to TBT (+)/LDL (-)/DHT (-) group (0.69 ± 0.16, 0.30 ± 0.02 and 0.34 ± 0.03-fold variation to TBT group, Figure 12 C).

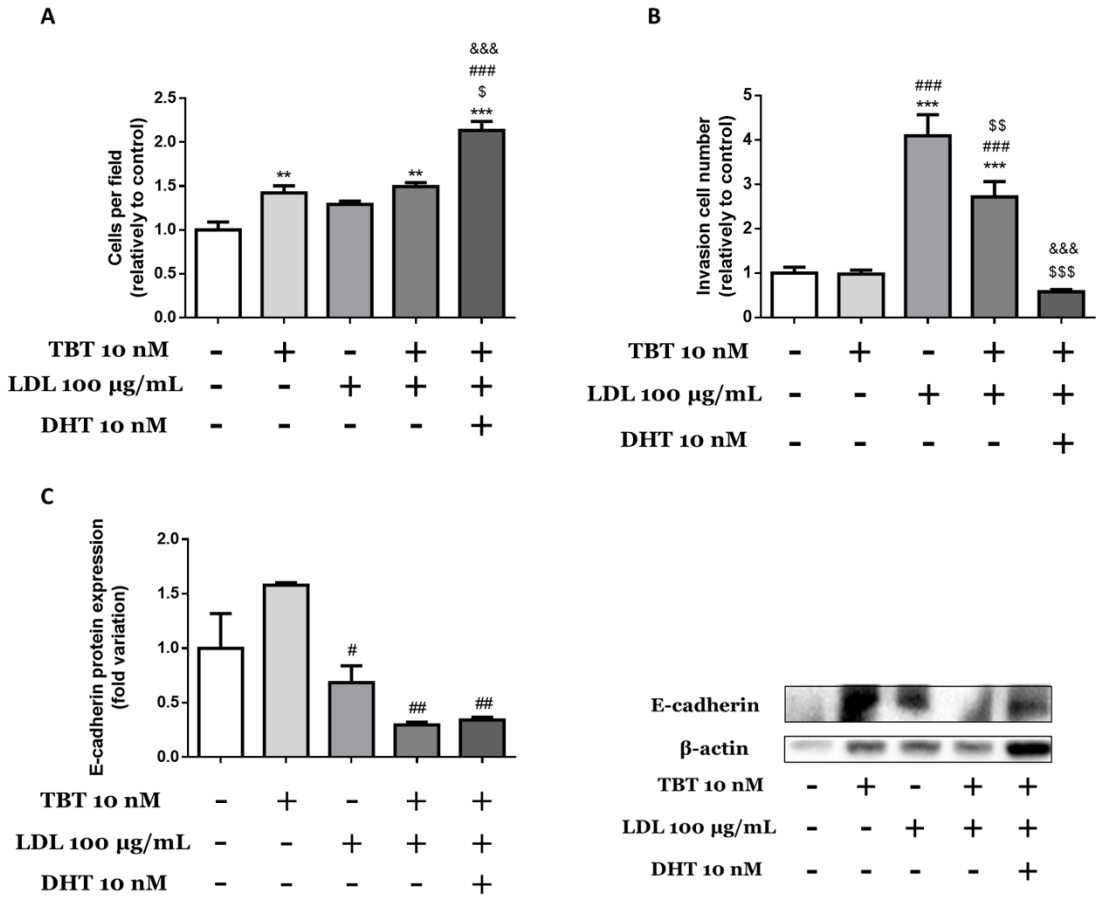

Figure 13. Effect of TBT (10 nM), LDL (100 µg/ml) and DHT (10 nM) on migration (A), invasion (B) and on the expression of E-cadherin (C) in LNCaP cells after 48 hours of treatment. Migration (A) and invasion (B) of LNCaP cells was determined by trans-wells assays in uncoated chambers and in Matrigel coated chambers respectively. The upper chamber contained serum free medium and LNCaP cells in the presence or absence of TBT, LDL and DHT. Lipid depleted medium in the lower chamber was used as chemoattractant. Data are expressed as the mean number of migrating cells per 20x magnification field (10 fields were assessed for each experimental condition). (C) Protein expression was analysed by WB after normalization with β-actin. All results are expressed as fold-variation relative to the control group Error bars indicate mean ± S.E.M (n=6). **p< 0.01, *** p< 0.001 when compared with the control group, when compared with the control group; # p<0.05, ## p< 0.01, ### p< 0.001 when compared with the control TBT (10 nM); &&& p< 0.001 when compared with the LDL (100 µg/ml); $ p< 0.05, $$ p< 0.01, $$$ p< 0.001 when compared with the LDL (100 µg/ml) + TBT (10 nM).

Chapter 5
Discussion and Conclusion

The present dissertation explored the effects of TBT on the viability, proliferation, and migration of human prostate cells. To infer about the TBT effects along PCa progression, the non-neoplastic epithelial PNT1A cells and the neoplastic LNCaP (androgen-sensitive) and PC3 (androgen-insensitive) cells were used in the different experiments. In addition, to ascertain whether obesity may change the TBT action, the effect of this obesogen in the presence of LDL cholesterol and DHT was investigated.

As a starting point, the effect of different concentrations of TBT on prostate cells viability was evaluated. In the non-neoplastic cell line, PNT1A, TBT decreased cell viability and cell mass as shown by the MTT and SRB assays, respectively (Figure 8 A and D). On the other hand, TBT increased the viability of neoplastic cell lines, LNCaP and PC3, in a time-dependent manner (Figure 8 B, C, E and F). The increase in PCa cells growth follows the observations in the literature using other EDCs with androgen-like activity, like organotins [117, 171-173]. In addition, other reports showed the promotion of LNCaP cells viability under exposure to 100 nM TBT, with the activation of AR-mediated transcription [117]. In breast cancer cells, an increased viability with TBT treatment was shown, which involved the action of the RXRs pathway and PPARγ subtype signaling [117, 174-177]. Herein, we reported noticeable differences on the effect of TBT influencing the viability of neoplastic and non-neoplastic prostate cells. As indicated, TBT decreased the viability of PNT1A and increased the viability of LNCaP cells (Figure 8). These results are extremely relevant pointing out a distinct effect of TBT in the normal prostate epithelium and cancer cells.

Taking into account the results of TBT on cell viability, the concentration of 100 nM and the exposure time for 48 hours were selected for analysing proliferation, migration, and invasion in LNCaP cells. Moreover, the concentration selected is in agreement with the range of TBT levels found in human blood after chronic exposure to check the effect of TBT (10-100 nM) [178, 179].

In the last decades, fundamental research and the empowerment of methodological approaches have disclosed the great complexity of cancer biology. Several genes that control cell proliferation and cell cycle and their effects on the establishment of the malignant phenotype have been discovered. Importantly, the main stimulators of prostate cell proliferation are androgens. The AR modulates the expression of AR target genes that are mainly associated with the regulation of cell cycle, survival and growth [180-183]. Considering the existent literature indicating the androgenic activity of TBT, we decided to evaluate the effects of TBT on AR expression (Figure 9 C). AR expression was increased with TBT treatment in the androgen-responsive LNCaP cells (Figure 9 C),

which was followed by changes on LNCaP cells proliferation and altered expression of key regulators of cell survival.

An increase on LNCaP cells proliferation was found with TBT stimulation (Figure 9 A and B), which follows Yamabe, Hoshino [117] previous findings showing an increase in the number of TBT-treated PCa cells.

The effect of TBT in modulating the behavior of LNCaP cells was supported by changes in the protein fingerprint of key targets of survival and oncogenic pathways. The Akt pathway plays a major role in the survival of PCa cells and has been associated with poor clinical outcomes [184-187]. However, TBT had no effect on the expression of p-AKT in LNCaP cells (Figure 12 C).

The ERK pathway is another important pathway involved in the promotion of cell proliferation and survival [188, 189]. The expression of active p-ERK was not changed in LNCaP cells after TBT treatment (Figure 9 C). However, in breast cancer cells, TBT increased the levels of p-ERK [177, 190, 191]. Nevertheless, the relationship between PCa and the ERK pathway is controversial. There are studies that demonstrate a decline in ERK activity in advanced tumors, whereas others have shown the activation of this pathway [192, 193].

c-Myc is an oncogene commonly overexpressed in PCa, which has an important role on cancer progression and establishment of the CRPC phenotype [194-196]. It is also recognized the coordinated action of c-Myc and the AR in the development of PCa and breast cancer [194, 197, 198]. We showed here that the expression of c-Myc increased in LNCaP cells in response to TBT (Figure 9 C). These results indicate that TBT has oncogenic properties, stimulating proliferation of PCa, which can be related with an increase on c-Myc and AR expression [117].

The stroma is able to sustain PCa invasion due to the release of factors capable of digest the extracellular matrix (ECM) [199], leading to the migration, invasion and spread of cancer cells to other tissues. Over time, cancer cells acquire the capability to migrate and change their position within tissues or between different organs, a process called migration [200]. Invasion is the cell movement, which is accompanied by a restructuring of basement membrane, and cells must modify its shape and interact with the ECM [201].

Besides the promotion of cell viability and proliferation, TBT also increased the migration and invasion capability of LNCaP cells (Figure 10 A and B). Moreover, the increase on the migration and invasion in response to TBT treatment was underpinned by the altered expression of an important EMT marker, E-cadherin (Figure 10 C). E-cadherin is a key molecule in cell-to-cell adhesion, and its loss is associated with PCa metastasis and chemoresistance [202-204]. There are other crucial EMT markers, such as vimentin and N-cadherin, but their protein expression was undetectable (data not

shown). Indeed, it has been described that LNCaP cells show low levels of vimentin [205, 206], being negative for N-cadherin [207].

Besides the androgenic properties, TBT is known as obesogen leading to the increase of adipocyte number, size and differentiation [208, 209]. TBT also changes important metabolic pathways related with hormone regulation of appetite and satiety [210]. TBT and its metabolites are degraded over time and then excreted, mainly through urine and feces, but also through bile. However, it is dependent on the degree of exposure, and exposure to high concentrations causes accumulation in the adipose tissue and liver [211]. This fact underpins the other aim of this thesis, hypothesizing that the effects of TBT may vary with cholesterol availability. This is highly important as obesity has been associated with the aggressiveness of PCa and poor prognosis [85-87]. Therefore, the next step of this dissertation was to assess the effect of a lower concentration of TBT (10 nM) in cell fate in the presence of LDL-cholesterol and DHT. The rationale for presence of DHT was to mimic the androgens levels on man, while LDL-cholesterol was used to simulate hypercholesterolemia and obesity-like conditions. 10 nM TBT increased LNCaP cells viability (Figure 11 B), which was augmented by the presence of LDL (Figure 11 A, B and C), and the effect was amplified upon exposure to DHT (Figure 11 A, B and C). We also found that the combined action of TBT and LDL promoted cell proliferation, an effect that was increased in the presence of DHT (Figure 12 A and B). In fact, our research group previously showed that DHT increased LNCaP cell viability and proliferation in conditions of high LDL availability [212]. Other studies demonstrated that the presence of androgens potentiate the effects of LDL on AR-positive-LNCaP cells, which was supported by the evidence that the androgens increased LDL uptake [213-216]. Importantly, cholesterol is the precursor for steroidogenesis and thus may fuel intratumoral androgen synthesis, accelerating the growth of prostate tumors [217]. With the information obtained, we may suppose that a cooperative relationship exists between TBT, LDL and androgens promoting PCa cell growth. Moreover, high availability of LDL is known to promote cell proliferation in various types of cancer, namely, breast cancer, squamous cell carcinoma of the esophagus, colorectal cancer, PCa, postmenopausal breast cancer, and gastric cancer [204-207, 218, 219]. It was also demonstrated the proliferation of breast cancer cells in a mouse model of hyperlipidemia [220], and that the inhibition of LDLr sensitized pancreatic cancer and PCa cells to chemotherapeutic drugs [95, 221]. All these results sustain the relevance of lipids and obesogenic compounds in cancer growth.

Thereafter, the effects of TBT, LDL and DHT in modulating LNCaP cells behavior were investigated. No changes were found on the protein fingerprint of key regulators of survival and oncogenic pathways such as Akt, p-Akt, MAPK, p-MAPK and c-Myc (Figure

12 C) [222]. Although some studies have demonstrated a relationship between high levels of LDL and the increased expression of the p-ERK pathway, in our study this relationship was not confirmed [223-225].

The results obtained also showed the stimulatory effect of TBT (10 nM) on LNCaP cells migration, which was potentiated by the presence of LDL and DHT (Figure 13 A). Interestingly, TBT had an inhibitory effect on the increased invasion seen in the LDL group, an effect further enhanced by the combination of TBT and DHT (Figure 13 B). In addition, E-cadherin expression was decreased upon the stimulus with LDL (Figure 13 C). At our knowledge, no other studies have evaluated the effect of TBT in the presence of LDL and DHT in PCa or other cancer types. These findings further support the idea that obesity acts as a potent inductor of cancer progression, since we verified that LDL promotes cell migration/invasion through the loss of EMT marker E-cadherin, a crucial molecule in cell-to-cell adhesion. Future studies will be necessary to clarify the discrepancy in the results obtained in migration/invasion with the presence of TBT and DHT in LDL conditions.

The effects of TBT promoting cell viability, proliferation, and migration/invasion of PCa cells even in lower concentrations are somehow alarming and allow to suppose that in the human body may contribute to worsening the aggressiveness of PCa. In addition, the effect of TBT can be more impactful in obesity conditions, which highlights the greater danger in obese PCa patients with the potentiation of development of metastasis.

Although the use of TBT has been banned, it remains in the environment, moving up in the food chain and with effects passing from one generation to another. Even so, the information concerning the liaison of TBT with human diseases, and specifically PCa, is scarce. For this reason, our results also strongly contribute to the improvement of scientific knowledge in the field and to raise aware about the possible consequences of exposure to this obesogen in PCa development/ progression.

Chapter 6

Future Perspectives

The present dissertation showed that TBT, an EDC with androgenic and obesogenic characteristics, promote proliferation, migration, and invasion of PCa cells, particularly LNCaP cells. These results were underpinned by the increased expression of c-Myc and AR in TBT treated cells. In the future it would be interesting to evaluate if these effects occur through the activation of the AR. Inhibition of AR could be performed using specific drugs for these receptors, such as enzalutamide, bicalutamide, flutamide, among others. Besides AR activation, we intend to study other mechanisms by which TBT may act, as RXRs and PPARγ signaling pathway activation. In addition, investigate the effects of TBT in another important cancer hallmarks of cancer, as apoptosis is also a future perspective.

Moreover, another important approach would be to study TBT effects in more complex models, like co-culture and *in vivo* models. This step is fundamental because TBT and other obesogens are partially metabolized in the liver and then accumulated in the liver and adipose tissue. In addition, it seems extremely important to study not only TBT, but also its metabolites partially metabolized in the liver. Taking into account the obesogen properties of TBT and its accumulation on adipose tissue, an experiment with complex co-culture models with adipocytes exposed with TBT interacting with PCa cells will be interesting.

Additionally, it was shown that a low concentration of TBT in LDL conditions promotes the proliferation and migration of LNCaP cells. However, we obtained quite intriguing results regarding cell invasion, and we did not obtain significant differences in different protein targets. In the future we will repeat the invasion protocol and some of the protein fingerprinting of key survival targets and oncogenic pathways such as c-Myc, p-Akt and p-MAPK.

In the future, new studies evaluating TBT effects in LDL conditions in non-neoplastic cells will be performed. Moreover, the exposure of prostate cells to TBT at lower concentrations over a long period of time will be performed, to mimic the real exposure to the obesogen.

Despite the important preliminary results obtained, the present thesis opens up a sea of unexplored hypotheses to clarify the importance of obesogens in cancer.

Chapter 7
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