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Endocrine disruptors, obesity, and prostate cancer: a metabolic perspective

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Dedicatória

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Resumo

O cancro da próstata é um dos cancros mais diagnosticados no homem e representa a quinta causa de morte por cancro. Atualmente, os fatores de risco descritos para o cancro da próstata incluem fatores endógenos, nomeadamente o envelhecimento, etnia, hormonas, história familiar, fatores genéticos e stresse oxidativo, ou exógenos, tais como dieta, obesidade, sedentarismo, estilo de vida e fatores ambientais. Recentemente, foi reportado que fatores extrínsecos contribuem com mais de 99,9% para o risco de desenvolvimento do cancro da próstata. Os fatores extrínsecos como o estilo de vida, a dieta ou os fatores ambientais, como radiação UV, carcinogénicos e exposição a pesticidas têm sido indicados na etiologia do cancro da próstata. Os disruptores endócrinos são um grupo de compostos que podem interferir com o sistema endócrino, que inclui alterações na produção, secreção, transporte, e/ou ação das hormonas. Existe um subconjunto de disruptores endócrinos que alteram o metabolismo energético favorecendo o armazenamento de lípidos e levando a uma predisposição para a obesidade, sendo por este motivo, designados obesogénios. O tributilestanho (TBT) é um disruptor endócrino amplamente utilizado em diversas ações humanas e disperso no meio ambiente, que possui propriedades obesogénicas e também androgénicas. O metabolismo é definido como um conjunto de processos bioquímicos em organismos vivos, sendo responsável pelo uso de nutrientes e produção de energia em humanos e outros organismos. A reprogramação metabólica é uma característica bem conhecida do cancro e vários estudos demostraram que as células do cancro da próstata têm a capacidade de reprogramar o metabolismo de modo a garantir a sua sobrevivência e possibilidade de metastizar. Contudo, a desregulação metabólica induzida pela obesidade ou obesogénios, nomeadamente o TBT, no metabolismo das células do cancro da próstata permanece altamente desconhecido. Deste modo, o objetivo desta dissertação foi avaliar o efeito do TBT na regulação do metabolismo glicolítico e lipídico das células do cancro da próstata e a influência de condições de obesidade nas ações do TBT. Assim, células da próstata não neoplásicas (PNT1A) e neoplásicas (LNCaP e PC3) foram estimuladas com TBT (10 ou 100 nM), LDL e/ou 5α-di-hidrotestosterona (DHT) por 48 horas. Posteriormente, o consumo de glucose, produção de lactato e atividade enzimática da lactato desidrogenase (LDH) foram determinados através de análise espectrofotométrica. A expressão proteíca de reguladores alvo do metabolismo glicolítico e lipídico foi analisada por Western blot. A quantificação do conteúdo lipídico (“lipid droplets” (LDs)) foi determinada através da coloração com Oil Red-O. O tratamento com 100 nM de TBT estimulou o fluxo glicolítico ao aumentar o consumo de glucose, a produção de lactato e a atividade da LDH nas células de cancro da próstata sensíveis a androgénios, LNCaP. Estes resultados foram suportados pelo aumento da expressão da enzima glicolítica fosfofrutocinase-

1 e do transportador de monocarboxilato 4. Para além disso, o tratamento com TBT também estimulou o metabolismo lipídico aumentando a expressão das enzimas acetil-CoA carboxilase e sintase de ácidos gordos nas células PNT1A e LNCaP, o que foi sustentado pelo aumento do conteúdo lipídico. O aumento dos LDs também foi observado em células LNCaP estimuladas com 10 nM de TBT. Esse efeito foi suprimido pela disponibilidade de LDL. De um modo geral, os resultados obtidos demonstram que o TBT induz a disrupção metabólica das células do cancro da próstata, estimulando o metabolismo glicolítico e lipídico, o que pode predispor para o desenvolvimento e progressão do cancro da próstata. Além disso, o TBT na presença de LDL desregula o metabolismo lipídico induzindo o uso de lípidos, o que pode contribuir para a aceleração da progressão do cancro.

Palavras-chave

Cancro da próstata; metabolismo; disruptores endócrinos; tributilestanho (TBT); obesidade

Resumo Alargado

O cancro da próstata é um dos cancros mais diagnosticados no homem e representa a quinta causa de morte por cancro. O cancro da próstata apresenta-se como uma patologia complexa que envolve vários mecanismos moleculares, que podem determinar o início da doença, não sendo hoje em dia totalmente compreendidos. Numa fase inicial da doença, as células tumorais são dependentes da ação estimuladora dos androgénios para a sua sobrevivência e proliferação. A descoberta da dependência androgénica das células foi o que permite ainda hoje em dia a utilização da terapêutica de privação androgénica, a qual tem por base a redução dos níveis circulantes de androgénios ou o bloqueio das suas ações. Contudo, usualmente o cancro progride para estadios mais agressivos, resistentes ao tratamento, sendo as células capazes de metastizar para locais distantes, nomeadamente para os gânglios linfáticos ou osso, sendo este estadio designado por cancro da próstata resistente à castração. Alguns estudos demonstraram que a incidência do cancro da próstata apresenta variações significativas entre as populações que vivem em diferentes áreas, e essas diferenças podem estar associadas a um conjunto de diferentes fatores de risco. Atualmente, os fatores de risco descritos para o cancro da próstata incluem fatores endógenos, nomeadamente o envelhecimento, etnia, hormonas, história familiar, fatores genéticos e stresse oxidativo, ou exógenos, tais como a dieta, a obesidade, o sedentarismo, o estilo de vida e os fatores ambientais. A obesidade e a hipercolesterolemia têm sido associadas à agressividade e progressão mais rápida do cancro da próstata. Recentemente, foi reportado que fatores extrínsecos contribuem com mais de 99,9% para o risco de desenvolvimento do cancro da próstata. Os fatores extrínsecos como o estilo de vida, dieta ou os fatores ambientais, como radiação UV, carcinogénicos e exposição a pesticidas têm sido indicados na etiologia do cancro da próstata. Os disruptores endócrinos são um grupo de compostos que podem interferir com o sistema endócrino, que inclui alterações na produção, secreção, transporte, e/ou ação das hormonas. A exposição aos disruptores endócrinos pode aumentar a incidência e prevalência de várias doenças, como cancro, diabetes, obesidade e infertilidade. Existe um subconjunto de disruptores endócrinos que alteram o metabolismo para beneficiar o armazenamento de lípidos, levando a uma predisposição à obesidade, designado de obesogénios. O tributilestanho (TBT) é um disruptor endócrino amplamente utilizado em diversas ações humanas e disperso no meio ambiente, que possui propriedades obesogénicas e também androgénicas. Os estudos com este disruptor começaram nos anos 70 no qual foi descrito um fenómeno designado ‘’imposex’’ nos gastrópodes, sendo este efeito um dos mais bem conhecidos deste composto. O TBT parece atuar através da ativação do RXR-PPARγ, promovendo a adipogénese e o armazenamento de lípidos. Além disso, sabe-se que este composto induz a proliferação das células do cancro da próstata.

O metabolismo é definido como um conjunto de processos bioquímicos em organismos vivos, sendo responsável pelo uso de nutrientes e produção de energia em humanos e outros organismos. As células cancerígenas utilizam fontes alternativas de energia de acordo com as suas necessidades, como glucose, lactato e ácidos gordos. De forma a satisfazer as altas taxas proliferativas e necessidades energéticas as células cancerígenas usam de modo prevalente a glicólise em detrimento da fosforilação oxidativa mesmo na presença de oxigénio, obtendo grandes quantidades de lactato e consequente acidificação do microambiente tumoral, contribuindo assim para a progressão do cancro. A reprogramação metabólica é uma característica bem conhecida do cancro e vários estudos mostraram que as células do cancro da próstata têm a capacidade de reprogramar o metabolismo para sobreviver e metastizar, conferindo uma vantagem adaptativa. A reativação metabólica ocorre em diversas vias metabólicas destacando-se a glicólise e o metabolismo lipídico. Contudo, o efeito de obesogénios, nomeadamente o TBT, no metabolismo das células do cancro da próstata permanece altamente desconhecido. Deste modo, o objetivo desta dissertação foi avaliar o efeito do TBT na regulação do metabolismo glicolítico e lipídico das células do cancro da próstata e a influência de condições de obesidade nas ações do TBT. Assim, células da próstata não neoplásicas (PNT1A) e neoplásicas (LNCaP e PC3) foram estimuladas com TBT (10 ou 100 nM), LDL e/ou 5α-di-hidrotestosterona (DHT) por 48 horas. Posteriormente, o consumo de glucose, produção de lactato e atividade enzimática da lactato desidrogenase (LDH) foram determinados através de análise espectrofotométrica. A expressão de proteínas de reguladores alvo do metabolismo glicolítico e lipídico foi analisada por Western blot. A quantificação do conteúdo lipídico (“lipid droplets” (LDs)) foi determinada através da coloração com Oil Red-O. O tratamento com 100 nM de TBT estimulou o fluxo glicolítico ao aumentar o consumo de glucose, a produção de lactato e a atividade da LDH nas células sensíveis a androgénios LNCaP. Estes resultados foram suportados pelo aumento da expressão da enzima glicolítica fosfofrutocinase-1 e do transportador de monocarboxilato 4. Para além disso, o tratamento com TBT também estimulou o fluxo lipídico aumentando a expressão das proteínas acetil-CoA carboxilase e sintase de ácidos gordos nas células PNT1A e LNCaP, o que foi sustentado pelo aumento no conteúdo lipídico. O aumento dos LDs também foi observado em células LNCaP estimuladas com 10 nM de TBT, no entanto esse efeito foi suprimido pela disponibilidade de LDL. De um modo geral, os resultados obtidos demonstram que o TBT contribuiu para a disrupção metabólica das células do cancro da próstata, estimulando o metabolismo glicolítico e lipídico, podendo predispor o desenvolvimento e progressão do cancro da próstata. Além disso, o TBT na presença de LDL desregula o metabolismo lipídico, induzindo o uso de lípidos pelas células tumorais, o que pode contribuir para a aceleração da progressão do cancro. Assim, estas descobertas suportam os dados existentes que ligam a obesidade, os androgénios e o

cancro da próstata. Ressaltam ainda a importância que pode ter a exposição ambiental a obesogénios em doentes de cancro da próstata obesos e com níveis de LDL aumentados.

Abstract

Prostate cancer (PCa) is one of the most diagnosed cancer in men and represents the fifth leading cause of cancer death. In the current state the risk factors for PCa include endogenous factors, namely aging, ethnicity, hormones, family history, genetic factors and oxidative stress, or exogenous, such as diet, obesity, physical inactivity, lifestyle and environmental factors. It was recently reported that extrinsic factors contribute more than 99.9% to the risk of PCa development. Extrinsic factors like lifestyle, dietary factors or environmental factors like UV radiation, carcinogens and pesticide exposure have been implicated in the etiology of PCa. Endocrine-disrupting chemicals (EDCs) are a group of compounds that can interfere with the endocrine system, which includes alterations in hormone production, secretion, transport, and/or action. There is a subset of EDCs that alter the metabolism to benefit the storage of lipids, leading to a predisposition to obesity, called obesogens. Tributyltin (TBT) is an EDC widely used and dispersed in the environment, which has obesogenic and androgenic properties. Metabolism is defined as a set of diverse biochemical processes in living organisms, being responsible for the use of nutrients and energy production in humans and other organisms. The metabolic reprogramming is a well-known hallmark of cancer and several studies showed that PCa cells have the ability of reprogramming metabolism to survive and metastasize. However, the metabolic deregulation induced by obesity or obesogens, namely TBT, in metabolism of PCa cells remains unknown. Thus, the aim of this dissertation was to evaluate the effect of TBT regulating the glycolytic and lipid metabolism of PCa cells, and the influence of obesogenic conditions on TBT actions. Therefore, non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) human prostate cells were stimulated with TBT (10 or 100 nM), low-density lipoprotein (LDL) and/or 5α-dihydrotestosterone (DHT) for 48 hours. Then, glucose consumption, lactate production and the enzymatic activity of lactate dehydrogenase (LDH) were determined through spectrophotometric analysis. Protein expression of target regulators of glycolytic and lipid metabolism was analyzed by Western blot (WB). Lipid droplets (LD) quantification was determined by staining with Oil Red-O. The present results showed that the treatment with 100 nM TBT stimulated the glycolytic flux by enhancing glucose consumption, lactate production and LDH activity in androgen-sensitive LNCaP cells. These results were underpinned by the increased expression of the glycolytic enzyme phosphofructokinase-1 (PFK1) and monocarboxylate transporter 4 (MCT4). In addition, TBT treatment also stimulated lipid synthesis by increasing the expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) proteins in PNT1A and LNCaP cells, which was supported by the increase in lipid content. This increase in LDs was also observed in LNCaP cells stimulated with 10 nM TBT. This effect was suppressed by the availability of LDL-

cholesterol. Altogether, the present findings demonstrate that TBT contributes to the metabolic rewiring of PCa cells, stimulating glycolytic and lipid metabolism and contributing for PCa development and progression. In addition, TBT in the presence of LDL disrupts lipid metabolism inducing the usage of lipids, which may contribute to the progression of cancer.

Keywords

Prostate cancer; metabolism; endocrine disruptors; tributyltin (TBT); obesity

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List of Abbreviations

ABHD5	Alpha-beta hydrolase domain containing 5
Ac-CoA	Acetyl-Coenzyme A
ACAT1	Acetyl-CoA acetyltransferase 1
ACC	Acetyl-CoA carboxylase
ADT	Androgen deprivation therapy
AFS	Anterior fibromuscular stroma
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
AR	Androgen receptor
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMI	Body mass index
BPA	Bisphenol A
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CPT1	Carnitine palmitoyltransferase 1
CRPC	Castration-resistance prostate cancer
CS	Citrate synthase
CS-FBS	Charcoal-stripped FBS
CZ	Central zone
DES	Diethylstilbestrol
DGAT	Diacylglycerol acyltransferase
DHT	5α-dihydrotestosterone
EDCs	Endocrine-disrupting chemicals
ER	Estrogen receptor
F-1,6-P	Fructose 1,6-bisphosphate
F-6-P	Fructose-6-phosphate
FA	Fatty acid
FASN	Fatty acid synthase
FBS	Fetal bovine serum
G-6-P	Glucose-6-phosphate
GLUT	Glucose transporter
HIF-1	Hypoxia-inducible factor 1

HK	Hexokinase
HR	Hormone receptor
LD	Lipid droplet
LD-FBS	Lipid depleted FBS
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDLr	Low-density lipoprotein receptor
MCT	Monocarboxylate transporter
OXPHOS	Oxidative phosphorylation
PAP	Prostatic acid phosphatase
PCa	Prostate cancer
PCBs	Polychlorinated biphenyls
PDH	Pyruvate dehydrogenase
PET/CT	Positron emission tomography computed tomography
PFA	Paraformaldehyde
PFK	Phosphofrutokinase
PI3K	Phosphoinositide 3-kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PK	Pyruvate kinase
PKM2	Pyruvate kinase isoenzyme M2
PPAR	Peroxisome proliferator-activated receptor
PSA	Prostatic-specific antigen
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
PZ	Peripheral zone
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHBG	Sex hormone-binding globulin
SREBPS	Sterol regulatory element-binding proteins
TBT	Tributyltin
TCA	Tricarboxylic acid
TPT	Triphenyltin
TZ	Transition zone
WB	Western Blot

Chapter 1

Introduction

1.1. Brief description of prostate anatomy and physiology

The human prostate is considered the main accessory gland of the male reproductive system [1]. It is a small firm structure with the size of a walnut and is located just below the urinary bladder, in front of the rectum and surrounding the prostatic urethra and the two ejaculatory ducts (Figure 1).

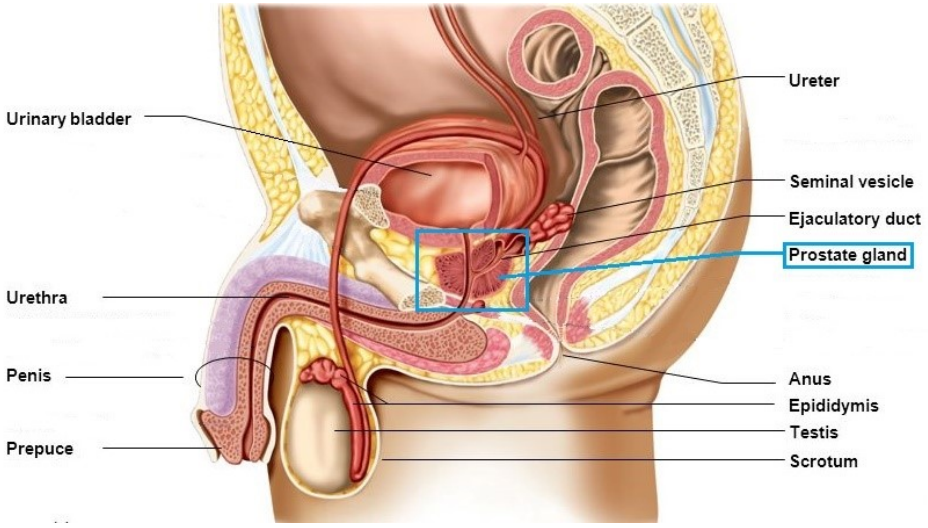

Figure 1. Localization of the prostate gland. The prostate is located anterior to the rectum, inferior to the bladder, dorsally to the symphysis pubis and surrounding the prostatic urethra and the two ejaculatory ducts (adapted from [2]).

The human prostate is composed of an apex, a base, and anterior, posterior, and inferior-lateral surfaces. Mostly it is constituted by stroma and glands that are firmly fused within a pseudocapsule. The inner layer of the prostate capsule is composed of smooth muscle and has an outer layer covering of collagen [3, 4].

McNeal and colleagues proposed other anatomic distribution, dividing the human prostate into four distinct regions, the central zone (CZ), the small transition zone (TZ), the peripheral zone (PZ), and the anterior fibromuscular stroma (AFS) or stroma (Figure 2). PZ represents approximately 70% of the prostate and is considered as the region most susceptible to development carcinomas, chronic prostatitis and post inflammatory atrophy [3, 5]. CZ, branches anteriorly from the prostatic urethra to encircle the ejaculatory duct, corresponds approximately 25% of the prostate volume in young adults and has a very low incidence of prostate cancer (PCa) [4, 6]. The TZ comprises around 5% of the prostate and surrounds the urethra proximal to the ejaculatory ducts [1]. In addition, approximately 25% of PCa arises from this zone and is characterized by the site of benign prostatic hyperplasia (BPH) [4, 7, 8]. The AFS zone is formed by smooth and fibrous muscular elements and it has the function of cover the glandular tissue and the exterior of the prostate [3, 9].

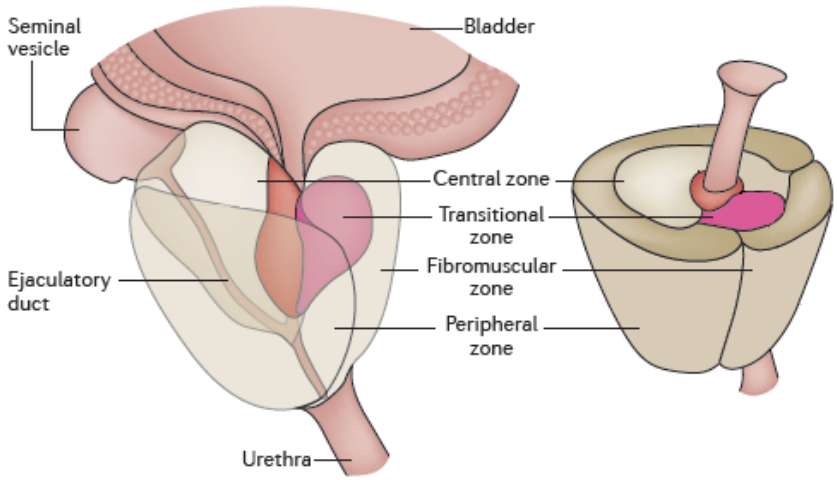

Figure 2. Schematics representations of the human prostate anatomy. The prostate gland is divided in four distinct zones, the central zone, the transition zone, the peripheral zone and anterior fibromuscular stroma. The central zone accounts for approximately 25% of the glandular tissue of the prostate gland and expands surround the ejaculatory ducts to the base of the bladder. The transition zone represents approximately 5% of the glandular tissue. The peripheral zone comprises about 70% of the gland, corresponding to the majority of the glandular tissue and covers the lateral and posterior aspects of the prostate. And finally, the stroma is make up of smooth and fibrous muscular elements (adapted from [10]).

The stromal compartment has the main function of ensure the appropriate microenvironment for the epithelium, but it also provides the microenvironment and signals to retain or restore prostate homeostasis in healthy conditions or during regeneration processes [1, 11].

On the other hand, the epithelium is composed of secretory epithelial cells, stem cells, basal cells and neuroendocrine cells [12]. The epithelial cells are responsible for the secretion of the prostatic fluid. This fluid integrates approximately one-fifth to one-third of the volume of ejaculate [11, 13-16]. The prostatic fluid is a thin and milky alkaline secretion, which contributes to neutralize the acidic environment of duct deferent and female vagina. It has in its composition citrate, zinc and the kallikreins, which include prostate-specific antigen (PSA) that is a glycoprotein usually expressed by prostate tissue and used as an indicator of PCa. These factors are crucial to controls the sperm maturation, semen liquefaction, the clotting cycle and sperm motility [1, 15, 17-19]. The stem cells are characterized by their capacity for multilineage differentiation, self-renewal and replicative quiescence in the adult prostate and these cells sit in niches, on the basement membrane at a very low frequency [20]. Basal cells are located on the basement membrane, are relatively undifferentiated and lack secretory activity [21]. Finally, neuroendocrine cells are amongst localized in the basal cell compartment and express secreted neuropeptides and other hormones involved in the promotion of prostate growth [22].

1.2. Etiology of PCa

1.2.1. Epidemiology

PCa is the second most common cancer diagnosis (1,276,106 new cases/per year), and the fifth leading cause of cancer death worldwide in men (358,989 deaths/per year) [19, 23]. It is estimated that until 2040 there will be 2,293,818 new cases, with a small variation in mortality, that is, an increase of 1.05% [19]. Incidence rates of PCa are high in Australia / New Zealand, Northern and Western Europe and North America, particularly in the United States, while Asian and African countries have the lowest incidence rates. On the other hand, mortality rates are high in the Sub-Saharan Africa regions and the Caribbean, whereas the lowest rates were recorded in central-south, east and southeast Asia and north Africa [19, 23]. In 2018, PCa was the most diagnosed male cancer, with an estimated incidence of around 460,000 cases and mortality was around 107,000 deaths in Europe [24]. In Portugal, in the same year, PCa was the most frequent cancer among men, presenting 6610 new cases, and was the third leading cause of cancer death in men, with 1880 deaths [25].

1.2.2. Risk factors for PCa

Risk factors for PCa can be classified as endogenous, namely aging, ethnicity, hormones, family history, genetic factors and oxidative stress, and exogenous, such as diet, obesity, physical inactivity, lifestyle and environmental factors [26]. The exact etiology of PCa remains unknown and the only risk factors recognized to be intimately associated with the incidence of PCa are age, African-American race and the genetic risk.

Age is considered the highest risk factor for the development of PCa [27]. PCa is frequently diagnosed in elderly men [19], being diagnosed about 75% of PCa in men older than 65 years [28]. Ethnicity seem to contribute to the prevalence of PCa. African-American men have the highest incidence, more mortality and probability to develop the pathology earlier in life. These differences have been associated with socioeconomic conditions and biologic factors, including the hormone levels [19, 29]. Considering the familiar antecedents, the risk to develop PCa is increased in men with family history. In first-degree relative the risk is 2- to 3-fold higher, and the risk is 5- to 11-fold higher if two or more first-degrees is affected [30-32]. Several genetic studies reported that a big familial predisposition may be responsible for 5–10% of PCa [33]. Various PCa susceptibility genes have been identified, for instance, RNASEL, ELAC2, MSR1, OGG1, CHEK2, BRCA2, PON1, GDF15, MIC-1 and TLR4, and recently NEAT1, FOXA1, SPOP, ETS, CDH12 and ANTXR2 [34-37]. Some of these genes encode proteins, that are involved in cellular defense against oxidative stress and inflammation [35]. Alterations in cellular proliferation, metabolic profile and also apoptosis were also reported [38, 39].

Extrinsic factors have also been implicated in the etiology of PCa. An important study described that extrinsic factors can contributed more than 99.9% to the development of PCa [40]. Environmental factors such as exposure to UV radiation or carcinogens, or factors related to eating habits and lifestyle are examples of these contribution [40, 41]. In addition, pesticide exposure contributes to the development of PCa, in which chronic or intermittent exposures to these compounds may contribute to higher rates of this type of cancer [42, 43]. Moreover, prostate is an organ susceptible to infectious agents, through urine and sexual activity. So, it is reasonable to assume that several infectious agents can promote inflammatory processes and consequently increase the risk to develop PCa [44, 45]. Epidemiological and biological studies suggest that inflammation is behind the high-grade or aggressive prostate tumors and ultimately metastatic spread [46, 47]. Also, inflammation seems to contribute to the onset and progression of cancers, since it is characterized by an infiltration of cells that release reactive oxygen species (ROS), reactive nitrogen species and pro-inflammatory cytokines, causing DNA damage, cell injury and cell death. Diet and lifestyle are two other factors that might play an essential role in the development and progression of PCa [48]. In fact, obesity is linked to aggressive and advanced PCa [49, 50]. A relationship between obesity and PCa will be detailed in section 1.5 of this dissertation. High levels of alcohol consumption can also have a relationship with the risk of PCa [51, 52]. On the other hand, several other diet products have been identified as having a protective role in PCa [48, 53]. High selenium consumption is related with reduced risk for develop PCa in 50 to 60 % [53, 54]. Tomatoes contain high levels of lycopene which has potent antioxidant properties and may be involved in the reduction of PCa risk [48, 55].

1.2.3. Diagnosis options for PCa

The principal methods to detect PCa include, digital rectal examination, which is a physical exam, combined with the PSA blood test screening and followed by transrectal ultrasound-guided biopsy [56]. The rectal exam consists of a systematic inspection in order to screen for dysfunctions and in the case of the detection of an irregularity or nodule, is an indicator for biopsy [57]. However, this examination is limited to detect the initial stages of the PCa. Human prostatic acid phosphatase (PAP) was the first serum biomarker used to monitor PCa, however it presented insufficient sensitivity [58]. This biomarker was the most important screening marker until the emergence of the PSA detection [59]. PSA was later referred as the ideal biomarker, which improved the detection of early-stage PCa, being still the most used diagnostic marker despite its limitations with its low specificity [60, 61]. Approximately 75% of positive PSA tests are false positives. Therefore, for the elimination of false negatives and false positives of the PSA test, a biopsy is essential for diagnosis [62].

There are other important techniques to aid in the diagnosis of PCa, including the imaging techniques based on the detection of cancer metabolites. However, it cannot be used as primary techniques because of limited accuracy. One example is positron emission tomography computed tomography (PET/CT) with the incorporation of ^{18}F-fluorodeoxyglucose, a glucose analogue [63, 64]. However, this technique has a limited sensitivity in PCa, showing a high glycolytic profile only in advanced stages [63]. Magnetic resonance imaging also detects, locate and define the local extent of PCa. It is recommended as an additional imaging in the case of patients presenting a negative biopsy and suspected primary PCa [65]. Its approach differs according to the stage of the disease and has been used to detect localized PCa, recurrent or residual disease after radiation therapy or surgery [66].

1.2.4. PCa development

The steady-state characteristic of normal prostatic epithelium to develop PCa is an imbalance of excessive proliferation and reduced apoptotic rates in epithelial cells [67]. Malignant transformation of prostate cells occurs through multiple processes (Figure 3). The prostatic epithelium can be damaged by inflammation, infection and/or exposure to carcinogens which can lead to the formation of proliferative inflammatory atrophy (PIA). This leads to a formation of histological lesions, called prostatic intraepithelial neoplasia (PIN) [68, 69]. This state of neoplasia is described by the appearance of dysplasia of prostate luminal epithelial cells and by the loss of distinct basal and secretory layers [70-72]. PIN can precede to the localized PCa and later progress to locally invasive adenocarcinoma, metastasize to distant sites, mainly to the lymph nodes or bone, and therefore to the progression of the disease to a castrate-resistant state [73].

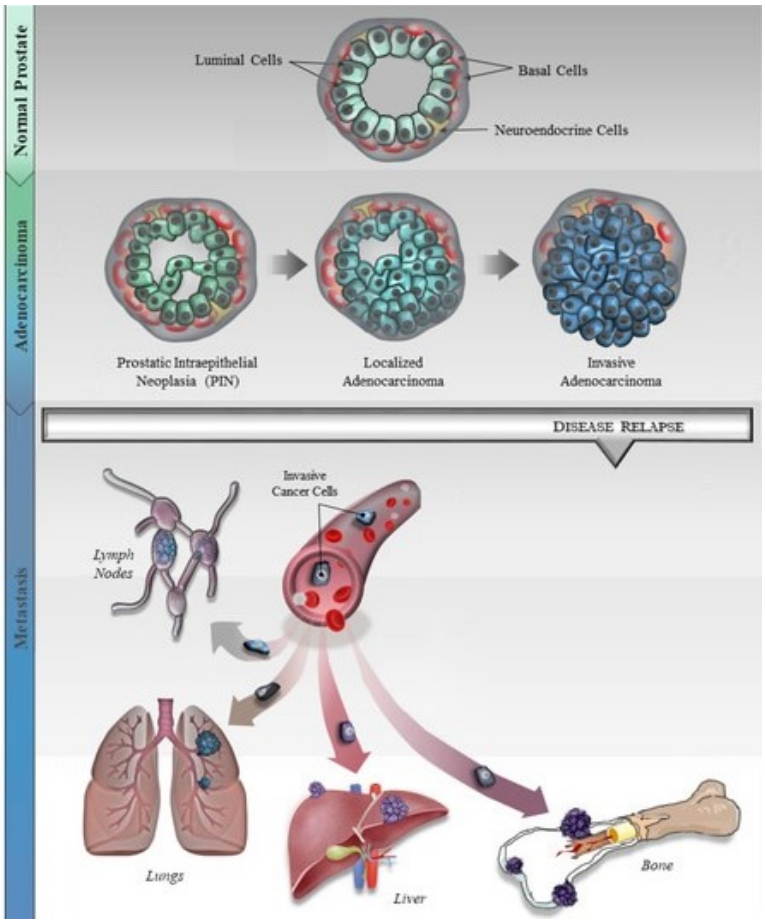

Figure 3. A generalized overview of PCa development. The illustration describes the progression of PCa from normal epithelial cells (basal, luminal and NE cells) to PIN that can precede to localized adenocarcinoma and finally to metastatic cancer (adapted from [74]).

In addition to structural and morphological changes, the development of PCa has been associated with molecular events [75].

Androgens are primary regulators of normal prostate, playing a critical role in male sexual development and in the physiology of the prostate. Furthermore, the androgens have also a crucial role in the regulation of PCa cell growth and proliferation. The two main androgens in men are testosterone, which is mainly produced by Leydig cells on the testis, and 5α-dihydrotestosterone (DHT), produced from testosterone by the action of 5α-reductase enzyme. Testosterone circulates mainly linked to sex hormone-binding globulin (SHBG), but free or unbound testosterone is the most bioavailable and active form [76]. In the course of androgen-dependent progression, PCa cells depend on the androgen receptor (AR) as the primary mediator of growth and survival [76-79]. This receptor is a member of the nuclear receptor superfamily that acts as a ligand-dependent transcription factor [80]. AR is activated by the biding of androgens, thus allow it to bind DNA and recruit co-regulators, inducing or repressing downstream gene transcription [81]. The AR signaling pathway has been linked to early growth of PCa, metastatic disease, development of hormonal resistance and disease

relapse, and in most cases of PCa, the AR is expressed through all stages of development [82]. Androgen deprivation therapies (ADT) through the ablation of AR function by ligand depletion and/or by use of AR antagonists are the first line of therapeutic intervention, since AR is a critical effector of PCa development and progression [78]. Despite in most tumors initially therapy is effective, PCa eventually progresses into an incurable and lethal stage of the disease, characterized by the presence of PCa cells resistant to castration [83]. Continuous administration of ADT makes PCa cells resistant to treatment and thus makes them able to survive and metastasize even in the absence or at very low levels of androgens [84, 85]. In this state, castration-resistant prostate cancer (CRPC) is established.

In addition to the functions already established for androgens in the control of survival and growth of PCa, androgens have also been identified as important metabolic regulators, modulating glycolysis and the use of lipids for PCa cells [39, 86-88]. Metabolic dysregulation is one of the distinct tributes of cancer cells and is crucial for the development of cancer.

1.2.5. Metabolic reprogramming in PCa

Actually, it has been widely accepted that cancer cells display metabolic specificities that contribute to tumor development and progression. Transformed cancer cells acquire the ability to reprogram the metabolism, changing the action of metabolic substrates and redox homeostasis [88]. The alterations in metabolism is described for different macromolecules, namely carbohydrates, proteins, lipids and nucleic acids [89].

The pioneer studies of Otto Warburg demonstrated that cancer cells increased the glucose consumption, but instead of the complete oxidation of glucose in the mitochondria, cancer cells preferentially produce lactate, promoting high rates of glycolysis as a rapid way to maximize the energy consumption. Also, acidification of the microenvironment by the accumulation of lactate at extracellular space, gives an advantage to tumor cells relatively to the immune cells. This phenomenon is considered a distinguishing feature of aggressive tumors [90-94]. Glycolysis is less efficient in quantity of adenosine triphosphate (ATP) generated per glucose molecule consumed, however is more faster than oxidative phosphorylation (OXPHOS), ensuring the high energy demands [95, 96]. Moreover, other metabolic substrates are needed to support the biomass production namely glutamine, lactate, pyruvate, ketone bodies and free fatty acids (FA) [97].

The prostate has a peculiar and unique metabolism that supports the sperm functionality. Prostate cells are characterized by the production of citrate, a metabolite that is important to maintain the sperm viability [98]. In prostate cells citrate is the final product of tricarboxylic acid (TCA) cycle [88, 99]. The citrate production and secretion occur due to the inhibition of

m-aconitase enzyme, which is responsible for oxidation of citrate [99, 100]. This inhibition results from the high accumulation of intracellular concentration of zinc [101]. In addition, there is a decrease in OXPHOS due to intense citrate production instead of its oxidation [99, 100]. This inefficient metabolism cannot meet the energy requirements for the fast growth of PCa cells. So, the metabolism of these cells is reprogrammed in order to have an efficient and energy-generating metabolism during their initial transformation. In PCa cells the zinc levels are very low and these leads to the reactivation of m-aconitase and the TCA cycle [102]. Advanced stages of PCa have an increase in metabolic activity, using glucose, FA and glutamine as energetic substrates.

In cancer cells the glycolytic process begins with the glucose uptake from extracellular space to intracellular space, through glucose transporters (GLUTs) family members. GLUTs family members, mediate glucose transport across the membrane, controlling the first limiting step in the rate of glucose metabolism [103, 104] (Figure 4). GLUT1 and GLUT3 are the main isoforms associated with cancer cells, with increased glucose uptake [105, 106]. Regarding PCa, GLUT1 is the main subtype of GLUTs associated with this cancer. GLUT1 is overexpressed in PCa in association with Gleason scores, and GLUT1 inhibition increase the production of ROS and induction of apoptosis of PCa cells [107-110]. GLUT1, GLUT3 and GLUT12 have been related to increased glucose uptake and also to glycolytic activity of PCa cells [39, 87, 111, 112]. GLUT3 levels is increased in androgen-sensitive LNCaP cells, while GLUT12 is overexpressed in androgen sensitive and CRPC cell models [112]. Glucose is converted to pyruvate through a chain of glycolysis reactions. Several enzymes are involved in glycolysis, three of which catalyze irreversible reactions, namely hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) [113]. Initially, due to the action of HK, glucose is phosphorylated and metabolized to glucose-6-phosphate (G-6-P) [114] (Figure 4). HK2 expression and activity is essential to the development of the glycolytic phenotype and also for cancer progression, namely in PCa [115, 116]. HK2 is overexpressed in PCa and significantly correlated with the Gleason score [117]. Subsequently, G-6-P is converted to fructose-6-phosphate (F-6-P), which is then phosphorylated into fructose 1,6-bisphosphate (F-1,6-P) through the action of PFK1 [118] (Figure 4). In normal cells, pyruvate is a substrate for the TCA cycle, while in cancer cells, pyruvate is reversibly converted to lactate by lactate dehydrogenase (LDH) [97] (Figure 4). LDH-5 is overexpressed in several cancers, namely in PCa [119, 120]. Moreover, lactate has been shown to play a role in cell proliferation and survival of cancer cells, including in PCa [39, 121-123]. Monocarboxylate transporters (MCTs) family is composed by 14 members, in which MCT1, MCT2 and MCT4 have been demonstrated to transport monocarboxylates such as lactate, pyruvate and ketone bodies, in diverse tissues, namely in PCa [124, 125] (Figure 4). These three isoforms have been implicated in the progression of PCa. MCT1 is expressed in both normal and malignant prostate [125]. The expression of MCT2 increases from normal

gland to PIN and *in situ* carcinoma, whereas MCT4 is only expressed in cancer tissues [125]. MCT1 and MCT2 transport a wider range of substrates, and contrarily, MCT4 is specifically associated with the export of lactate in cancer cells with high glycolytic rate [88]. Late-stage PCa cells presented active glycolysis, resulting in an accumulation of lactate as a byproduct of excessive anaerobic metabolism, creating a toxic environment for normal cells adjacent to malignant cells. In this way, cancer cells express high levels of MCTs in order to ensure the efflux of intracellular lactate [93]. A relationship between the MCT1/MCT4 lactate shuttle in PCa cells and stromal cells, namely cancer-associated fibroblasts was reported [121]. Fibroblasts have been involved in tumor progression and are referred as important consumers of glucose and active producers of lactate [121, 126]. Neighboring cancer cells establish a relationship with fibroblasts through MCT1 and thus have a higher use of lactate [121].

On the other hand, pyruvate can be also oxidized to acetyl-coenzyme A (Ac-CoA), in mitochondria, by pyruvate dehydrogenase (PDH) (Figure 4). Ac-CoA can be metabolized into citrate by condensation with oxaloacetate, by the action of citrate synthase (CS) (Figure 4). Citrate can remain in the TCA cycle or, on can be metabolized in cytosol by ATP citrate lyase in order to produce substrates for *de novo* lipogenesis, acetylation reactions or else production of ketone bodies [97].

Lipids are essential structural components of cell membranes, act as second messengers in intracellular signaling and are important sources of energy. Uptake, synthesis and oxidation of FA contribute significantly to cancer cell survival, proliferation, differentiation and metastasis [127, 128]. FA uptake occurs through the fatty acid transporter CD36, which is present in the plasma membrane [129] (Figure 4). High expression of CD36 is positive related with lower survival rates and development of metastases [130, 131]. There are few studies that detailed the role of this transporter in PCa cells. However it is known that the silencing of this transporter reduces FA uptake, cell proliferation and migration, decreasing the development and progression of primary prostate tumors [131].

Inside the cell, FA can undergo β-oxidation or, alternatively, be stored in lipid droplets (LDs). Carnitine palmitoyltransferase 1 (CPT1) catalyzes the first and limiting step of β-oxidation (Figure 4). This enzyme conjugates FA with carnitine, transporting them to the mitochondria where the acylcarnitines will be later oxidized [129]. CPT1A is overexpressed in PCa in relation with benign tissues, specifically in high-grade tumors [132]. The presence of CPT1A was crucial to PCa growth and cell invasion [132, 133]. If intracellular FA content exceeds the cell needs, they can be stored in LDs [134] (Figure 4). Several enzymes are involved in the storage process, namely diacylglycerol acyltransferase (DGAT) [135] (Figure 4). In contrast, the alpha-beta hydrolase domain containing 5 (ABHD5), an activator co-enzyme of adipose triglyceride lipase regulated the lipolysis [134-137]. In PCa, the LDs formation is stopped with the DGTA1

inhibition, otherwise ABHD5 inhibition promoted the LD accumulation [138]. Moreover, the ABHD5 loss is related to the aggressiveness of PCa [139]. The accumulation of LDs is a common feature of human PCa [138, 140, 141], and it was associated with the development of high-grade tumors and metastases [142].

In non-neoplastic cells, FA synthesis is almost non-existent. However, it is frequently activated in PCa cells. Acetyl-CoA carboxylase (ACC) is the first limiting enzyme in FA synthesis, in which it metabolizes Ac-CoA to malonyl-CoA [129] (Figure 4). Subsequently, the enzyme fatty acid synthase (FASN) is responsible for the synthesis of long chain saturated FA from malonyl-CoA [143-148] (Figure 4). High levels of FASN are described in PCa, being strongly correlated with tumor progression [149, 150]. *In vitro* studies have found that FASN overexpression increased the proliferation and growth of PCa cells [151, 152], whereas, its inhibition reduced cell proliferation, induced caspase-dependent apoptosis and the production of ROS [152].

Cholesterol is one of the essential components of cell membranes. In addition, it is also crucial in steroidogenesis, being a precursor of steroid hormones biosynthesis [153]. There is a balance between the synthesis, absorption and storage of cholesterol under normal conditions. Several epidemiological studies showed a positive relationship between hypercholesterolemia and risk for developing PCa, accelerated progression to CRPC and, also appearance of bone metastases after ADT [154-156]. The low-density lipoprotein receptor (LDLr) is a plasma membrane protein that facilitates the uptake of LDL via endocytosis, thereby providing an extracellular source of cholesterol for the PCa cells [157] (Figure 4). The high LDL-cholesterol availability and the LDLr cholesterol have been involved in breast cancer cell growth, in cell lines and a mouse model of hyperlipidemia [158, 159]. In PCa the impact of the availability of LDL and LDLr is still unexplored, however it was showed that LDLr mediated the cholesterol uptake in PCa cells [160].

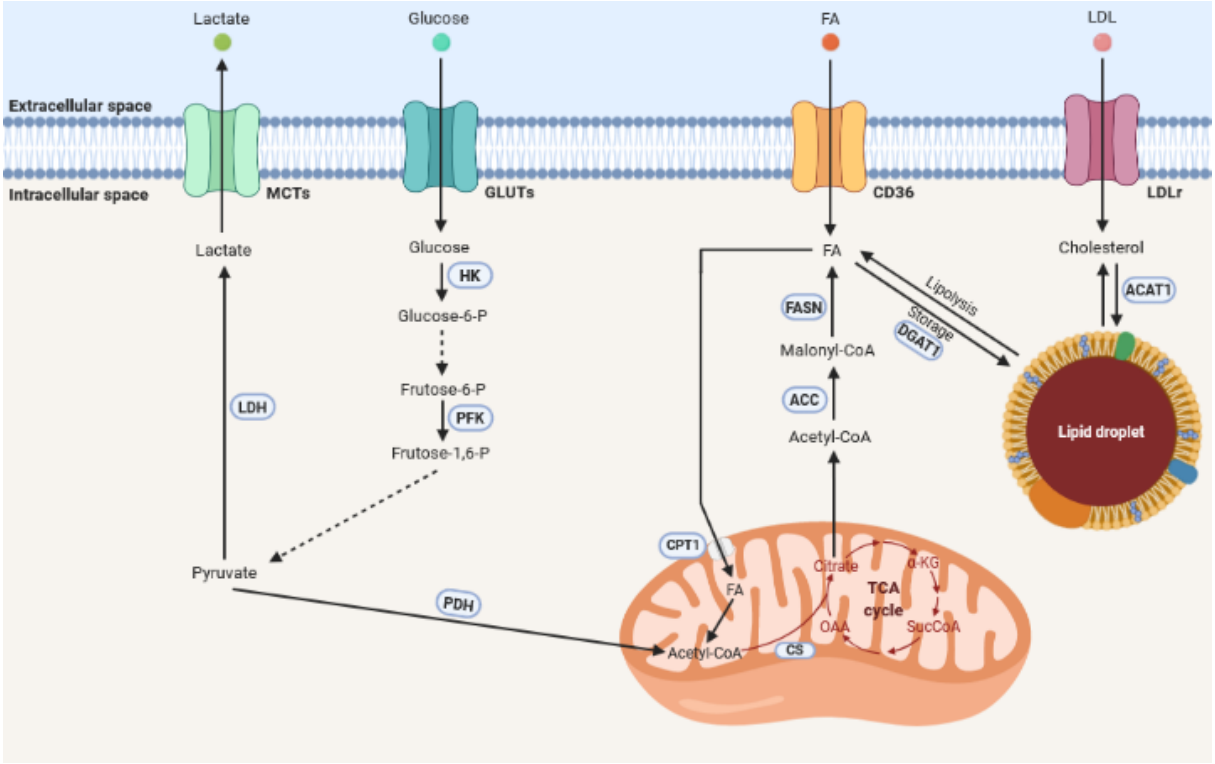

Figure 4. Metabolic adaptations in cancer cells. Glucose enters into the cell through various glucose transporters (GLUTs). In the cytoplasm, in a process called glycolysis, glucose undergoes several modifications mediated by a tandem activation of enzymes, namely hexokinase (HK), and phosphofructokinase 1 (PFK1). The product of glycolysis, pyruvate, through the enzyme pyruvate dehydrogenase (PDH) in mitochondria, can be converted into acetyl-CoA. Cancer cells preferentially use the conversion of pyruvate into lactate, by the activity of lactate dehydrogenase (LDH). Next, lactate is exported to the extracellular space through monocarboxylate transporters (MCTs). Fatty-acid (FA) uptake, oxidation and biosynthesis in the interplay of carcinogenesis. FA uptake occurs through the CD36 transporter. FA oxidation starts with conjugation with carnitine by carnitine palmitoyltransferase 1 (CPT1), allowing its transport to the mitochondria generating acetyl-CoA. Acetyl-CoA by the action of citrate synthase (CS), can be converted into citrate. Citrate enters the TCA cycle to generate energy in the form of ATP or otherwise, is involved in FA synthesis. Acetyl-CoA is metabolized by acetyl-CoA carboxylase (ACC) in malonyl-CoA, which is then converted into FA by the action of the enzyme fatty acid synthase (FASN). However, FA can also be stored in lipid droplets through storage enzymes, namely diacylglycerol acyltransferase (DGAT1), when these are not used by proliferating cells. On the other hand, lipid droplets can undergo lipolysis and thus convert monoacylglycerols to FA. Low-density lipoprotein (LDL) enters into the cells through the low-density lipoprotein receptor (LDLr) and is an extracellular source of cholesterol for cells. Cholesterol may undergo esterification through the enzyme acetyl-CoA acetyltransferase1 (ACAT1) and then stored in lipid droplets. Legend: α-KG: α-ketoglutarate; SucCoA: succinyl-CoA; OAA: oxaloacetate.

Non-transformed cells regulate the mitogenic signaling that directs cell growth and division, for the purpose of maintaining a balance between cell proliferation and death [161]. Several signaling pathways are altered in cancer cells, which seem to be responsible for the metabolic changes previously mentioned [162, 163]. Oncogenes and tumor suppressor genes that regulate the metabolic adaptations of cancer cells have been described, namely the AMP-activated protein kinase (AMPK), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), phosphatase and tensin homolog (PTEN), sterol regulatory element-binding proteins (SREBPs), p53, hypoxia-inducible factor 1 (HIF-1) and c-Myc.

AMPK is a central energy sensor, able to detect imbalances in the ratio of AMP/ATP [164-166]. Phosphorylation events mediated by AMPK suppress the metabolic functions that consume ATP, such as FA, sterols, glycogen and protein synthesis [144]. AMPK was shown to regulate glucose and lipid metabolism in PCa cells. The regulation of glucose metabolism by AMPK was crucial to sustain PCa cell survival and avoid apoptosis under glucose deprivation [167]. *De novo* lipogenesis was also recognized as an essential metabolic pathway downstream of AMPK activation. AMPK has been shown to suppress this pathway, decreasing the concentration of malonyl-CoA [168, 169]. Increased AMPK phosphorylation has been found to inhibit ACC activity, blocking the production of malonyl-CoA and consequently reducing FA synthesis [138].

One of the most commonly transformed signaling pathways in human cancers is the PI3K pathway, which is activated by mutations in tumor suppressor genes, like PTEN, mutations in the complex components PI3K or by aberrant signaling of receptor tyrosine kinases [170, 171]. Moreover, PI3K signaling phosphorylate AKT and the activation of this pathway influences a variety of cellular biological functions, such as cell growth, proliferation, survival and metabolism [172]. The loss of PTEN, and consequently the increase of AKT activity, was identified in cases of PCa and associated with the development of CRPC and poor clinical outcome [173-175]. Recently, a study found that PTEN deletion increased glycolysis, glutaminolysis, FA synthesis and β-oxidation in PCa cells [176].

The activation of AKT was associated with the accumulation of metabolites from aerobic glycolysis [177]. In the addition, the inhibition of AKT reduced aerobic glycolysis in PCa cells [178]. The activation of PI3K/AKT together with PTEN loss were related with the accumulation of cholesteryl esters in LDs in advanced and metastatic PCa which had an impact on cell proliferation [142]. Moreover, the absence of PTEN and AKT activation resulted FA *de novo* synthesis increasing the FASN expression and activity [142, 179, 180].

SREBPs are a family of transcription factors that regulate the expression of genes related with cholesterol and FA synthesis [181, 182]. Increased cholesterol biosynthesis caused by SREBP-2, induced proliferation, invasion and migration of PCa cells [183]. It has also been shown that SREBP increased the expression of FASN and stearoyl-CoA desaturase in PCa cells [184-187]. Deletion of PTEN together with the reactivation of the MAPK pathway increased the expression of SREBP-1 and the activation of the lipogenic pathway in PCa cells [188]. Moreover, it was described that transcriptional activity of SREBP promoted the accumulation of LDs in PCa cells [187]. However, the mechanism related still needs to be disclosed.

The most common mutated tumor suppressor gene p53 is recognized in the control of cell cycle, proliferation, DNA damage and repair, but also in the regulation of metabolic genes [189, 190]. p53 suppressed the transcription of GLUTs and HK, and additionally, its deficiency has been shown to increase the expression of MCTs [191-193]. P53 was also related with lipid

metabolism regulation, mediated by SREBP-1 [194]. It was found that p53 binds directly to the promoter region of SREBP-1 and thus suppressed its expression [194]. Still, the connection of p53 with metabolic reconfiguration and the lipid biosynthesis pathway in PCa cells needs confirmation.

HIF-1 and MYC-dependent pathways are other signaling pathways involved in the regulation of cellular metabolism. The HIF-1 heterodimeric transcription factor is activated by hypoxic stress and has an essential role maintaining the cellular oxygen homeostasis [195, 196]. HIF-1 regulates the expression of m-aconitase and also LDH [197], contributing to the reactivation of the TCA cycle and promoting the establishment of the glycolytic phenotype in PCa cells. In addition, HIF-1 can also modulates the expression levels of GLUTs and HK. Some studies showed that HIF-1 inhibition causes the reduction of GLUT1 expression [198, 199] and, in addition, hypoxia or anoxia conditions were associated with increased levels of HK2, eventually by HIF-1 regulation [200]. HIF-1 also regulates the metabolic reprogramming of both stromal and PCa cells, with the development of dependency on lactate as an energy substrate [201].

c-Myc oncogene is commonly overexpressed in PCa cases, being important for cancer progression. Some studies have found that c-Myc regulates glycolysis in PCa models inducing HK2, pyruvate kinase isoenzyme M2 (PKM2) and also LDHA expression [202, 203]. Moreover, overexpression of c-Myc also increased the glycerolipid metabolism and FA and cholesterol synthesis [177, 183].

1.2.6. PCa therapy

There are several possible therapies for PCa, which differ according with the stage of the disease. The options include radical prostatectomy, prostate brachytherapy and ADT for local PCa [204, 205]. In the case of local tumors that have not spread beyond the gland, it is recommended to monitor using a strategy called Watchful Waiting [206]. Radiotherapy, chemotherapy and hormonal therapy are the therapies which can be used in the case of metastatic PCa [207]. ADT is the principal treatment for androgen-sensitive primary tumors and metastatic PCa. ADT suppresses the production of testosterone or the action of androgens by inhibiting the AR. This therapy is effective at initial phases but frequently upon some months, resistance to therapy occurs and consequently the stage of CRPC emerges. In this stage, other treatment options, based in chemotherapy appeared. Mitoxantrone, Docetaxel, Cabazitaxel are examples of drugs in the treatment of CRPC. However, the treatment for CRPC was mainly palliative, and overall survival rates are in best of the cases on average 16-18 months [208]. Some therapies based on the use of metabolic inhibitors for PCa treatment are in pre-clinical and clinical trials [209]. Despite the advances made in the last years, is necessary

to continue the investigation about the molecular and cellular mechanisms that are involved in the various stages of PCa, namely in CRPC, and determine new and effective therapies.

1.3. Endocrine disrupting chemicals (EDCs)

1.3.1. Generalities of EDCs

The term endocrine-disrupting chemicals (EDCs) or endocrine disruptors was firstly introduced in 1991, at a Wingspread conference [210]. Year after year, new compounds are recognized as potential endocrine modulators, and nowadays, there are around 1,000 chemicals designated as EDCs [211].

Numerous organizations have created their own definitions regarding EDCs (Table 1). All these definitions described EDCs as substances that alter the processing of natural hormones or endocrine functions. In general, the EDCs are natural or man-made chemicals that can interfere with the endocrine system, which includes alterations in hormone production, secretion, transport, and/or action [212].

Table 1. Definitions of EDCs for three different agencies (adapted from [213]).

AGENCY	YEAR	DEFINITION
U.S. Environmental Protection Agency	1996	*"an agent that interferes with the synthesis, secretion, transport, binding, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior".*
European Union	1996	*"an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. A potent endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism".*
World Health Organization	2002	*"exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations".*

EDCs are ubiquitous in the environment and therefore, there are several forms of exposure to EDC, which can occur from air, water, dust, and food via ingestion, inhalation, skin and placenta [214]. Most EDCs are lipophilic and can cross physiological barriers. They can be detected in human adipose tissue and human biological fluids (serum, urine, milk and amniotic fluid) [215]. These compounds are present in daily life of consumer products and also in industrial manufacturing processes [216], in a wide variety of products such as pesticides,

herbicides, fungicides, flame retardants, surfactants, plastics, protectors products, cosmetics and care products. Some of the EDCs do not persist in environment and in the body and are degraded in the environment, by sunlight, bacteria and chemical processes [217]. Some EDCs are highly persistent with half-lives in years [218].

1.3.2. Classification

EDCs are highly heterogeneous and can be classified according to the action, origin or source. Thus, according to the mode of action these substances can be grouped into xenoestrogens, xenoandrogens, antiestrogens or antiandrogens. Occasionally, the same compound may have more than one of these properties. Xenoestrogens is a group of EDCS that mimic estrogenic action, thus, through binding to the estrogen receptor (ER), as agonists [219-222]. In the case of xenoandrogens, represents a set of substances that can interrupt endocrine homeostasis, acting as agonists and mimic the actions of androgens by binding to AR [223, 224]. Antiestrogens and antiandrogens are the class of EDCs, which antagonize ER and AR, respectively [225, 226]. The according to the origin, it can be natural, like phytoestrogens, or synthesized, namely chemical compounds, which include industrial solvents, pesticides, lubricants, plasticizers, pharmaceuticals, and preservatives, such as polychlorinated biphenyls (PCBs), dioxins, phthalates, diethylstilbestrol (DES), and bisphenol A (BPA) [227]. Concerning the source, it can be classified as natural and artificial hormones (phytoestrogens and contraceptive pills, respectively), as drugs with hormonal side effects (metoprolol and naproxen), or as industrial and household chemicals (plasticizers, phthalates and fire retardants) [217, 228].

1.3.3. Mechanism of action

EDCs are compounds that can act through different mechanisms, directly in endocrine system or indirectly at systemic level changing the concentration of endogenous hormones. The different mechanisms include: (1) Binding to a hormone receptor (HR) leading to activation or inhibition of the signaling pathway, (2) interactions with downstream signaling pathway of HR, (3) stimulation or (4) inhibition of endogenous hormone biosynthesis, (5) binding to the circulating hormone-binding protein, (6) stimulation or inhibition of synthesis or degradation of hormone-binding proteins, (7) stimulation or (8) inhibition of HR expression (Figure 6).

In the case of the first mechanism (EDC #1), EDCs exhibit a hormone-type mechanism of action, that is, those capable to bind and to activate a HR. As an example of this mechanism we can mention the case of xenobiotics interacting with hormone nuclear receptors [229]. Regarding the second mechanism (EDC #2), disruptors are able to bind to receptors like

hormones, antagonizing endogenous hormone action. PCBs suppress transcription by inhibiting the binding of T3 to the thyroid receptor [230]. The most common interaction is with receptors for steroid hormones, such as estrogens, androgens and adrenal hormones, peroxisome proliferator-activated receptors (PPAR), aryl hydrocarbon receptors and thyroid receptors [213]. In the third mechanism (EDC #3), EDCs presented structures that are widely different compared to hormones, interacting with hormone signaling pathway components downstream of receptor activation. An example is plasticizer di-(2-ethylhexyl)-phthalate, that act via the PPAR and affecting downstream pathways on AMPK, ERK1, ERK2 and ACC activation. Relatively to mechanisms 4 and 5 (EDC #4 and EDC #5), not directly regulate hormone receptors, but disturb endogenous hormone(s) biosynthesis or degradation [229]. An example of mechanism 4 is BPA, in which at a low dose can inhibit adiponectin secretion in human adipocytes [231-233]. One of the possible examples of mechanism 5 is the case of parabens, which inhibit 17β-hydroxysteroid dehydrogenase and consequently estrogen degradation. [234]. Regarding mechanisms 6 and 7 (EDC #6 and EDC #7), several hormones are transported by association with proteins presented in blood, in particular the hydrophobic ones, such as steroids and thyroid hormones. In general, EDCs are hydrophobic and can compete with small hydrophobic hormones for these transport proteins. In the case of mechanism 6, various EDCs can disturb directly the hormone-binding transport proteins, competing with the endogenous hormones in blood. An example of this mechanism is the case of EDCs that interact with SHBG or α-fetoprotein [235, 236]. Regarding mechanism 7, EDCs affect the biosynthesis or degradation of hormone-binding transport proteins. Polybrominated diphenyl ethers (PBDEs) is an example, acting in the downregulation of the transport protein transthyretin [230]. Concerning mechanism 8 (EDC #8), EDCs interfere with endocrine homeostasis, through the stimulation of endogenous hormone receptors. BPA stimulate leptin receptor expression in ovarian cancer cells *in vitro* [232]. Finally, in mechanism 9 (EDC #9), compounds can also inhibit expression of the receptor. BPA can regulate AR expression *in vivo* and *in vitro* studies in breast or prostate cancer cases [229].

In the next chapter of this thesis, we will give special attention to a subset of EDCs that alter the metabolism to benefit the storage of lipids, leading to a predisposition to obesity, called obesogens.

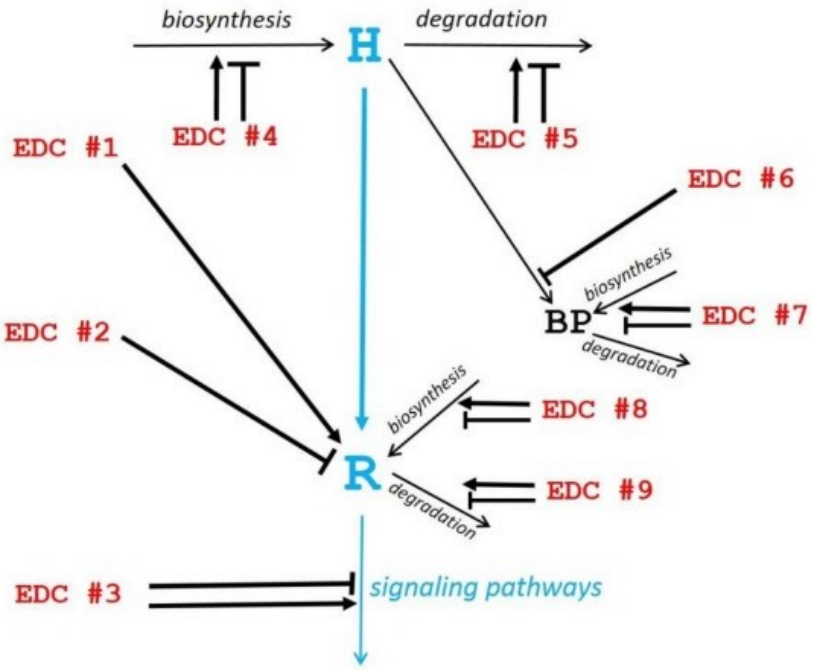

Figure 5. Scheme of the mechanisms of action of EDCs. In blue, the physiological hormonal mechanism is shown. In red, the different EDC mechanisms of action, EDC # 1 to EDC # 9, are shown, and the black arrows point to the site of action (⊣ inhibition, → stimulation) (Adapted from [229]).

1.4. Obesogens

1.4.1. History and classification

Some of the classes of EDCs can interfere with regulatory function in adipocyte actions. Due to the ability to create imbalances in body weight, promoting fat storage, these compounds are called obesogens [237-241]. The term obesogen was referenced by Grün and Blumberg in 2006 [210]. Actually, several new obesogenic chemicals were classified [211, 242].

Obesogens are classified as xenobiotics, present in diet and/or in the environment that improperly regulate and promote the accumulation of lipids and adipogenesis [243]. Not all EDCs are obesogens, just as not all obesogens are EDCs, as is the case of refined sugars, which may not meet the strict definition of an EDC, yet are obesogens [244].

Obesogens include non-steroidal estrogens, parabens, phthalates, bisphenols, organotins and polychlorinated biphenyls. Obesogen exposure alone may not be enough to develop obesity and interactions with other factors, for example diet, may be critical [244]. The sources of obesogens are extremely diverse and are summarized in Figure 7.

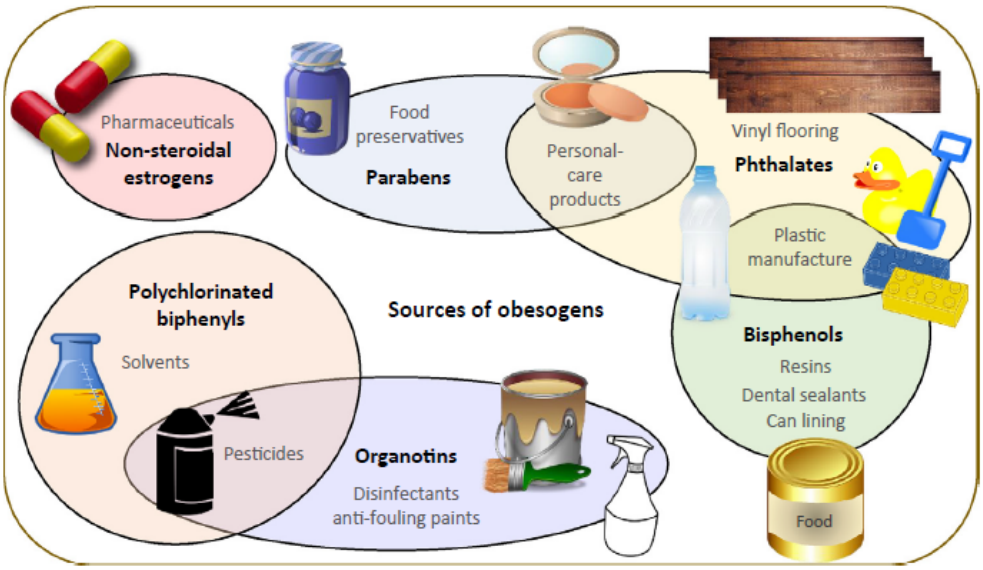

Figure 6. Sources of obesogens. Each chemical class (bold) is shown along with the most common exposure source(s), namely industrial and common consumer products [216].

1.4.2. Mechanisms of action

Actually, 50 obesogens have already been identified [242], however little is known about the mechanisms of action. Obesogens can mimic or partially mimic natural hormones and thus have undesirable biological effects. These compounds can bind to receptors in the cell membrane, cytosol or the nucleus thus affecting cellular responses, peptide hormones or gene expression [245]. Obesogens interferes with adipocytes modifying the nuclear transcriptional regulators, in lipid flux and/or adipocyte proliferation/differentiation, namely the PPARα, PPARδ and PPARγ and steroid hormone receptors [212, 246]. The main regulator of adipogenesis is PPARγ, a member of the nuclear receptor superfamily, and its activation provides adipocyte differentiation and/or the induction of lipogenic enzymes [228, 247]. Organotin compounds, particular tributyltin (TBT) and triphenyltin (TPT), are the best known compounds that interacts with the nuclear receptor superfamily, particularly PPARγ [228].

TBT is considered the principal model to study obesogenic activity [248]. TBT was widely used as biocides in anti-fouling paints for ships in the mid-1960s on a global scale, and is banned in 2008 [249]. Despite current and future restrictions, the disruptor persists in the environment, since this compound persists in sediments [250]. TBT can remain in the ecosystem over tens of decades [251]. Studies on TBT started in the 1970s, in which the phenomenon called imposex in gastropods was reported [252]. This is one of the most well-known effects of this compound and is considered the best example of EDC in wildlife. This phenomenon is characterized by the imposition of male sexual organs on females, altering sexual development and reproduction [253, 254].

Human exposure of TBT is through dietary intake, however, possible effects of dermal and inhalation exposure are not ruled out [255]. The presence of TBT is described in animals [256, 257] and human tissues [258, 259], including the placenta [260]. In an epidemiological study that investigated the effects of gestational exposure to TBT, weight gain up to three months of age was observed in newborns [260]. Most studies of this compound carried out in fish, comprised environmentally relevant doses, in the range of approximately 1–100 ng/l. TBT increased body weight, adipose tissue mass, triglycerides and cholesterol [261-265]. Moreover, studies in mice and rats with high doses, in the range of 0.5–500 μg/kg body weight, also an increase in body weight [266-270], adipose tissue mass [269, 271-273] and leptin levels [273]. Recent studies have also documented the transgenerational inheritance of obesogenic effects [272, 273]. TBT acts as agonist of PPARγ and retinoid X receptor (RXR), with high affinity comparable to endogenous ligands. Furthermore, when these compounds are administered in pre-adipocyte cell lines, it results in adipocyte differentiation through PPARγ [274]. Moreover, TBT, at high doses, can inhibit aromatase activity in adipose tissue directly and thus lead to reduced estradiol levels and down-regulation of ER target genes. At moderate to high doses, TBT inhibits the activity of 11β-hydroxysteroid dehydrogenase 2 leading to a decreased inactivation of cortisol [228].

1.5. The Relationship of Obesity with Prostate Cancer: a Link with obesogens?

Obesity occurs when energy intake, mainly stored in the form of triglycerides, exceeds energy expenditure [275, 276]. Obesity is a multifactorial disease, that is influenced by several factors, namely behavioral, environmental and genetic, diet, stage of development, age, physical activity and genetic alterations [277, 278]. Clinically, obesity is defined based on the body mass index (BMI), which is determined by the division of weight, in kilograms, by height in meters square (kg/m^2). The World Health Organization establishes overweight and obesity as abnormal or excessive fat accumulation [279]. Adipose tissue is responsible for energy store in the form of lipids, in order to maintain physiological homeostasis. Adipocytes act as endocrine cells, thereby releasing growth factors, pro-inflammatory chemokines, free FA and hormones [280-282]. A large number of adipocytes and its pro-inflammatory capacity can potently create a good microenvironment to tumor development [281].

Obesity is considered a leading cause of cancer for around 13-20% of all obesity-related cancers, which include breast cancer in postmenopausal, ovary, endometrium, colon, esophagus, gallbladder bile, pancreas, kidney and prostate [283]. Obesity provides several endocrine, metabolic and inflammatory alterations, that can promote the progression of PCa

[284]. Some examples include: i) Modifications in lipid metabolism and dyslipidemia; ii) Development of pre-diabetes characterized by insulin resistance and mild hyperinsulinemia; iii) Alteration in adipokine secretion; iv) Development of a subclinical proinflammatory state; v) Changes in various endocrine cascades, which includes growth hormone/insulin-like growth factor 1, renin-angiotensin system and steroid hormones [285].

Epidemiological studies connected EDC exposure with an increased incidence and prevalence of several diseases, such as breast, prostate and testis cancer, diabetes, obesity and infertility [222]. There are many evidence from animal and epidemiology models studies that some EDCs may influence the progression or development of PCa [286-288]. These compounds can interfere with estrogen signaling, either through interaction with ERs or through influencing steroid metabolism [289]. In addition, some of these EDCs are also considered obesogens, which inadequately regulate lipid metabolism and adipogenesis [228, 290], being also correlated with obesity, metabolic syndrome and type 2 diabetes [291, 292].

Many epidemiological studies pointed a relationship between BPA exposure with an increased risk of overweight or obesity [216]. This disruptor promotes obesity and obesity-related complications (insulin resistance, high blood pressure and dyslipidemia) [293]. Early life exposure to this disruptor, can potentially represent a carcinogen, and may increase the susceptibility to hormonal carcinogenesis in the prostate [294, 295]. The effect of BPA on AR mutation (AR-T877A) was observed in human PCa cells [296]. 1 nM of BPA activated AR-T877A and consequently, led to cell cycle progression and cell proliferation in the absence of androgen. However, BPA had no effect on wild-type AR [297]. In other study, the exposure to a low dose of BPA in neonatal rats was able to increase the incidence of cancer and the presence of PIN lesions after adult estrogen exposure [298].

PCBs are a class of fat-soluble compounds, with estrogenic or antiandrogenic activity, and can bioaccumulate in human body fat deposits. In an epidemiological study workers exposed to large levels of PCBs presented a strong exposure-response relationship for PCa mortality [228].

DES is other EDC, that exposed during the neonatal period, had a strong association with increased body weight [293]. In rodents, it was found that after prenatal exposure to DES, there was an increased susceptibility to carcinogenesis in adulthood [299-302]. During the pregnancy the maternal exposure to DES, consequently, provides in more extensive prostatic squamous metaplasia in human male offspring relative to that observed only with maternal estradiol [303]. So, men exposed to this compound *in utero* may later in life have an increased risk for development of prostatic disease [304]. However, the exact mechanism of action of this compound in PCa remains unclear.

Some epidemiological studies indicated that chronically exposure to pesticides in agricultural burden potentiate the risk of PCa development [305, 306]. TBT, has been shown to disrupt main regulators of adipogenesis and lipogenic pathways *in vivo*, and its exposure *in utero* provides a high accumulation of lipids in adipose tissue, liver and testis of neonate mice [293]. TBT and TPT were also described as regulators of androgen-dependent transcription and cell proliferation in human PCa cells [307].

Despite the reports demonstrating a relationship between some obesogens and PCa development, very few data are accessible demonstrating a relationship with TBT and the metabolism of PCa cells.

Chapter 2

Aim of the Thesis

The metabolism deregulation is recognized as a hallmark of cancer, contributing to PCa development and progression. The inhibition of cancer cell metabolism has been suggested as a possible therapeutic option, and so a deep knowledge on the factors and conditions that alter cancer metabolism is crucial. Obesity and high-fat diet are among the exogenous factors that have been associated with the aggressiveness, recurrence, and lower survival rates of PCa. However, the mechanisms connecting obesity with the aggressiveness of PCa remain to be fully detailed. In addition, obesity has been associated with aggressiveness, recurrence, and lower survival rates of PCa. Nevertheless, the detailed mechanisms connecting obesity with the aggressiveness of PCa remain unknown. Interestingly, obesogens, a set of chemical substances able to interfere with the synthesis and action of natural hormones, have been implicated in metabolic syndrome and obesity, which raises the curiosity about their influence in PCa. TBT is an EDC widely used and dispersed in the environment that has been demonstrated to present obesogenic and androgenic properties. TBT is an obesogen that was widely used and dispersed in the environment due to its agroindustry applications and the utilization in ships' anti-fouling paints. Currently, it has been demonstrated that this compound presents obesogenic and androgenic properties. Moreover, TBT seems to induce PCa cells proliferation [307]. Altogether, the existent findings lead us to hypothesize that TBT may promote metabolic alterations in prostate cells that may favor the development of PCa. The present dissertation aims to:

- Evaluate the effects of TBT altering the glycolytic and lipid metabolism of non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) human prostate cells;

- Characterize the effect of TBT in deregulating prostate cancer cells metabolism in obesity conditions.

Chapter 3

Material and methods

3.1. Cell lines

The non-neoplastic prostate cell line, PNT1A, and two neoplastic prostate cell lines, LNCaP and PC3 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The PNT1A cell line is a human post-pubertal prostate epithelial cell line. LNCaP cells were derived from lymph nodules metastasis of PCa, express the androgen receptor and are androgen-sensitive [308]. On the other hand, PC3 cells come from bone metastasis and are non-responsive to androgens [309]. Therefore, the LNCaP and PC3 cell lines have being widely used as *in vitro* models of an early androgen-sensitive stage of PCa, and a late and castration-resistant stage, respectively [39, 87, 310].

3.2. Cell culture and treatments

Both neoplastic and non-neoplastic cells were cultured and maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in RPMI-1640 phenol red culture medium (R6504, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 1% penicillin-streptomycin (A5955, Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS) (F7524, Sigma-Aldrich, St. Louis, MO, USA). Passaging was routinely performed with trypsin (T3924, Sigma-Aldrich, St. Louis, MO, USA). The stimuli of the three different cell lines were carried out in T-25 flasks (SPL Life Sciences). The cells were seeded and at a 60% confluence, the culture medium was replaced by phenol red-free RPMI 1640 medium (R8755) containing 5% charcoal-stripped FBS (CS-FBS, F6765, Sigma-Aldrich, USA) or 1% lipid-depleted FBS (LD-FBS, Biowest, Riverside, MO, USA). The cells were maintained for additional 24 hours and, then, exposed to 100 nM of TBT for 48 hours. Alternatively, cells were exposed to TBT (10 nM) in the presence of LDL (100 μg/ml) and/or DHT (10 nM) for 48 hours. Finally, 1 ml of medium was collected for each duly identified eppendorf, for measurement of extracellular metabolites. The cells were trypsinized and harvested for protein analysis.

The TBT stock solution (T50202, 100g, Sigma-Aldrich) was dissolved in ethanol, resulting in a 10 mM TBT solution. All TBT working solutions through dilutions were prepared from the stock solution in medium.

3.3. Total protein extraction

Total proteins were isolated from the cells of the three cell lines. The samples were resuspended in an appropriate volume of radioimmunoprecipitation assay (RIPA) (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 (Sigma-Aldrich) and 10% PhosSTOP

(Roche, Mannheim, Germany) and kept on ice for 20 minutes and sometimes mixed in vortex. Subsequently, the samples were centrifuged at 14,000 rpm, at 4°C for 20 minutes and then, total proteins, present in supernatant, were collected to new duly identified eppendorfs. The protein concentration was quantified by the Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher, Rockford, USA) [311]. This kit contains two reagents, the Reagent A, a carbonate buffer containing BCA reagent, and the reagent B, a cupric sulfate solution. The working reagent consists of a ratio of 1 part of reagent B to 50 parts of reagent A. In a 96-well plate, it was introduced 1 µL of protein sample, 19 µL of milli-Q water and 80 µL of prepared working kit reagent. For the blank, the protein volume was changed by the same volume of RIPA buffer. The plate was incubated for 1h at 37°C, in the dark and the absorbance was measured spectrophotometrically using the xMark™ microplate absorbance spectrophotometer (Bio-Rad) at 562 nm. To obtain the calibration curve, the bovine serum albumin (BSA) was used as standard with several dilutions, starting with a concentration of 2mg/mL. Finally, the total protein concentration was determined according to the Lambert-Beer law using the standard curve before prepared.

3.4. Western Blot analysis

Total proteins (25µg) extracted from the non-neoplastic and neoplastic human prostate cell lines, were denatured at 100°C for 5 minutes and resolved in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed for 1 hour at 180 V, at room temperature. After activation of the polyvinylidene difluoride (PVDF) membranes, proteins were transferred and this process was carried out at 4°C, at 750 mA or 1 A for 2 hours. Then, membranes were blocked with 5% skimmed dried milk for 1 hour at room temperature and later incubated overnight at 4°C, with constant agitation, with rabbit anti-GLUT2 (1:500, H-67, sc-9117, Santa Cruz Biotechnology), rabbit anti-PFK1 (1:1000, H-55, sc-67028, Santa Cruz Biotechnology), rabbit anti-LDH (1:10000, ab 52488, EP1566Y, Abcam), rabbit anti-MCT4 (1:1000, H-90, sc-50329, Santa Cruz Biotechnology), rabbit anti-CD36 (1:400, ab 64014, Abcam), rabbit anti-ACC (1:1000, #3662S, Cell Signaling Technology), rabbit anti-FASN (1:1000, C20G5, #3180S, Cell Signaling Technology), and mouse anti-CPT1A (1:1000, 8F6AE9, ab 128568, Abcam) primary antibodies. A mouse anti-β-actin monoclonal antibody (1:10000, A1978, Sigma-Aldrich) was used for protein loading control in all Western Blot (WB). After washing with constant agitation, the membranes were incubated for 1 hour with the goat anti-rabbit IgG HRP-linked (1:10000, #7074S, Cell Signaling Technology) or m-IgGk BP-HRP (1:20000, sc-516102, Santa Cruz Biotechnology) secondary antibodies. Subsequently, membranes were washed again, 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 detected and quantified by the volumetric analysis tool of Image Lab 5.1 software (Bio-Rad, Hercules, CA, USA) and normalized by division with the respective β-actin band density.

3.5. LDH activity assay

The enzymatic activity of LDH in the three different cell lines was determined using a commercial assay kit (Spinreact, Girona, Spain). Protein extracts were added to a previously prepared kit reagent, in proportion of 1:150, in a 96-well plate. This plate was incubated at 37°C for 1 minute using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The initial absorbance was measured, and then consecutive absorbance readings were acquired at 1 minute intervals for 3 minutes. The readings were obtained at 340 nm with a constant temperature of 37°C. The LDH activity is directly proportional to the variation of absorbance over 3 minutes in each sample. The acquired activities were calculated according the concentration of the protein, and the results being expressed as fold variation in relation to the control group.

3.6. Quantification of glucose and lactate

The concentration of glucose and lactate in the culture medium of untreated and TBT-treated prostate cells with or without LDL and/or DHT, was assessed by means of spectrophotometric analysis using commercial assay kits (Spinreact, Girona, Spain).

Glucose assay consists in the oxidation of the glucose present in the samples by the glucose oxidase present in the prepared reagent according to the kit. In this reaction, hydrogen peroxide is formed, which will react with phenol and aminophenazone present in the prepared reagent, and in this way form a quinone, which is detected by the appearance of a red/violet color, allowing the quantification of glucose spectrophotometrically. For glucose quantification, 1 µL of cell culture medium collected at 0 and 48 hours after the addition of stimulus was placed in a 96-well plate. Culture medium samples were mixed with 100 µL of a prepared kit work reagent and incubated at 37°C for 10 minutes. The absorbance values were measured at 505 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). Similarly, lactate quantification was carried out by mixing 1 µL of cell culture medium with 100 µL of a prepared kit work reagent and incubated at 37°C for 5 minutes. The absorbance was read at 505 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). This assay relies on the oxidation of lactate present in each sample by the lactate oxidase present in the work reagent. This oxidation forms pyruvate and peroxide, and subsequently, by the action

of peroxidase, will be transformed into quinone, developing a red/violet color that is measured spectrophotometrically.

The glucose consumption and lactate production of the three different lines in response to TBT treatment with or without LDL and/or DHT were determined by comparison with the metabolite content in the culture medium samples at 0 hours and normalized to the total amount of protein in each experimental group.

3.7. Oil Red-O assay

PNT1A cells (50000 cells/well), LNCaP (200000 cells/well) and PC3 (50000 cells/well) seeded in 12-well plates (VWR) were exposed to TBT (10 or 100 nM), LDL (100 µg/ml) and/or DHT (10 nM) for 48 hours. Then, cells were fixed with 4% Paraformaldehyde (PFA) for 30 minutes. Cells were washed twice with distilled water and rinsed with 60% isopropanol for 5 minutes. After washing, cells were stained with Oil Red-O solution (O1391, Sigma-Aldrich, St Louis, MO, USA) for 15 minutes. Representative images of the microscope were acquired. Quantification of lipid content was performed by diluting the dye with 100% isopropanol for 5 minutes with gentle agitation, and the absorbance was subsequently read in 96-well plate at 492 nm, using the xMark™ microplate absorbance spectrophotometer (Bio-Rad).

3.8. Statistical analysis

Statistical significance of differences between controls and treatments groups was evaluated by unpaired T-test or one-way ANOVA, followed by Tukey post-test, using GraphPad Prism v6.00 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when $p<0.05$. All experimental data are shown as mean ± standard error of the mean (S.E.M).

Chapter 4

Results

4.1. Glycolytic metabolism of PCa cells is stimulated by TBT

The effect of TBT (100 nM) on glycolytic metabolism of non-neoplastic, PNT1A, and neoplastic, LNCaP and PC3 prostate cells was evaluated. The glucose consumption and lactate production were evaluated through biochemical spectrophotometric assays. Treatment with 100 nM of TBT for 48 hours enhanced the glucose consumption in LNCaP cells by approximately 1.58-fold variation relatively to control ($p<0.01$, Figure 7A), whereas no changes were observed in the other cell lines (Figure 7A). Regarding the lactate production, a significant increase after TBT treatment was observed in LNCaP and PC3 cells, by respectively, 1.63-fold and 1.31-fold variation relatively to the control groups ($p<0.05$, Figure 7B). In TBT-treated PNT1A cells, no significant changes were observed (Figure 7B).

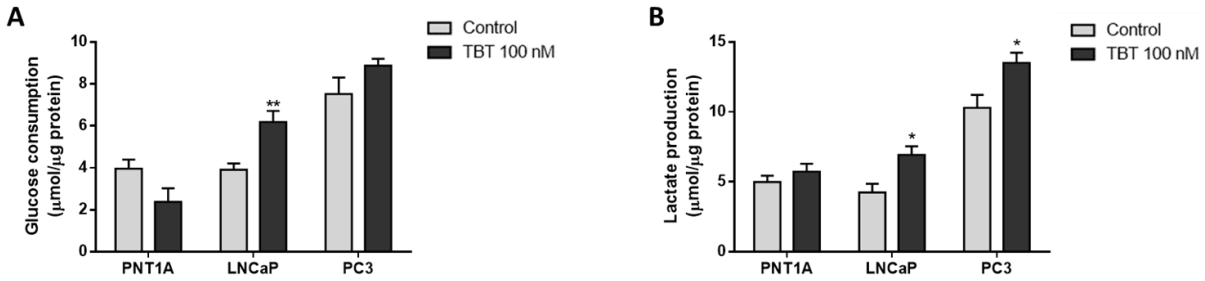

Figure 7. Glucose consumption (A) and lactate production (B) in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cell lines after treatment with 100 nM of TBT for 48 hours, obtained by spectrophotometric assays. Errors bars indicate mean ± S.E.M (n=6)**.** * $p<0.05$; ** $p<0.01$.

The expression of key targets of glycolytic metabolism, GLUT2, PFK1, LDH and MCT4 was also evaluated in the non-neoplastic and neoplastic cell lines after treatment with 100 nM of TBT for 48 hours. Glucose consumption is mediated by the uptake of glucose through GLUTs, namely GLUT2. The expression of GLUT2 was significantly increased in PNT1A cells after treatment with TBT (1,5 ± 0,11-fold change vs control, $p<0.05$, Figure 8A). In LNCaP and PC3 cells, a significant decrease after TBT treatment was observed (0.66 ± 0.05, 0.81 ± 0.04-fold change vs. control, respectively for LNCaP and PC3, Figure 8A). One of the rate-limiting steps in glycolysis, is the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate by PFK1, being a crucial step to study the metabolization of the internalized glucose. In LNCaP cells stimulated with TBT a significant increase in the expression of this enzyme was verified by approximately 1.94-fold variation comparatively to the control group ($p<0.05$, Figure 8B). Regarding PNT1A and PC3 cells exposed to TBT, no significant alterations were observed (Figure 8B).

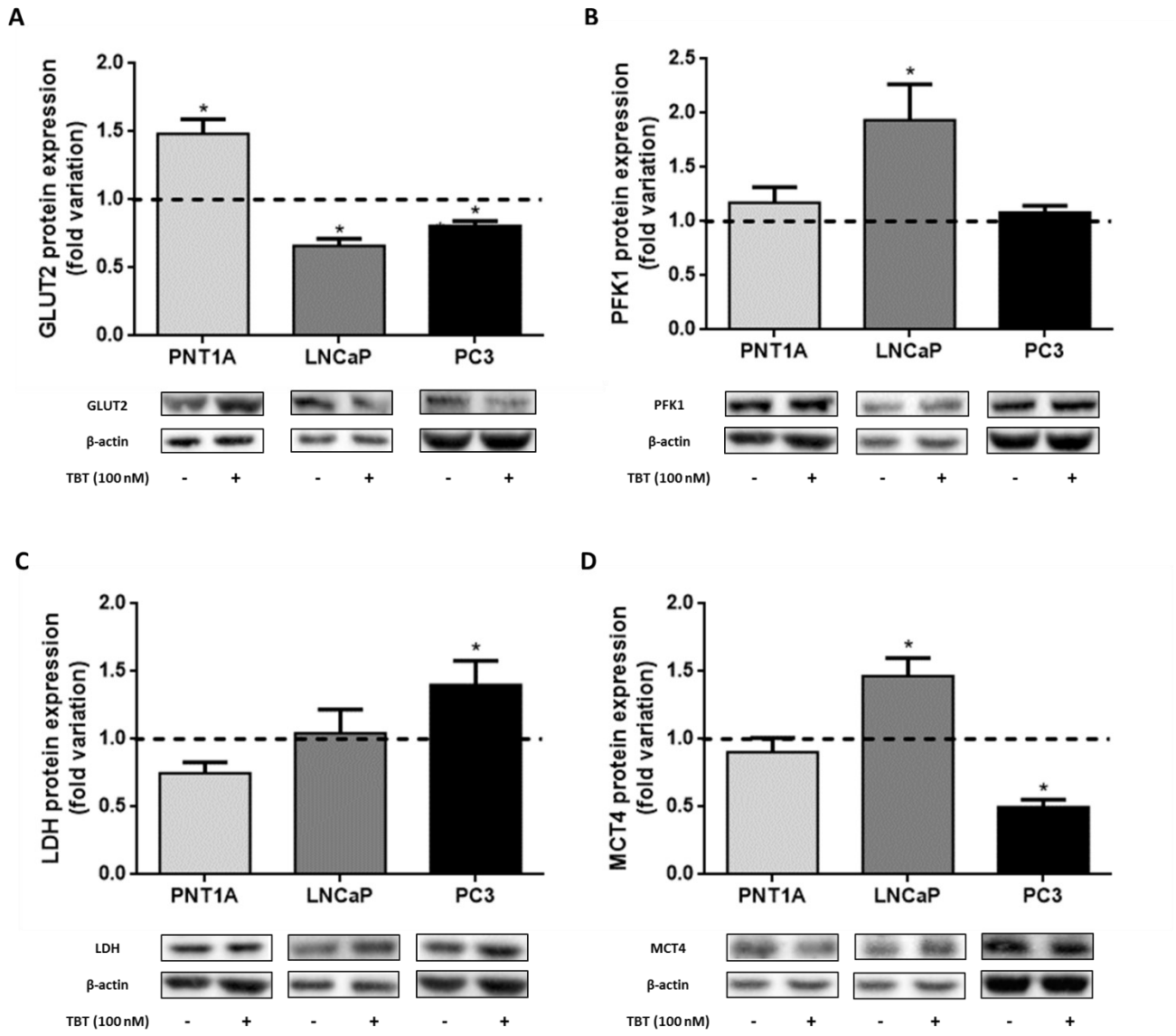

Figure 8. Expression of GLUT2 (A), PFK-1 (B), LDH (C) and MCT4 (D) in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cell lines after TBT (100 nM) stimulation for 48 hours obtained by WB analysis. Representative immunoblots for GLUT2, PFK1, LDH and MCT4 are shown, respectively, below the graph in panels. Results are expressed as fold-variation relatively to control group (dashed line) after normalization with β-actin. Error bars indicate mean ± S.E.M (n=6). * $p<0.05$.

LDH is the enzyme responsible for the conversion of the end-product of glycolysis pyruvate into lactate. TBT significantly increased the LDH expression in PC3 cells (1.40 ± 0.18 fold-change vs control, $p<0.05$, Figure 8C). Moreover, the activity of this enzyme was significantly increased in LNCaP cells (2.15 fold-change vs control Figure 9). No alterations on the expression and activity of LDH were observed in the other cell lines evaluated. The lactate produced is exported to the extracellular space through specific members of the monocarboxylate transporter family. Here, we found that MCT4 expression was significantly increased upon TBT treatment in LNCaP cells (1.47 ± 0.13 fold-change vs. control, $p<0.05$, Figure 8D). However, a significant decreased of MCT4 expression was verified in PC3 cells

treated with TBT (0.5 ± 0.06 fold-change vs. control, p<0.05, Figure 8D). In PNT1A-treated cells no significant alterations was observed (Figure 8D).

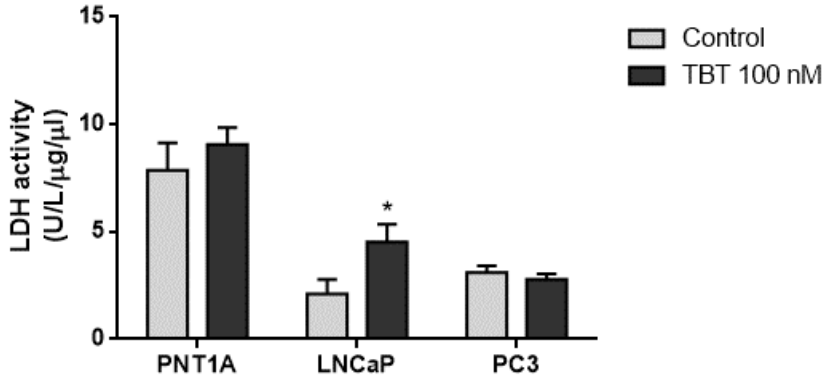

Figure 9. LDH enzymatic activity in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after treatment with 100 nM of TBT for 48 hours, determined by spectrophotometric assay. Error bars indicate mean ± S.E.M (n=6). * p<0.05.

4.2. TBT stimulates PCa cells fatty acid metabolism

In order to evaluate the effect of 100 nM TBT in the FA metabolism regulators, CD36, ACC, FASN and CPT1A expression levels were analyzed in non-neoplastic and neoplastic human prostate cell lines. The protein expression of the fatty acid transporter, CD36 was significantly decreased in LNCaP cells (0.78 fold-change vs. control, p<0.05, Figure 10A). No significant changes were found in PNT1A and PC3 cells with TBT treatment (Figure 10A). ACC is an enzyme responsible for converting Ac-CoA to malonyl-CoA with implications in lipid synthesis. Treatment of PNT1A and LNCaP cells with TBT significantly augment the expression of ACC protein (3.32 ± 0.85- and 4.30 ± 0.64 fold-change vs. control, p<0.01, respectively, Figure 10B), and no changes were observed in PC3 stimulated cell lines comparatively with the control. FASN is a crucial enzyme that catalyzes FA synthesis. TBT treatment significantly increased the FASN expression levels in all tested cell lines (1.5 ± 0.07, 2.2 ± 0.14, 1.7 ± 0.2fold-change vs. control, respectively for PNT1A, LNCaP and PC3, Figure 10C). CPT1A, a rate-limiting component in the carnitine-dependent transport of FA across the inner mitochondrial membrane, was significantly increased in non-neoplastic PNT1A cells stimulated with TBT (about 2.4 fold-change vs. control, p<0.05, Figure 10D), and a decrease on its expression was observed in PC3 cell line treated with TBT (0.72 ± 0.06 fold-change vs. control, p<0.05, Figure 10D).

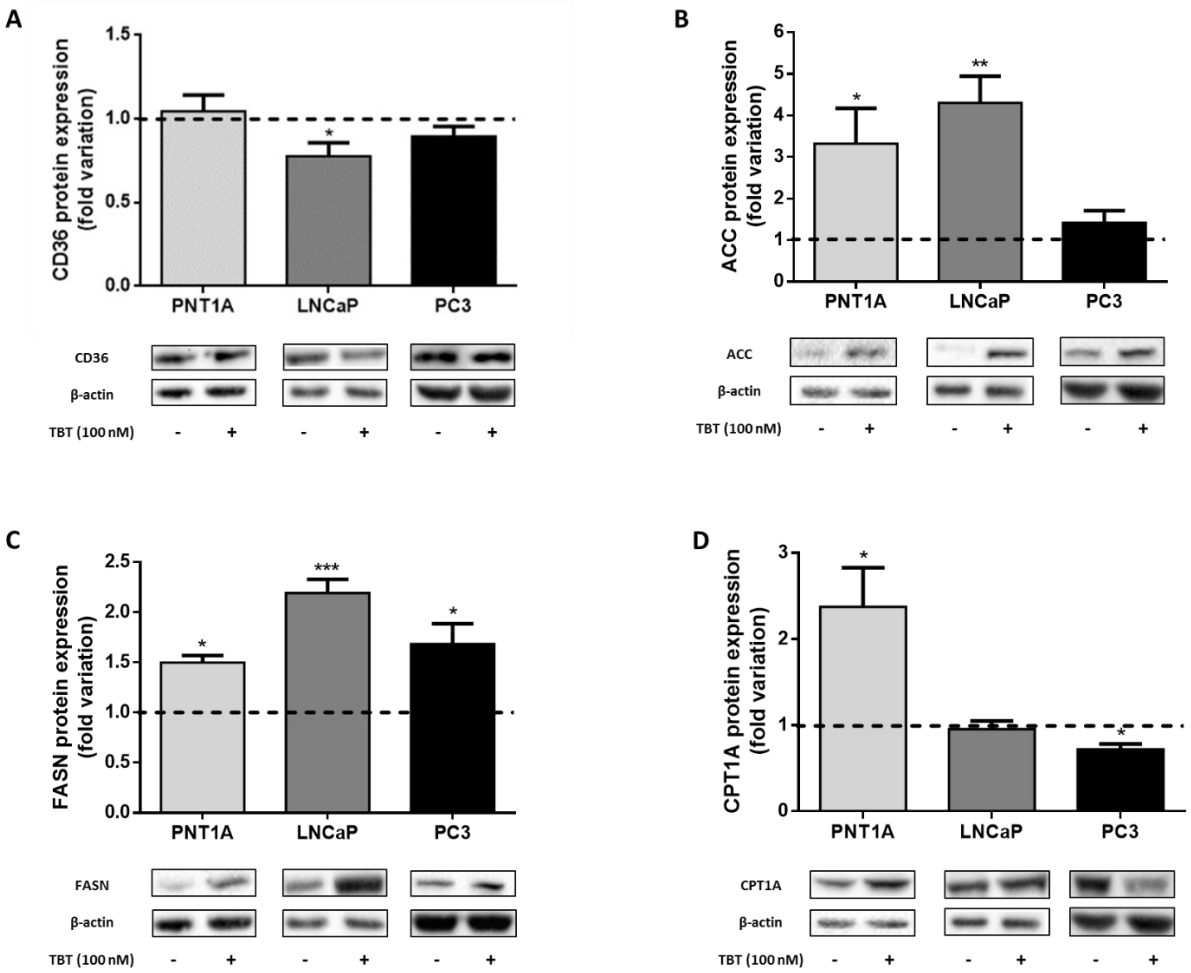

Figure 10. Expression of CD36 (A), ACC (B), FASN (C) and CPT1A (D) in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cell lines after TBT (100 nM) stimulation for 48 hours, obtained by WB analysis. Representative immunoblots for CD36, ACC, FASN and CPT1A are shown, respectively, below the graph in panels. Results are expressed as fold-variation relatively to control group (dashed line) after normalization with β-actin. Error bars indicate mean ± S.E.M (n=6). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Accumulation of FA in LDs is a feature of cancer cells, being an additional source of FA from lipolysis [312]. Treatment with 100 nM of TBT increased the relative number of LDs compared to untreated groups in PNT1A and LNCaP cells (1.24 ± 0.06, 1.21 ± 0.04 fold-change vs. control, respectively, Figure 11). However, in PC3 cells after TBT stimulation, the number of LDs decreased (0.85 ± 0.02 fold-change relatively to control, Figure 11).

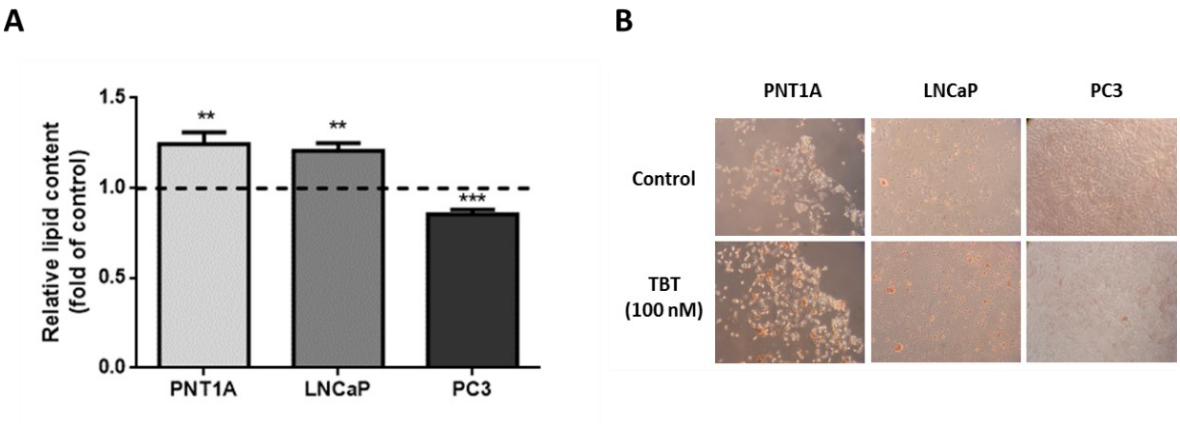

Figure 11. LD staining and quantification in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cell lines after TBT (100 nM) stimulation for 48 hours, determined by the Oil Red-O assay. Representative images of Oil Red staining are shown in the right panel (B). Error bars indicate mean ± S.E.M (n=6). ** p<0.01; *** p<0.001**.**

4.3. LDL-cholesterol availability suppresses the effect of TBT on PCa cells lipid metabolism

Some studies have demonstrated an association between obesity and hypercholesterolemia with more aggressive stages of PCa [155, 313-316]. Furthermore, the androgens play an essential role in the regulation of PCa cells growth and proliferation [76]. In this context, we evaluated if the treatment with 10 nM of TBT, 100 μg/ml of LDL and 10 nM of DHT affected glycolytic and lipid metabolism in neoplastic LNCaP cells. Concerning glycolytic metabolism, no changes on glucose consumption and lactate production were seen in LNCaP cells in all conditions (Figure 12).

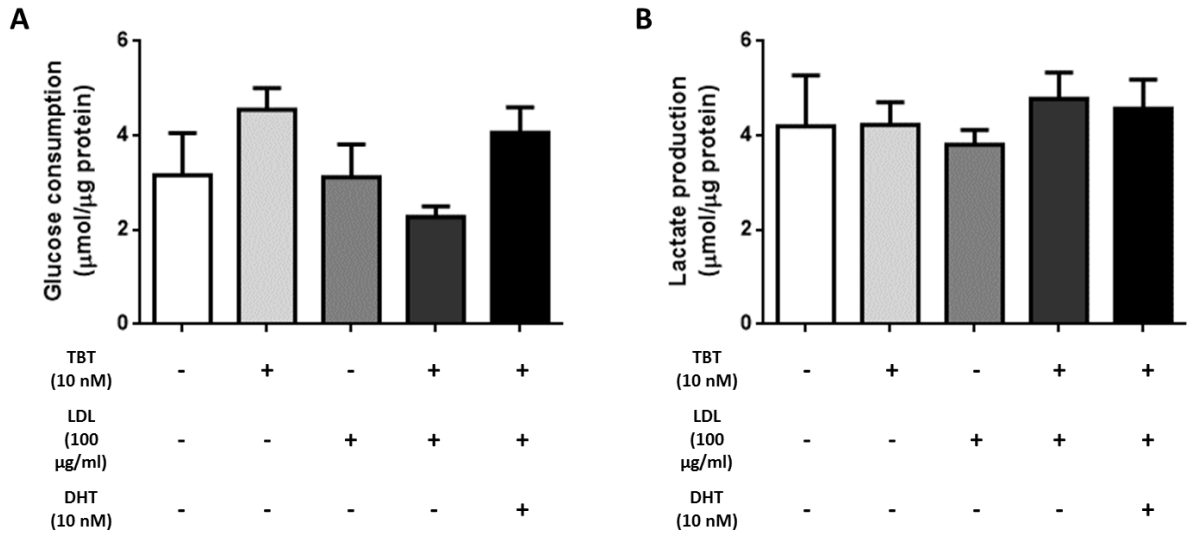

Figure 12. Effect of TBT, LDL, DHT on glucose consumption (A) and lactate production (B) in LNCaP cells obtained by spectrophotometric assays. Cells were treated with TBT (10 nM), DHT (10 nM) and/or LDL (100 µg/ml) for 48 hours. Error bars indicate mean ± S.E.M (n=6).

Relatively to the effect of TBT combined with LDL and DHT in the lipid metabolism of LNCaP cells, the expression of the regulators FASN and CPT1A was evaluated. In lipid-depleted conditions, the treatment with 10 nM of TBT significantly stimulates FASN expression (2.0 ± 0.16-fold change vs. control, $p<0.001$, Figure 13A). LDL did not affect FASN expression levels, but its presence suppressed the TBT-stimulatory effects over FASN (Figure 13A), an effect more evident in the presence of DHT. Regarding CPT1A expression levels, there was a significant increase in LNCaP cells stimulated with TBT (1.9 ± 0.1-fold change vs control, $p<0.01$, Figure 13B). LDL stimulate the expression of CPT1A, however it was not verified an additive effect with TBT (Figure 13B).

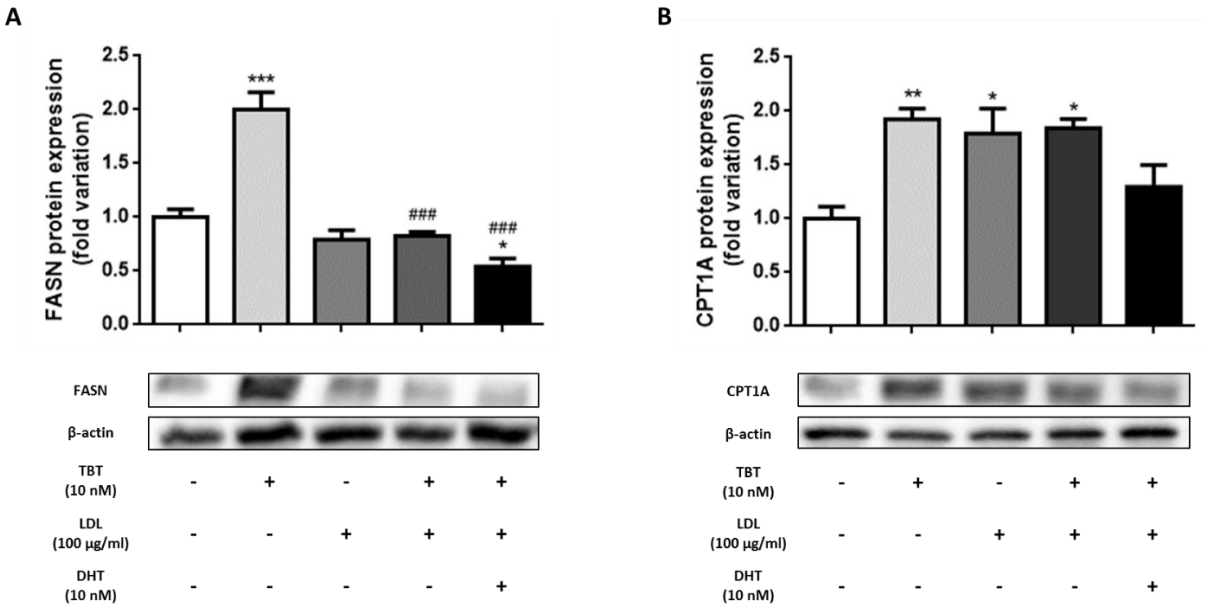

Figure 13. Effect of TBT, LDL and DHT treatment on the expression of FASN (A) and CPT1A (B) in LNCaP cells. Cells were treated with TBT (10 nM), DHT (10 nM) and/or LDL (100 µg/ml) for 48 hours. Protein expression was analyzed by WB after normalization with β-actin Results are expressed as fold-variation relatively to 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. ### $p<0.001$ when compared with the TBT group. Representative immunoblots for FASN and CPT1A are shown, respectively, below the graph in panels A and B.

Finally, it was evaluated if TBT-treatment and LDL availability modified the LNCaP cells capability for storing neutral lipids (Figure 14). The relative number of LDs in TBT-treated LNCaP cells was significantly increased (1.25 ± 0.04 fold-change, $p<0.01$). However, this mobilization is no longer visible in the presence of LDL, an effect that was amplified in the presence of DHT (Figure 14).

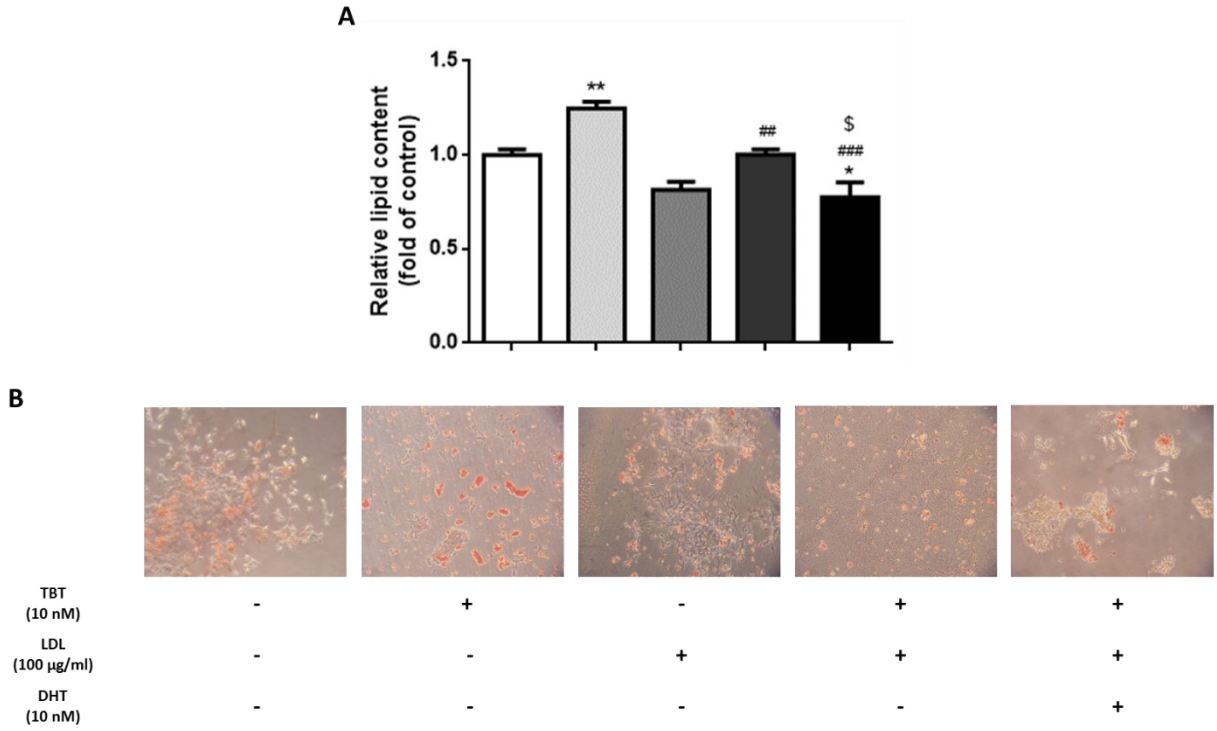

Figure 14. Effect of TBT, LDL, DHT in LD staining and quantification determined using the Oil Red-O assay in LNCaP cells. Cells were treated with TBT (10 nM), DHT (10 nM) and/or LDL (100 µg/ml) for 48 hours. Representative images of Oil Red staining are shown in the below panel (B). Error bars indicate mean ± S.E.M (n=6). * $p<0.05$; ** $p<0.01$ when compared with the control group. ## $p<0.01$; ### $p<0.001$ when compared with the TBT group. $ $p<0.05$ when compared with the TBT + LDL group.

Chapter 5

Discussion and Conclusion

The present dissertation investigated the effect of the obesogen TBT in deregulating the glycolytic and lipid metabolism of non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) human prostate cell lines. An initial MTT assay demonstrated that 100 nM TBT stimulated PCa cell viability for 24 and 48 hours of treatment (Data not shown). Moreover, TBT concentrations between 10 and 100 nM were reported in human blood [317, 318]. Integrating the effects observed on cell viability and the information available at the literature, the concentration of 100 nM TBT was first selected to analyze the effects on PCa metabolism.

Cancer cells present high glycolytic activity, which is mainly characterized by the increased glucose uptake [90, 92, 319]. Firstly, we evaluated glucose consumption in prostate cells after TBT treatment and a significant increase in glucose consumption was observed in neoplastic LNCaP cells treated with 100 nM TBT for 48 hours (Figure 7A). Glucose uptake from the extracellular media is mediated by GLUTs family members (namely, GLUT1-3 and 12) [87, 320]. GLUT2 is associated with highly glycolytic cells in liver, intestine and kidney cells [321-323]. However, augmented expression of GLUT2 has also been described in cancer cells [324-326]. Considering these points, our results demonstrated a high glucose uptake observed in LNCaP cells exposed to TBT leading to a decrease in GLUT2 expression in LNCaP-treated cells (Figure 8A). These results suggest that other GLUTs as GLUT1, 3 or 12 may promote the entrance of glucose into the cells. In fact, it is known that glucose consumption is altered by TBT, essentially by the disruption of translocation mechanism mediated by GLUT1 in human embryonic carcinoma cell line [327]. In our group it was already demonstrated under *ex vivo* conditions that TBT reprograms glucose metabolism and expression of GLUT1 in Sertoli cells [328]. In the case of prostate cells, GLUT1, GLUT12 and GLUT3 are the most well-characterized transporters, being overexpressed in PCa and responsible for the uptake of glucose in cancer cells [87, 111, 112, 322, 329]. In addition to these facts, it is known that androgens stimulate glycolytic flow in PCa cells by modulating the expression of GLUT1, GLUT3 and GLUT12 together with the glucose uptake in LNCaP cells [39, 86, 87, 112].

After glucose entrance in the cells, a sequence of reactions occurs under the responsibility of several enzymes. PFK1 is one of the key targets in this process by promoting the irreversible conversion of F-6-P to F-1,6-P, a rate-limiting step of glycolysis. The expression of PFK1 was previously reported in prostate cells [39]. A significant increase in PFK1 expression was observed in LNCaP cells stimulated with 100 nM TBT (Figure 8B). This is the first report of the influence of TBT in the disruption of PCa cells metabolism, namely, affecting PFK1 expression levels, However, in other cell types, namely in Sertoli cells, TBT was shown to decreased PFK1 expression [328]. Moreover, this enzyme was also positively regulated by androgens in neoplastic LNCaP cells [87]. This increase in PFK1 expression would mean an augmented capacity of LNCaP cells to produce pyruvate through the glycolysis pathway.

Pyruvate is the final product of glycolysis, which can be transported to the mitochondria or converted into lactate by the activity of LDH. The conversion of pyruvate to lactate is less efficient in terms of ATP generated, however it is faster in the production of energy, and is the preferred route of rapidly dividing cells, namely cancer cells [95, 96]. Consequently, increased pyruvate is expected to be followed by augmented lactate production that is then exported into the extracellular space by the MCTs.

A significant increase in lactate production was observed in both neoplastic cell lines used in the study (LNCaP and PC3) after treatment with 100 nM TBT for 48 hours (Figure 7B). This increase was accompanied by the augmented activity (LNCaP cells, Figure 9) and increased expression (PC3 cells, Figure 8C) of LDH. No other studies investigated these TBT actions in PCa cells, but a report in Sertoli cells demonstrated that TBT decreases the LDH expression and lactate production [328]. LDH protein has two isoforms, LDHA and LDHB. LDHA is responsible for catalyzing the reduction of pyruvate in lactate and is overexpressed in many cancers such as prostate, pancreatic and gastric cancer [120, 330-332]. LDHB is responsible for catalyzing the conversion of lactate into pyruvate [333] and its suppression or loss of expression has been demonstrated as an initial and crucial event in the development and progression of prostate, breast (ductal carcinoma *in situ*) and pancreatic cancers [331]. LDH protein has been shown to be regulated by several genes and proteins [330, 331], such as androgens and c-Myc [87, 334]. Interestingly, our group also showed that TBT increased the expression of c-Myc in LNCaP cells (data not shown), being a possible mechanism by which TBT influences LDH expression and activity.

Following the glycolytic process and concerning the MTC4 transporter, its expression was increased in LNCaP cells and decreased in PC3 cells after TBT-stimulation (Figure 8D). In neoplastic LNCaP cells, the effects of TBT in lactate production may result from the increased expression of MCT4. Concerning PC3 cells, the increase on lactate production may be associated with other MCTs, for example MCT1. Normally, MCT4 and MCT1 are the principal lactate transporters and are generally overexpressed in cancers [335, 336]. MCT4 has been associated with glycolytic cells, and it was demonstrated to be upregulated in PCa cells [337, 338]. In addition, the increased expression of MCT4 and MCT1 was related with the progression of PCa and a poor prognosis of the disease [39, 201, 339-341]. The expression of MCT4 was also increased by androgens in LNCaP cells [87].

Taking into account the obesogenic effects of TBT, the effect of TBT in deregulating lipid metabolism, and the influence of this EDC in LDL-cholesterol conditions modulating the PCa cell metabolism were also investigated in this thesis. Firstly, was analyzed the effect of TBT on the expression of CD36, ACC, FASN and CPT1A (Figure 10A, 10B, 10C and 10D).

In normal adult human tissues, *de novo* FA synthesis is usually suppressed and the expression of lipogenic proteins is maintained at low levels, with dietary lipids being preferably used for their energy needs. On the other hand, cancer cells are able to synthesize large amounts of *de novo* FA and cholesterol. In addition, they shunt glucose-derived carbons from the TCA cycle to the cytosol in order to feed FA synthesis [342]. *De novo* FA synthesis mainly depends on two important enzymes, ACC and FASN. ACC catalyzes the conversion of Ac-CoA to malonyl-CoA and is the first limiting enzyme in FA synthesis. Thereafter, FASN synthesizes long chain saturated FA from malonyl-CoA. The expression and activity of both ACC and FASN are up-regulated in many cancers, including PCa [342]. High levels of FASN are also strongly correlated with PCa progression, more aggressive phenotypes and CRPC bone metastasis [149, 150]. Moreover, FASN and ACC are known targets of androgens actions regulating lipid *de novo* synthesis in PCa cells. In this dissertation, we demonstrated that TBT increased ACC and FASN expression levels in PNT1A and LNCaP cells (Figure 10B and 10C). These results predict that TBT may stimulate FA synthesis, upregulating the expression of lipogenic enzymes, which therefore may promote PCa development.

The FA transporter CD36 is a major transporter for FA uptake. CD36 is overexpressed in pancreatic, gastric, ovarian and PCa and has an important role in oncogenic signaling and therefore in cancer progression [131, 343-346]. Our results showed a significant decrease in CD36 expression in neoplastic LNCaP cells after stimulation with 100 nM TBT (Figure 10A). As previously described TBT has obesogenic properties and also androgenic, and interestingly, our research group showed similar results on CD36 expression with androgens stimulation [347-350]. In the intracellular space, CPT1A is an enzymatic complex in β-oxidation, that allows the translocation of FA to mitochondrial matrix in a rate-limiting step. The products of lipid oxidation enter the TCA cycle to generate ATP [129]. CPT1A is overexpressed in PCa in regard to benign tissues, specifically in high-grade tumors [132]. In non-neoplastic PNT1A cells treated with TBT, there was a significant augment in CPT1A expression levels (Figure 10D), an effect not observed in PCa cell lines.

Alternatively, incorporated or excessive FA can be stored in reservoirs called LDs. In situations of lipid deprivation, FA can be recruited by lipolysis [141, 142]. Beyond that, the excessive accumulation of esterified cholesterol in LDs was associated with the development of high-grade prostate tumors and metastases [142]. This development was linked with the accumulation of cholesteryl ester that was driven by loss of tumor suppressor PTEN, upregulation of the PI3K/AKT/mTOR pathway and activation of SREBP and LDLr [142]. Herein, TBT increased the lipid content (LDs) in PNT1A and LNCaP cells (Figure 11). Our results significantly pointed TBT as an inductor of lipid synthesis and a promotor of lipid storage in both non-neoplastic and neoplastic cell lines. Previous results of our research group

and others, indicated that also androgens increase the expression levels of FA synthesis regulators in neoplastic LNCaP cells [350]. In addition, androgens have been shown to increase lipogenesis and lipid content in PCa cells, mainly neutral lipids, and phospholipids and free cholesterol which are stored in LDs and in membranes respectively [351].

In the second part of this dissertation, the effect of lower TBT concentrations (10 nM) in regulating glycolytic and lipid metabolism in LNCaP PCa cells was analyzed. Due the fact that obesity and hypercholesterolemia have been linked to the aggressiveness of PCa [155, 313-316], and the known obesogenic activity of TBT [349], the influence of LDL-cholesterol availability on obesogen TBT actions was also investigated. Due to the parallelism of TBT actions in comparison to DHT, it seemed essential to mimic the junction of the two in the different lipid conditions.

Our results showed that in neoplastic LNCaP cells treated with a lower TBT concentration (10 nM), no alterations were observed in the glycolytic metabolism of PCa cells. However, there was a significant increase in the expression levels of FASN and CPT1A (Figure 13A and 13B), which was underpinned by a significant increase in lipid content, even in lipid depleted conditions (Figure 14). However, the TBT-induced lipid synthesis and LDs accumulation was suppressed by the availability of LDL-cholesterol, an effect even more evident in the presence of DHT (Figure 14). These findings indicated that high LDL availability turn-off the production of FA by PCa cells, a result that was previously seen in our research group in LNCaP cells treated with DHT in the presence of LDL [350]. However, it is known that LDL combined with DHT increases the relative number of LDs in relation to the LDL-untreated groups in LNCaP cells [350]. Importantly, the increase on CPT1A expression in TBT conditions occurs independently of the presence of LDL.

Mechanistically, TBT actions in promoting adipogenesis and lipid storage seem to occur through the activation of RXR-PPARγ [237, 240, 274]. Nevertheless, TBT interferes also with the metabolism of androgens, managing to induce the imposex in marine snails [348]. In fact, some similarities between TBT and androgen actions were observed. Moreover, increased expression of the AR expression was found in LNCaP cells after treatment with TBT (our research group results, not shown). Interestingly, others authors demonstrated that the expression of endogenous AR target genes, namely the prostate specific antigen, but not the receptor expression, was increased by TBT (100 nM) and TPT (1 nM) [307]. On the other hand, flutamide, an androgen antagonist, did not inhibit the action of TBT and TPT activating the AR. These results demonstrated the ability of TBT and TPT to activate androgen-responsive genes independently of AR in mammalian cells [307]. Therefore, more studies are needed to understand the mechanisms by which TBT acts.

In conclusion, our results demonstrated that high TBT concentration (100 nM) stimulated glycolytic metabolism in neoplastic cell lines, increasing glucose uptake and lactate production as well as the expression of glycolytic regulators mainly in androgen-sensitive LNCaP cells. The increase in the glycolytic pathway and lactate production is associated with the acidification of the tumor microenvironment, and the exported lactate may possible be used by tumor cells as an alternative fuel source contributing to the onset and progression of tumors. It was also observed that TBT disrupted lipid metabolism in prostate cells, with both low and high TBT concentrations (10 and 100 nM) stimulating the expression of target regulators of lipid synthesis. TBT increased the synthesis of FA in both neoplastic LNCaP and non-neoplastic PNT1A cells, which was accompanied by the accumulation of lipids (LDs). Overall, the results obtained suggest that TBT may be a causative agent in prostate carcinogenesis by disrupting the metabolism of human prostate cells. The stimulation of the glycolytic and lipid pathway could drive the metabolic changes associated with the progression of PCa. At least for our knowledge, this is the first study showing the effect of TBT in deregulating the metabolism of non-neoplastic or neoplastic cell lines, which can contribute to development or progression of PCa. In addition, the outcomes of this dissertation also contribute to support the link between obesity and PCa progression, and highlight for the strongest negative effects of environmental exposure to obesogens in PCa patients with obesity.

Chapter 6

Future Perspectives

The outcomes of this dissertation allowed us to conclude that TBT, with its androgenic and obesogenic properties, disrupted the metabolism of human prostate cells, stimulating the glycolytic and lipid metabolism and thus may contribute to PCa development.

In a near future, it will be pertinent to analyze the expression levels of GLUTs 1 and 3 and MCT1 with TBT stimulation (100 nM). We also expect to study the expression of ACC and CD36 in the presence of TBT (10 nM), LDL and DHT. In addition, other metabolic pathways that are known to be involved in PCa progression, as mitochondrial or glutamine metabolism should be analyzed. To complement these results, it will be opportune to carry out the quantification of FA and ATP content. Real-time cell measurements, such as the mitochondrial respiration and medium acidification using the Seahorse equipment will also be considered. It also seems interesting to study the effects of TBT in obesity conditions in non-neoplastic cells (PNT1A) and to see if there is a harmful combination for the development of a carcinogenic phenotype.

Since TBT accumulation site is in the adipocytes, it will be pertinent to study a complex model of adipocytes exposed to TBT and in co-culture with PCa cells. Also, the effect of a long-exposure to TBT, alone or in obesity conditions, in prostate cells could represent an experimental design closer to what happens with human exposure.

Despite this, much remains to be explored such as the mechanism action of TBT. The activation of RXR-PPARγ and also other androgen-responsive genes could be evaluated.

Finally, after *in vitro* analysis using human prostate cell lines, all the effects of TBT must be subsequently analyzed *in vivo* using PCa mouse models as the transgenic adenocarcinoma of the mouse prostate model (TRAMP).

Chapter 7

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