UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Prostate Cancer Metabolism in the Interplay of Obesity and Estrogens Actions

(Versão Final Após Defesa Pública)

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

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Covilhã, outubro de 2018

Dedicatória

Dedico esta dissertação às pessoas mais importantes da minha vida, os meus pais e a minha irmã.

“Happiness is only real when shared”, Christopher McCandless.

Agradecimentos

Queria começar por agradecer à minha orientadora Doutora Professora Sílvia Socorro por, em primeiro lugar, ter confiado em mim para realizar este trabalho. Estou grata por todo o seu empenho, apoio e por ter estado sempre por perto para me ajudar. Os seus conselhos e sugestões impulsionaram este trabalho e permitiram-me crescer. Foi um prazer trabalhar consigo e das melhores decisões que tomei.

Agradeço também à minha co-orientadora Doutora Cátia Vaz por toda a paciência que teve comigo ao longo deste ano, por todos os ensinamentos de laboratório e pelo carinho que demonstrou. Um obrigado especial pela companhia e ajuda que disponibilizou nos fins de semana e feriados para estar a meu lado a criar este trabalho. Para além de mentora, uma amiga.

Este trabalho tornou-se possível também graças aos meus colegas de grupo e de laboratório a quem deixo uma palavra de agradecimento. Uma palavra especial à Marília Figueira, Henrique Cardoso, Tiago Carvalho e Sara Correia que foram incansáveis comigo e ajudaram-me tanto nas crises como nas conquistas. Obrigada à Marília por ter criado uma amizade tão boa entre nós, por me ter apoiado em todos os westerns complicados, e por me apelidar "mini-Marília". Ao Henrique, que a cada passo que dei me deu confiança e força para continuar. Ao Tiago, pela boa disposição e alegria logo pela manhã, pelas piadas e por toda a ajuda quando as coisas pareciam não ter sentido. À Sara, pelo carinho e pelas palavras amigas que todos precisamos. Um grande obrigada.

Um especial agradecimento às pessoas mais importantes da minha vida, os meus pais e a minha irmã, que sempre me apoiaram a seguir os meus sonhos e a lutar por aquilo que acredito. Por confiarem em mim, pela paciência quando as coisas não estavam tão bem e pelos mimos, ainda que por vezes à distância. Tudo o que sou hoje devo-vos a vocês.

À Alice e à Bia, um agradecimento por se tornarem a minha família da Covilhã. Viver convosco foi das melhores experiências e, sem vocês, muitos dos choros e risos teriam sido passados sozinha. Obrigada por me transmitirem calma e amor e por continuarem sempre por perto quando preciso.

Aos meus amigos da Universidade, Maggie, Inês, Ana, Leonor, Mafalda, Diogo e Rúben, um obrigado gigante. Passámos juntos esta etapa e partilhámos o bom e o mau. Foram o meu pilar e agradeço-vos o apoio e o carinho e também as aventuras. Lembraram-me que a vida não é só trabalho e tornaram estes anos memoráveis.

Agradeço aos meus amigos de Viseu, Sofia, Catarina, Sara, André e Filipe que me ensinaram que na vida é possível ter grandes e longas amizades, amizades para a vida. Que apesar de estarmos longe, é sempre possível arranjarmos um tempinho para nós. Obrigada por todo o vosso carinho e força.

Por fim, e não menos importante, um obrigado ao meu companheiro, ao Rui. Por me ter levantado quando estava em baixo e por ter celebrado comigo quando tudo corria bem. Por partilhar as minhas conquistas, por crescer lado a lado comigo e pelo apoio incondicional desde o primeiro dia. Sem ti, isto não seria possível.

Um muito obrigado a todos!

Resumo

A capacidade das células tumorais reprogramarem o seu metabolismo emergiu, nos últimos anos, como um dos principais "hallmark" do cancro. De forma a satisfazer as suas necessidades energéticas, as células cancerígenas reorganizam a sua atividade metabólica, aumentam a taxa de glicólise e, dessa forma, conseguem manter os altos níveis de biossíntese de lípidos e outras macromoléculas, assim como as altas taxas de proliferação. Esta grande exigência energética das células cancerígenas é traduzida pela ativação da glicólise anaeróbica, mesmo na presença de oxigénio, o que resulta numa elevada produção de lactato. O excesso deste metabolito aumenta a acidez extracelular, suprime a autoimunidade do hospedeiro e favorece a invasão e divisão das células cancerígenas. Para além da glicose, os ácidos gordos são outra fonte energética das células e a sua via oxidativa e de síntese parecem estar aumentadas nas células cancerígenas.

O papel dos androgénios como promotores do desenvolvimento e progressão do cancro da próstata é bem conhecido. Para além disso, em cancros hormono-dependentes, como cancro da mama e da próstata, as hormonas esteroides foram identificadas como importantes moduladores de vias metabólicas das células cancerígenas. Estudos anteriores do nosso e de outros grupos de investigação demonstraram o papel dos androgénios como estimuladores do cancro da próstata ao regularem o consumo da glucose e a produção de lactato, e caracterizam o perfil metabólico de linhas celulares de próstata não-neoplásica, PNT1A, e de linhas neoplásicas, LNCaP e PC3. No entanto, nos últimos anos, os estrogénios também tem sido implicados na carcinogénese da próstata.

A obesidade é uma epidemia a nível global caracterizada por uma quebra no normal funcionamento do tecido adiposo sendo associada a um estado de hiperestrogenismo. Para além disso, a obesidade tem sido identificada como um fator para um aumento da agressividade tumoral e de mau prognóstico no cancro da próstata.

O presente trabalho teve como objetivo avaliar o papel do 17β-Estradiol (E_2) na modulação do metabolismo glicolítico e lipídico das linhas celulares de próstata humana de forma a perceber a sua ação como estimulador do desenvolvimento e progressão do cancro.

Para isso, linhas celulares de próstata humana não neoplásicas (PNT1A) e neoplásicas (LNCaP) foram mantidas em cultura na presença ou ausência de 0,1; 1 e 100 nM de E_2 durante 24, 48 e 72 horas. A concentração de 1 nM e o tempo de tratamento de 48 horas foram as condições selecionadas para avaliação dos efeitos do E_2 no metabolismo glicolítico e lipídico nos diferentes modelos celulares em estudo (PNT1A, LNCaP e PC3). A expressão proteica e a atividade dos moduladores alvo destes processos foram analisados por Western Blot e ensaios bioquímicos.

Os resultados obtidos revelam que o tratamento com E_2 estimulou o fluxo glicolítico ao aumentar o consumo de glucose e a produção de lactato nas células PNT1A, LNCaP e PC3. Estes

resultados foram suportados pelo aumento da expressão ou atividade dos transportadores de glicose e enzimas glicolíticas. Para além disso, o tratamento com E_2 também aumentou a expressão de proteínas envolvidas na incorporação de lípidos e na sua ß-oxidação, assim como na síntese. Efeitos estes que foram observados em todas as linhas celulares (neoplásicas e não-neoplásicas), o que é demonstrativo da forte ação do E_2 na regulação do metabolismo lipídico.

Para concluir, os resultados obtidos sugerem que o E_2 pode ter um papel no desenvolvimento e progressão do cancro da próstata ao estimular a via glicolítica e lipídica quer nas células não-neoplásicas quer neoplásicas. Além disso, as evidências deste trabalho suportam os estudos que defendem o papel causador do E_2 no cancro da próstata. Mais ainda, a relação entre estrogénios, obesidade e o cancro da próstata pode ser estabelecida uma vez que o hiperestrogenismo pode aumentar a probabilidade de desenvolvimento e invasão tumoral ao potenciar a reprogramação metabólica das células cancerígenas.

Palavras-chave

Cancro da próstata, estrogénios, LNCaP, metabolismo glicolítico, metabolismo lipídico, PC3, PNT1A

Resumo Alargado

A capacidade das células tumorais de reprogramarem o seu metabolismo emergiu, nos últimos anos, como um dos principais "hallmark" do cancro. De forma a satisfazer as suas elevadas necessidades energéticas e de forma a aumentar a sua sobrevivência e proliferação, as células cancerígenas são capazes de reorganizarem a sua atividade metabólica para criarem uma vantagem adaptativa num ambiente tumoral. Entre essas adaptações, as células aumentam a taxa de glicólise e, dessa forma, conseguem manter os altos níveis de biossíntese de lípidos e outras macromoléculas, assim como as altas taxas de proliferação. Esta grande exigência energética das células cancerígenas é traduzida pela ativação da glicólise anaeróbica, mesmo na presença de oxigénio, o que resulta numa elevada produção de lactato. Dessa forma, é privilegiada a via glicolítica em vez da fosforilação oxidativa, um processo denominado, Efeito de Warburg. A via glicolítica depende, em primeiro lugar, da incorporação da glicose pelos transportadores de membrana GLUTs e a sua continuação é assegurada pela primeira enzima glicolítica, a hexocinase, que aprisiona a glicose dentro da célula ao convertê-la em glucose-6-fosfato. Toda a sequência de reações bioquímicas seguintes culminam na produção de piruvato. Nas células cancerígenas, a conversão do piruvato em lactato pela lactato desidrogenase (LDH) é privilegiada, mesmo em ambiente anaeróbio. O excesso de lactato aumenta a acidez extracelular e, consequentemente, suprime a autoimunidade do hospedeiro e favorece a invasão e divisão das células cancerígenas. Para além da glicose, os ácidos gordos são outra fonte energética das células. O piruvato proveniente da glicólise pode também ser convertido a acetyl-CoA pela piruvato desidrogenase, o que é essencial para iniciar a via de síntese de ácidos gordos. A biossíntese e oxidação de ácidos gordos são duas vias que parecem estar aumentadas nas células cancerígenas.

As células crescem e dividem-se quando fatores de crescimento ativam vias de sinalização que controlam a progressão do ciclo celular. Mutações em proto-oncogenes e proteínas supressoras de tumores têm impacto nessas vias de sinalização, alterando o metabolismo, aumentando os processos bioenergéticos e a síntese de macromoléculas, e promovendo a divisão das células cancerígenas. Vias como PI3K/Akt, AMPK, a sinalização pelo HIF e MYC e a atividade do p53 estão associadas à reprogramação metabólica das células cancerígenas.

O papel dos androgénios como promotores do desenvolvimento e progressão do cancro da próstata é bem conhecido. Em cancros hormono-dependentes, como cancro da mama e da próstata, as hormonas esteroides foram identificadas como importantes moduladores das vias metabólicas das células cancerígenas. Estudos anteriores do nosso e de outros grupos de investigação demonstraram o papel dos androgénios como estimuladores do cancro da próstata ao regularem o consumo da glicose e a produção de lactato, e caracterizaram o perfil metabólico de linhas celulares que mimetizam os vários estados da doença (linha não-neoplásica, PNT1A, e linhas neoplásicas, LNCaP e PC3).

Os estrogénios, embora sejam essencialmente conhecidos como hormonas femininas, estão presentes no organismo masculino, ainda que em quantidades pequenas. Nos últimos anos, os estrogénios também têm sido implicados na carcinogénese da próstata, embora não se conheçam totalmente os mecanismos de atuação.

A obesidade é uma epidemia a nível global caracterizada por uma quebra no normal funcionamento do tecido adiposo que é acompanhada por uma expansão aberrante do mesmo. Os adipócitos do tecido expandido libertam mais hormonas sendo criado um estado de hiperestrogenismo que pode levar ao desenvolvimento de síndromes metabólicas ou cancros. A obesidade tem sido identificada como um fator para um aumento da agressividade tumoral e de baixo prognóstico no cancro da próstata.

O presente trabalho teve como objetivo avaliar o papel do 17ß-Estradiol (E_2) na modulação do metabolismo glicolítico e lipídico das linhas celulares de próstata humana, de forma a perceber a sua ação como estimulador do desenvolvimento e progressão do cancro.

Para isso, linhas celulares de próstata humana não neoplásicas (PNT1A) e neoplásicas (LNCaP) foram mantidas em cultura na presença ou ausência de 0,1; 1 e 100 nM de E_2 durante 24, 48 e 72 horas. Estes ensaios preliminares de avaliação do consumo de glucose e produção de lactato permitiram escolher a concentração de 1 nM e o tempo de tratamento de 48 horas para avaliação dos efeitos do E_2 no metabolismo glicolítico e lipídico nos diferentes modelos celulares em estudo (PNT1A, LNCaP e PC3). A expressão proteica e a atividade dos moduladores alvo destes processos foram analisados por Western Blot e ensaios bioquímicos.

Os resultados obtidos revelam que o tratamento com E_2 estimulou o fluxo glicolítico ao aumentar o consumo de glicose e a produção de lactato nas células PNT1A, LNCaP e PC3. Estes resultados foram suportados pelo aumento da expressão ou atividade dos transportadores de glicose e enzimas glicolíticas. No entanto, verificaram-se algumas variações entre linhas celulares, mas que não se revelaram significativamente estatísticas. Para além disso, o tratamento com E_2 também aumentou a expressão de proteínas envolvidas na incorporação de lípidos e na sua ß-oxidação, assim como na síntese. Efeitos estes que foram observados em todas as linhas celulares (neoplásicas e não-neoplásicas), o que é demonstrativo da forte ação do E_2 na regulação do metabolismo lipídico. Uma vez mais, verificaram-se variações entre linhas celulares. Nas células PNT1A e LNCaP, a expressão do CD36 aumentou após o estímulo com E_2. Mas, na linha celular neoplásica PC3 foi observada uma diminuição da expressão deste transportador, o qual é responsável pela incorporação de lípidos.

Para concluir, os resultados obtidos sugerem que o E_2 pode ter um papel no desenvolvimento e progressão do cancro da próstata ao estimular a via glicolítica e lipídica quer nas células não-neoplásicas quer neoplásicas. Além disso, as evidências deste trabalho suportam os estudos que defendem o papel causador do E_2 no cancro da próstata. Mais ainda, a relação entre estrogénios, obesidade e o cancro da próstata pode ser estabelecida uma vez que o hiperestrogenismo pode aumentar a probabilidade de desenvolvimento e invasão tumoral ao potenciar a reprogramação metabólica das células cancerígenas.

Abstract

Cancers have a common ability of reprogramming energy metabolism, which is known as a hallmark of cancer. In order to satisfy their needs, cancer cells reorganized metabolic activity upregulating glycolysis rate, which allows cells to maintain high biosynthesis levels of lipids and other macromolecules, sustaining high proliferation rates. The high energy demands of cancer cells are fulfilled by anaerobic glycolysis, even in the presence of oxygen, which results in high rates of lactate production. The excess of lactate exported to the extracellular medium increases acidity and suppresses host anticancer immunity, which favours cancer cells growth and invasion. Besides glucose, fatty acids are another important energetic source, and its oxidation and biosynthesis seems to be augmented in cancer cells.

Androgens are well-known drivers in development and progression of PCa. In addition, in hormone-dependent cancers, such as breast and prostate cancer, steroid hormones have been identified as important modulators of metabolic pathways. Our research group and others have demonstrated the role of androgens as stimulators of PCa by modulating glucose consumption and lactate production, as well as the distinct metabolic profile between non-neoplastic cell line, PNT1A, and neoplastic cell lines, LNCaP and PC3. However, in the last years, estrogens had also been implicated in the carcinogenesis of the prostate, despite some studies defend their protective effect.

Obesity is a worldwide epidemic characterized by a disruption in adipose tissue that is associated with a stage of hyperestrogenism. In addition, obesity has been identified as a factor for aggressiveness and poor prognosis of PCa.

The present work aims to evaluate the role of 17β-Estradiol (E_2) on modulating the glycolytic metabolism and lipid metabolism in human prostate cell lines, and to understand its action as stimulator of the development and progression of PCa.

Non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) human prostate cell lines were cultured in presence or absence of 0,1; 1 e 100 nM de E_2 for 24, 48 e 72 h. The 1 nM concentration and a treatment period of 48 h were the conditions selected to evaluate the effect of E_2 on glycolytic and lipid metabolism in all cell lines under study. Protein expression and activity of target modulators of these biological processes were assessed by means of Western blot analysis and biochemical assays.

The obtained results showed that treatment with E_2 augmented glucose consumption and lactate production in PNT1A, LNCaP and PC3 cell lines. These results were underpinned by the increased expression or activity of glucose transporters and glycolytic enzymes. Besides that, E_2-treatment increased the expression of lipid regulators in all cell lines, which demonstrate its action regulating lipid metabolism.

To conclude, the obtained results showed that E_2 might have a role in the development and progression of PCa by stimulating the glycolytic and lipid metabolism in both non-neoplastic and neoplastic cells. Moreover, the evidence gathered hereom follow the studies that defend

the causative role of E_2 in PCa. Finally, a relationship between estrogens, obesity and PCa might be likely established since hyperestrogenism may increase the odds for tumour development and invasion by potentiating the metabolic reprogramming of cancer cells.

Keywords

Estrogens, glycolytic metabolism, lipid metabolism, LNCaP, PC3, PNT1A, prostate cancer

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List of Abbreviations and Acronyms

ACC	Acetyl coenzyme A carboxylase
Ac-CoA	Acetyl coenzyme A
ADT	Androgen deprivation therapy
AKR	Aldo-keto reductase
AKT	Protein kinase B
ALT	Alanine transaminase
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ANT	Adenosine nucleotide translocators
AR	Androgen receptor
ArKO	Aromatase knockout
ATP	Adenosine triphosphate
BMI	Body mass index
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CPT	Carnitine palmitoyl transferase
CRPC	Castration-resistance prostate cancer
CS	Citrate synthase
CS-FBS	Charcoal-stripped fetal bovine serum
CZ	Central zone
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DHT	5α-dihydrotestosterone
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
E1	Estrone
E2	17β-Estradiol
E3	Estriol
ECM	Extracellular matrix
EDs	Endocrine disruptors
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
En	Enolase
EPCA	Early prostate cancer antigen

ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERß	Estrogen receptor beta
EZHZ	Enhancer of zeste homolog gene z
F-1,6-P	Fructose-1,6-biphosphate
F-2,6-P	Fructose-2,6-biphoshate
F-6-P	Fructose-6-phosphate
FA	Fatty acid
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FDA	Food and Drugs Administration
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
G-6-P	Glucose-6-phosphate
GAPD	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography mass spectrometry
GLS	Glutaminase
GLUT	Glucose transporter facilitator
GnRH	Gonadotrophin releasing hormone
GPR30/GPER	G protein-couple estrogen receptor
HIF1	Hypoxia inducible factor 1
HK	Hexokinase
HSD	Hydroxysteroid dehydrogenase
Hsp	Heat-shock protein
IGF	Insulin-like growth factor
KGF	Keratinocyte growth factor
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein-kinase
mCRPC	Metastatic castration-resistance prostate cancer
MCT	Monocarboxylate transporter
mHSP	Metastatic hormone-sensitive prostate cancer
MRI	Magnetic resonance spectroscopic imaging

mTOR	Mammalian target of rapamycin
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PAP	Prostatic acid phosphatase
PCa	Prostate cancer
PDGF	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PDPK1	PI3K-dependent kinase 1
PET	Positron emission tomography
PFK	Phosphofrutokinase
PGI	Phosphoglucoisomerase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
PI3K	Phosphatidylinositide 3 kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PIP2	Phosphatidylinositol-4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PK	Pyruvate kinase
PMSF	Phenylmethtylsulfonyl fluoride
PSA	Prostatic-specific antigen
PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
PTEN	Phospholipid phosphate and tensin homolog
PVDF	Polyvinylidene difluoride
PZ	Peripheral zone
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SCO2	Cytochrome c oxidase
SDR	Short-chain dehydrogenase/reductase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGLT	Na^+-coupled glucose transporter
StAR	Steroidogenic acute regulatory protein
T	testosterone
TCA	Tricarboxylic acid

TIGAR	P53-induced glycolysis and apoptosis regulator
TZ	Transition zone
uPA	Urokinase plasminogen activator
US	United States

I. INTRODUCTION

1. Brief Overview of Prostate Anatomy and Physiology

The prostate gland, common described as “walnut-shaped”, is the largest accessory gland of the male reproductive system with approximately 4 cm length and 2 cm width (Thapar and Titus 2014; VanPutte et al. 2013). It is located in the sub peritoneal compartment, posterior to the symphysis pubis, anterior to the rectum, and inferior to the urinary bladder, between the pelvic diaphragm and the peritoneal cavity and involving the urethra and the ejaculatory duct (Lee, Akin-Olugbade, and Kirschenbaum 2011; VanPutte et al. 2013). Anatomically, the prostate is oriented as having a base, an apex and the anterior, posterior, and inferior-lateral surfaces. The prostatic plexus is responsible for the nerve supply and the branches of the internal iliac artery are responsible for the arterial supply (Lee et al. 2011).

The first anatomic division of the prostate proposed by Lowsley divided the gland into lobes or regions: an anterior lobe, a posterior lobe, two lateral lobes, and one middle lobe (Lowsley 1912). Later, a new anatomical division was proposed by McNeal (McNeal 1968, 1981), comprising the central zone (CZ), the transition zone (TZ) and the peripheral zone (PZ) and the anterior fibromuscular stroma (Fig. 1). This zonal differentiation is made based on their different embryologic origins, histology, biological functions and susceptibility to pathologic disorders (Lee et al. 2011), as summarized in Table 1.

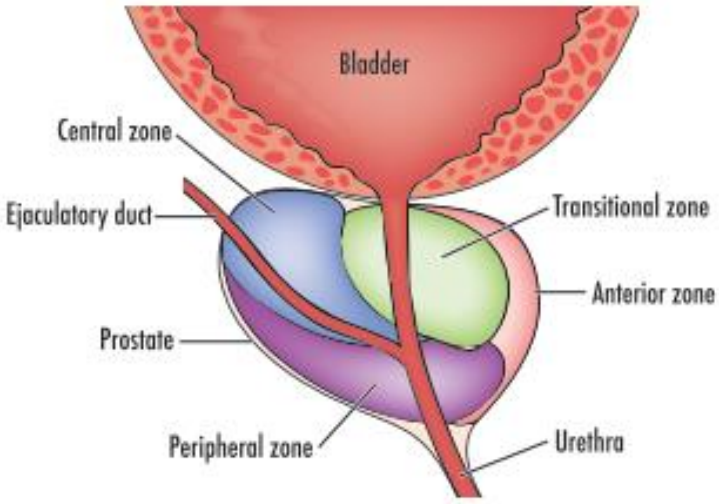

Figure 1. Zonal anatomy of the human prostate. The prostate is divided in four zones: the central zone, the transition zone and the peripheral zone. The central zone surrounds the ejaculatory duct whereas the transition zone surrounds proximal prostatic urethra. (in (Eylert MF 2012)).

The PZ comprises about 70 % of the mass of the glandular tissue of the prostate (Table 1). This zone is very susceptible to inflammation development of carcinomas with 70 % of all prostate cancer (PCa) arise in this region (Alves et al. 2018; Bartsch et al. 1979; McNeal 1988). The TZ corresponds to 5 % of the prostate and, being a fibromuscular site, is the site where occurs the development of benign prostatic hyperplasia (BPH), a disease of the fibromuscular

stroma (Lee et al. 2011). Last, the CZ, which correspond to 25 % of the prostate, is located at the base of the prostate between PZ and TZ. It has the lowest incidence of PCa (Lee et al. 2011; McNeal 1988) (Table 1).

Table 1: Characteristics of the different zones of human prostate gland (adapted from (Lee et al. 2011)).

	Central Zone (CZ)	**Transition Zone (TZ)**	**Peripheral Zone (PZ)**
Volume of normal prostate (%)	25	5	70
Embryonic origin	Wolffian duct	Urogenital sinus	Urogenital sinus
Stroma	Compact	Compact	Loose
Origin of prostatic carcinoma (%)	5	25	70
Benign prostatic hyperplasia (%)	-	100	-

The human prostate is composed by two histological components, the glandular or epithelial component and the surrounding non-glandular or fibromuscular stromal component, which are tightly fused within a pseudo capsule (Alves et al. 2018; Lee et al. 2011). The epithelium of the prostate consists of secretory epithelial cells, basal cells, stem cells and neuroendocrine cells (Frick and Aulitzky 1991). Basal cells form a layer between the secretory cells and the basement membrane (Marker et al. 2003). The inner layer of the prostate capsule is composed of smooth muscle fibres and the outer layer is covering of collagen (McNeal 1988).

The prostate is an exocrine gland and PZ has a secretory epithelium responsible for the production of the prostatic fluid (Thapar and Titus 2014). It secretes a thin and alkaline fluid which compose the seminal fluid (Bhavsar and Verma 2014). The prostatic secretions are important for semen coagulation and liquefaction and are involved in the coating and uncoating of sperm and in the interactions with the cervical mucus (Hayward and Cunha 2000). The prostatic fluid is alkaline in order to neutralize the acidic environment of duct deferent and female vagina (Thapar and Titus 2014). It has in its composition zinc, calcium, citrate, phosphate, citric acid, spermine, prostaglandins, cholesterol, seminin, clotting enzyme, profibrinolysin, acid phosphatase, prostatic acid phosphatase (PAP) and the prostatic-specific antigen (PSA). PAP and PSA are products of secretory cells used to monitoring the onset and progression of PCa (Frick and Aulitzky 1991).

Regarding prostate embryology, the growth and development of the prostate begin in fetal life and are complete at sexual maturity. Prostate starts developing from the epithelial invaginations of the urogenital sinus. This event requires androgenic hormones, which induce cell differentiation and growth of both prostate epithelium and stroma with their effects mediated by the nuclear androgen receptor (AR) (Lee et al. 2011). ARs are activated by

testosterone (T) or by a more potent androgen, 5α-dihydrotestosterone (DHT). After birth, serum T levels are low and the growth of prostate is inhibited. During puberty, serum T levels rise significantly leading to an increasing deoxyribonucleic acid (DNA) content and consequently, prostate growth and stromal and epithelial cell differentiation mainly in the PZ, because this is the androgen dependent (Fujikawa et al. 2005; Marker et al. 2003). In the age of late 40s, prostate starts enlarging which coincides with the hormonal unbalance and consequent development of adenomas in the TZ and hyperplasia (Fujikawa et al. 2005).

Moreover, estrogenic levels also have a role in prostatic development acting synergistically with androgens (Frick and Aulitzky 1991). TZ is an estrogen dependent zone because it develops in the age-related decline in androgen levels and consequently in an estrogen-dominant environment (Fujikawa et al. 2005). The activity of steroid hormone receptors also can be affected by peptide growth factors, like insulin-like growth factor (IGF), which can be alternative ways to promote development, growth and maintenance of human prostate (Gnanapragasam et al. 2000).

2. General Notes on Prostate Cancer

2.1 Epidemiology

PCa is the cancer with more new cases per year, the fifth leading cause of cancer death among men worldwide, and the second most prevalent after lung and bronchus cancer (Hsing and Chokkalingam 2006; Pernar et al. 2018). One in 9 men will be diagnosed with PCa during their lifetime and will eventually die from it (Siegel, Miller, and Jemal 2018). According with the American Cancer Society, 164,690 new cases of PCa are estimated in the United States (US) in 2018 with 29,430 of them maybe resulting in death (Siegel et al. 2018). Likewise, PCa incidence in Portugal has increased in the last years, a tendency expected to continue until 2020 (Pina et al. 2017). In 2017, PCa represented 22,4 % of all cancers diagnosed in Portugal, being predicted a rise to 29 % in 2020 (Pina et al. 2017). On the contrary, mortality is expected to decline in this period. (Wong et al. 2016).

Considering the socioeconomic status, health care access, screening programmes, lifestyle factors and geographic and genetic background, it is possible to find contrasts in the epidemiology of PCa between populations around the world (Hsing and Chokkalingam 2006; Pernar et al. 2018). An increase in the incidence of this disease started in the early 1990s because it was introduce the PSA blood screening test, enabling more and early diagnosis (Benedettini, Nguyen, and Loda 2008). The highest rates of incidence are reported in non-Hispanic people (higher in black non-Hispanic than white non-Hispanic), following the Hispanic, American Indian or Alaska natives and the lowest rates in Asia (Benedettini et al. 2008; Siegel et al. 2018). However, international differences in PCa diagnosis practices can be a contributor

to the variation in PCa incidence rates worldwide (Center et al. 2012). In contrast, PCa mortality rates has a tendency to decrease in countries of North America, Oceania and Western and Northern Europe due to an improvement in treatments and an earlier detection (Center et al. 2012).

2.2 Risk Factors

Different types of research have identified several risk factors for PCa, which are clustered in two major categories: genetic and environmental factors (Hsing and Chokkalingam 2006). When combined, these factors can lead to development of PCa.

The major risk for PCa is **age**. The incidence of this disease increases exponentially with advancing age (Siegel et al. 2018) with over 80 % of prostate tumours in the US diagnosed above the age 65 (Hsing and Chokkalingam 2006). The weight of prostate is relatively stable during adult life until the 40-45 years old beginning to rise slowly thereafter, leading, frequently, to BPH. This condition is characterized by the benign growth of the prostate, however it has been related with an increased risk of PCa (Aaron, Franco, and Hayward 2016; Vuichoud and Loughlin 2015). Another factor is **race or ethnic variation** with higher rates of cancer incidence in African-Americans and the lowest in Asian men (Hsing and Chokkalingam 2006). A practical example is the incidence of PCa among Korean-American immigrants in the United States comparing with native Koreans in South Korean. These have low incidence of the disease, but when they migrate to US a shift occurs, increasing the risk of developing cancer similar to Americans (Lee et al. 2007). This clearly shows that ethnicity is intimately related with the influence of **dietary habits and lifestyle**. A western diet, highly caloric and enriched in saturated fat (especially fat from red meat (Sinha et al. 2009)), and refined carbohydrates is associated with an increased risk of PCa (Bostwick et al. 2004; Giovannucci et al. 1993; Hsing and Chokkalingam 2006). Possible explanations for the threat of diet are i) the dietary fat increases the availability of estrogens and androgens (Hsing and Chokkalingam 2006), and ii) cooking of the meat forms carcinogenic products that can accumulate in prostatic fluid and promote the disease (Giovannucci et al. 1993). On the contrary, Asian and vegetarian diets with low fat and high fiber content are associated with lower levels of hormones and therefore, lower risk in developing PCa (Hsing and Chokkalingam 2006). Closely, with diet appear **obesity and physical activity**. In fact, there are many studies supporting the association between the higher incidence of PCa with obesity and physical inactivity (Hsing and Chokkalingam 2006), a relationship that will be detailed in section 5.2 of this dissertation. Alcohol (Sesso, Paffenbarger, and Lee 2001), smoking, diabetes and liver cirrhosis are others factors associated with the emergence of PCa (Hsing and Chokkalingam 2006).

Inflammation, triggered by infectious agents via urine and sexual activity (Kohnen and Drach 1979), estrogens and hormonal changes (Robinette 1988) or damaged epithelial cells that release antigens against the prostate itself (De Marzo et al. 2013) also has been linked with

prostate malignancy. It downregulates the prostate tumour-suppressor genes and potentiates cell hyperproliferation, stress oxidative and DNA damage (Klein, Casey, and Silverman 2006).

The relationship between PCa and **genetic factors** is more difficult to define. Since PCa is diagnosed at late age, it is difficult to obtain DNA samples from living affected men for more than one generation. The family nature of PCa was first reported by Morganti *et al*. (Morganti et al. 1956) in 1956, and was only more than thirty years thereafter, that Carter *et al*. (Carter et al. 1992), described a segregation analysis of PCa. They first reported that PCa may be attributed to an autosomal dominant inheritance but of a rare and highly penetrant high-risk allele (Carter et al. 1992). However, there are other studies that attribute other type of inheritance (Bratt 2002). The risk to develop PCa is increased in men with multiple affected first-degree relatives (Simard et al. 2003), and if a relative has been diagnosed at an early age (Bratt 2002). In general, it is difficult to identify PCa susceptibility genes. Nevertheless, ELAC2/HPC2, RNASEL/HPC1, MSR1/SR-A, CHECK2, BRCA2, PON1, OGG1, MIC-1 and TLR4 genes have already been indicated as candidates (Gillanders et al. 2004; Klein et al. 2006; Simard et al. 2003), and more recently NEAT1, FOXA1, SPOP, ETS, CDH12 and ANTXR2 (Wedge et al. 2018).

2.3 Diagnosis

The first line diagnosis of PCa is the physical examination by the digital rectal examination (DRE) combined with the PSA blood test screening (Kelly et al. 2017). PSA is a serine protease biomarker encoded by the prostate-specific gene kallikrein 3 (Thapar and Titus 2014) and an androgen-dependent gene that is believed to have a role in liquefying the seminal fluid (Lilja, Ulmert, and Vickers 2008). When the architecture of the prostate gland is disrupted by tumorigenesis, inflammation or enlargement of the prostate, PSA protein is secreted into the serum (Thapar and Titus 2014). Serum PSA levels higher than 4.0 ng/ml (traditional threshold level) are considered indicative of PCa (Lilja et al. 2008). However levels below that can also be present in PCa patients, leading to false negatives results and poor specificity (Dimakakos, Armakolas, and Koutsilieris 2014). Furthermore, this test isn't always reliable, as PSA secretion also is influenced by cases of prostatitis, urinary tract infections and BPH leading to false positives (Lima et al. 2016). This problem can be partially overcome by measurement of free vs total PSA ratio, which has been show to be useful to distinguish between PCa and BPH cases. Low ratios might indicate the existence of small foci of PCa that could not be detected by other method (Ito et al. 2003; Lilja et al. 2008).

Based on the results of the first line tests, more sophisticated diagnostic techniques should be used, as biopsy or imaging techniques (Center et al. 2012). Biopsy analysis allows a histological examination of the tumour and it is done when there is a suspicion of cancer (Heidenreich et al. 2011). However, because of its heterogeneity, the sample may not be representative and also lead to false results (DeMarzo et al. 2003; Siegel et al. 2018).

Positron emission tomography (PET) and magnetic resonance spectroscopic imaging (MRI) are non-invasive imaging technologies based on the detection of metabolic biomarkers and

important for PCa staging (Teicher, Linehan, and Helman 2012). MRI is used to identify local lesions within the prostate and guide the biopsy, due to the great anatomic information and resolution of the different prostate structures (Lindenberg et al. 2016). It has been used for detection of localized PCa, but it can also detect recurrent or residual disease after radiation therapy or surgery and indicate early metastases (Lindenberg et al. 2016). PET helps providing greater specificity to PCa staging, however, it depends on the presence of abundant target, like glucose, and it has better benefits in localized high grade tumours (Lindenberg et al. 2016; Teicher et al. 2012), which display a more glycolytic phenotype.

Because of the limitations of PSA as a biomarker, its use has been questioned over the years, and the identification of new molecular tools an intensive area of research. In the last years, several studies have focused their attention to found new biomarkers for PCa detection and monitoring. Knowing the unique metabolic profile of the prostate gland and the composition of the prostatic fluid or other biological fluids, it is possible to find alterations which can indicate the progression of the disease (Kelly et al. 2016, 2017; Lima et al. 2016). The development of metabolic biomarkers is a non-invasive method that allow screening levels of citrate, phosphocholine, amino acids, spermine, carnitines, sarcosine, lactate and alanine, which can be changed in this disease, making them promising candidates biomarkers (Kelly et al. 2017; Lima et al. 2016; Thapar and Titus 2014). Among other markers for management of PCa (reviewed in (Gaudreau et al. 2016)), are the prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), early prostate cancer antigen (EPCA), enhancer of zeste homolog gene 2 (EZH2), the urokinase plasminogen activator (uPA) and the combined use of TGF-β1 and IL-6 (Dimakakos et al. 2014). However, their application in the clinic has not been the reality. Thus, continuous investigation on proteomic and metabolomics to discovery more specific and sensitive biomarkers than PSA, allowing an additional help on diagnosis and an insight into the dysregulated metabolic pathways of PCa (Dimakakos et al. 2014; Kelly et al. 2017).

2.4 Therapy

The therapeutic approaches in PCa need to be based on the tumour's phase of progression or, in other words, if the tumour is dependent or not on androgens. In the case of localized disease, initial treatment might be radical prostatectomy, prostate brachytherapy or androgen deprivation therapy (ADT) (Keyes et al. 2013). With the progression of the disease, PCa can develop towards a metastatic stage called metastatic hormone-sensitive PCa (mHSPC) (Sonnenburg and Morgans 2018), or become resistant to ADT, and at this stage, the disease is named castration-resistance PCa (CRPC) (Carles et al. 2012). CRPC is reflected on a continuous rise of PSA levels, progression of a pre-existing disease or appearance of metastatic CRPC (mCRPC) (Gomella, Petrylak, and Shayegan 2014). In any case, in advanced stages of PCa, chemotherapy and hormonal therapy are the therapies envisaged (Carles et al. 2012).

The standard treatment for PCa is ADT, which is a hormonal therapy used in both non-metastatic and metastatic tumour (Sharifi, Gulley, and Dahut 2005). It aims to inhibit testicular T production or its action in order to suppress PCa progression (Lilja et al. 2008). For that purpose, it can lower androgen levels or block its action (Gomella et al. 2014).

For lowering androgens levels, surgical castration (for example bilateral orchiectomy) or chemical castration with luteinizing hormone-releasing hormone (LHRH) agonist/antagonists can be used (Tzortzis et al. 2017). Surgical castration can reduce circulating serum T by more than 90 % and chemical castration can supress serum T levels by supressing pituitary gland-testes axis (Niu et al. 2018). This decrease in T levels from the testes can improve men survival, however has side effects like metabolic changes as obesity, diabetes, dyslipidemia and cardiovascular disease (Tzortzis et al. 2017). Nevertheless, androgens can also be synthesized in the adrenal in the form of dehydroepiandrosterone (DHEA), maintain residual levels of androgens in serum, so it is important targeting these non-testicular androgens (Camporez et al. 2013; Labrie et al. 2005).

Once the progression of PCa to an aggressive stage has been confirmed, androgen depletion should be maintained (Carles et al. 2012). In order to target residual androgens, restrain androgens effects with additional treatment with antiandrogens can be a solution for both mHSPC and mCRPC. Nonsteroidal antiandrogens like bicalutamide, nilutamide and flutamide bind competitively the AR, blocking AR signalling (Singer et al. 2008).

Enzalutamide is another AR blocker, approved by Food and Drugs Administration (FDA), for treatment of mCRPC that overcome the agonist properties of the previous ones (Gomella et al. 2014; Niu et al. 2018; Sonnenburg and Morgans 2018).

Instead of inhibiting AR, and due to the ability of metastatic PCa to generate its own androgens, it is possible to prevent androgen biosynthesis in all sites in the body, including within tumour itself (Gomella et al. 2014). FDA have granted abiraterone as an androgen biosynthesis inhibitor for mCRPC (Gomella et al. 2014).

Hormone therapy depends on the heterogeneity of the tumour, becoming complicated to predict the responses to this treatment (Mukamel, Nissenkorn, and Servadio 1980). Combination of both ADT and chemotherapeutical agents, like docetaxel and carbazitaxel, was demonstrated to be effective on overall survival and quality of life of mCRPC, when specially targeted on androgen-independent cells (Isaacs 1984; Petrylak 2014). Besides, radiopharmaceuticals are also commonly used to treat PCa metastatic lesions on the bone (Gomella et al. 2014). Radium 223 is an example approved by FDA that has already demonstrated its capacity to improve survival and reduce bone pain with relative lack of toxicity among mCRPC patients (Den, Doyle, and Knudsen 2014).

Estrogens have also been used as hormonal therapy through its negative feedback actions on hypothalamus and pituitary, inhibiting adrenal androgen production but have the disadvantage of cardiovascular complications (Carles et al. 2012).

Emerging therapies for metastatic PCa includes i) novel antiandrogens for hormonal therapy but with greater potency and less central nervous system penetration (Sonnenburg and

Morgans 2018); ii) histone deacetylase inhibitors, which interfere with cell proliferation and tumour angiogenesis (Zarour and Alumkal 2010); and iii) polymerase inhibitor that leads to unrepaired single-strand breaks and, consequently, cell death (Sonnenburg and Morgans 2018; Zarour and Alumkal 2010). Moreover, despite PCa has not been considered an immunogenic tumour, recent discoveries have targeted it for immunotherapy (Gomella et al. 2014). Therefore, various approaches are being tested like i) recombinant vaccine that encode transgenes for PSA, which is designed to stimulate the host immune system against tumour cells expressing PSA (Zarour and Alumkal 2010); and ii) immune-regulating agents, that enables T cell activation and promote cell-mediated cytotoxicity and tumour regression (Sonnenburg and Morgans 2018; Zarour and Alumkal 2010).

2.5 Mechanisms Underlying the Development of Prostate Cancer

The adult prostate gland maintain a hormone-regulated balance between cell proliferation and death. The development of PCa relies on the disequilibrium in these processes, the prostate structures become less organized and ultimately the gland suffers an excessive enlargement (Bianco et al. 2002). With further growth, invasion of the surrounding tissues and metastization to the lymph nodes, brain, bladder and bone is likely to occur (Miller et al. 2003).

Inflammation has been shown to cause morphological alterations in the prostatic epithelium precursors of the pre-cancerous lesions that can progress to PCa (Fig. 2) (Bostwick et al. 2004; Brawer 2005), but the cells remain able to proliferate, a condition named proliferative inflammatory atrophy (PIA). This leads to a formation of histological lesions, named high-grade prostatic intraepithelial neoplasia (PIN) (De Marzo et al. 1998). This state of neoplasia can evolve and disrupt the basal layer and turning into a precursor to PCa (Brawer 2005). Evidences supporting this are the loss or the down-regulation of inhibitor of the cell cycle progression, p27, in the vast majority of PCa cases (Guo et al. 1997; De Marzo et al. 1998; Yang et al. 1998).

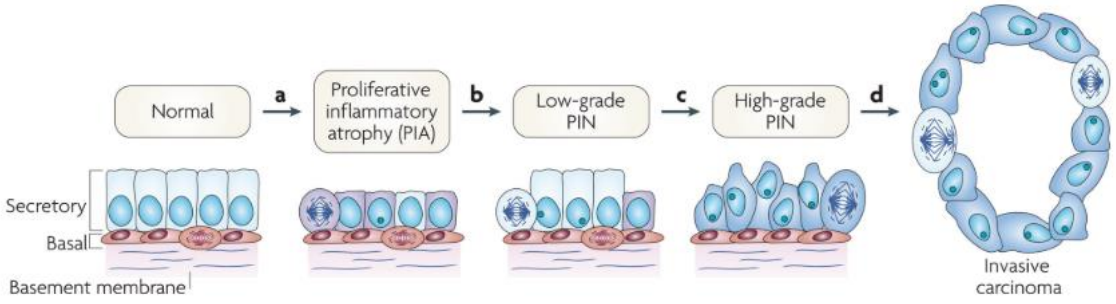

Figure 2. Cellular alterations in early prostate neoplasia progression. This process starts with infiltration of lymphocytes, macrophages and neutrophils. Then, phagocytes release reactive oxygen and nitrogen species causing DNA damage, cell injury and cell death, which trigger the epithelial regeneration. The downregulation of p27 and phosphatase and tensin homologue (PTEN) protein in luminal cells stimulates cell cycle progression. This favours the onset of genetic instability and the continued proliferation of genetically unstable luminal cells lead to progression towards invasive carcinomas. PIN – prostatic intraepithelial neoplasia (in (De Marzo et al. 2013)).

Besides the morphological and structural changes, the development of the disease has been associated with molecular events (DeMarzo et al. 2003) and alterations on cellular metabolism (Cutruzzolà et al. 2017; Massie et al. 2011; Vaz et al. 2012, 2016) (see Section 3 of this chapter).

At initial stages, development of PCa is modulated by the hormonal milieu, contrasting with the later stages of disease that are characterized by the resistance to hormonal modulation. Therefore, early-stages of PCa are hormone–dependent, or the so-called androgen sensitive PCa. The androgenic actions support prostate growth and development, and also apoptosis (Huggins 1967). In this way, dissemination of PCa depends on the androgens present in the body, such as Huggins and Hodges first confirmed (Huggins and Hodges 1941). In the presence of androgens, prostatic cancer cells have faster rates of proliferation than cell death, which enables continuous proliferation and growth (Isaacs 1984). This depends on the AR as the primary mediator (Pienta and Bradley 2006).

In case of early detection, androgen ablation or testicular surgical removal can be used as therapy, because it withdraws the main source of androgens and the ARs are not activated (Saraon, Jarvi, and Diamandis 2011). However, over time, there is a selection of cell subpopulations that can survive under environmental pressure and in absence of survival factors such as androgens resulting in a usually lethal form of disease called CRPC. These subpopulations, in order to survive, were resistant to androgen ablation or, after a period of growth arrest, adapted to the low androgen environment and resumed proliferation (Scher and Sawyers 2014). At this stage, ADT ceases to be effective and cancer progresses and metastasis happens (Saraon et al. 2011).

The mechanisms leading to androgen independence can be diverse and involve many pathways. The first one is named **Hypersensitive Pathway** where cells are not entirely independent of androgens because their responses are influenced by AR and androgens (Feldman and Feldman 2001). In this case, cancer cells develop the ability to use very low levels of androgens for growth due to increasing AR signalling by gene amplification or changes in the expression of coregulators or steroidogenic enzymes (Pienta and Bradley 2006; Saraon et al. 2011). **Promiscuous Pathway** refers to an androgen independence caused by genetic changes like missense mutations, which decrease the specificity of ligand binding (Feldman and Feldman 2001). Hence, aberrant activation of androgen signalling leads to AR activated by nonandrogenic steroid molecules or androgens antagonists in the circulation (Pienta and Bradley 2006). The third one, **the Outlaw Pathway**, depends on activation of AR in a ligand-independent mechanisms (Pienta and Bradley 2006), using growth factors overexpressed in some PCa like IGF-1, keratinocyte growth factor (KGF) or epidermal growth factor (EGF) (Hobisch, Kiocker, and Trapinan 1994). The fourth one, **the Bypass Pathway**, is a pathway which gives cells the ability to survive and inhibit apoptosis cascade independently of AR activation and absence of androgens (Feldman and Feldman 2001; Saraon et al. 2011). The last one is called the **Lurker Cell Pathway**, which defends that exists a small population of PCa stem cells or progenitor cells in tumour microenvironment, and even before tumorigenic event,

that are carcinogenic and do no express ARs, surviving to androgen-depletion therapy (Pienta and Bradley 2006).

3. Metabolic Alterations in Prostate Cancer

Sustained proliferative signalling, evasion of growth suppressors, resistance to cell death, acquisition of enabling replicative immortality, induction of angiogenesis, activation of invasion and metastasis, genome instability, inflammation, evasion of immune destruction and reprogramming of energy metabolism are the known hallmarks of cancer established since 2011 (Hanahan and Weinberg 2011). The rewiring of cancer cells metabolism has been since than a matter of huge interest with perspective of increasing knowledge considering the factors that drive tumour onset and progression, and use as a therapeutic approach. The general overview of cancer cells metabolic alterations and the specificities of PCa will be explored in this chapter.

Cancer cells have the distinctive feature of reprogramming their metabolism to promote and sustain growth, proliferation, and survival (Liberti and Locasale 2015). This characteristic was first reported by Otto Warburg, who observed that cancer cells have a large uptake and consumption of glucose, while displaying a preference for lactate production through anaerobic glycolysis, instead of the complete oxidation of glucose in the mitochondria, even in aerobic conditions (Warburg 1925). That led the conclusion that cancer cells could reprogram glucose metabolism to an “aggressive” metabolic phenotype known as the Warburg Effect (Warburg 1925, 1956).

The metabolism of glucose to lactate, in anaerobic conditions, is less efficient in terms of the adenosine triphosphate (ATP) generated per glucose molecule consumed (Heiden, Cantley, and Thompson 2009). However, cancer cells use the glycolytic metabolism to produce ATP before exposure to hypoxic conditions (Heiden et al. 2009). This generate large amounts of lactate and less ATP, but in a higher rate, thus ensuring the high energy demands (Liberti and Locasale 2015). If cancer cells were too efficient producing ATP, glycolysis would be inhibited, because key enzymes in limiting steps would be inhibited, and there would not be an energy source to the biomass of the tumour, so the cancer cells would stop growing (Eidelman et al. 2017; Zheng 2012). This become a selective advantage to overcome a nutrient depletion and promote cancer cell proliferation (Liberti and Locasale 2015), and create cancer cells capable of changing their metabolism to adapt to any microenvironment (Zheng 2012). However, overproduction of lactate can be toxic and as compensation, cancer cells highly express monocarboxylate transporters (MCTs), mainly MCT1 and MCT4, which export lactate to the extracellular medium reducing the intracellular lactate levels (Draoui and Feron 2011; Liberti and Locasale 2015) (Fig. 3).

However, cancer cells are influenced by the heterogeneity of the tumour mass and microenvironment: nutrient availability and distance from vasculature (Denicola and Cantley

2015; Fu et al. 2017; Zheng 2012). Hence, they will shift from an anabolic to a catabolic profile and vice-versa. In a nutrient rich environment, cancer cells are known to generate ATP aerobically via oxidative phosphorylation promoting differentiation (Denicola and Cantley 2015), whereas in nutrients and oxygen depleted environment, they adapt to alternative catabolic pathways like anaerobic glycolysis and autophagy, promoting proliferation (Denicola and Cantley 2015).

To support any bioenergetic pathways are necessary metabolic substrates, synthesized within tumour cells or taken up from circulation, which includes glucose, glutamine, lactate, pyruvate, ketone bodies and free fatty acids (FA) (Martinez-Outschoorn et al. 2016) (Fig. 3).

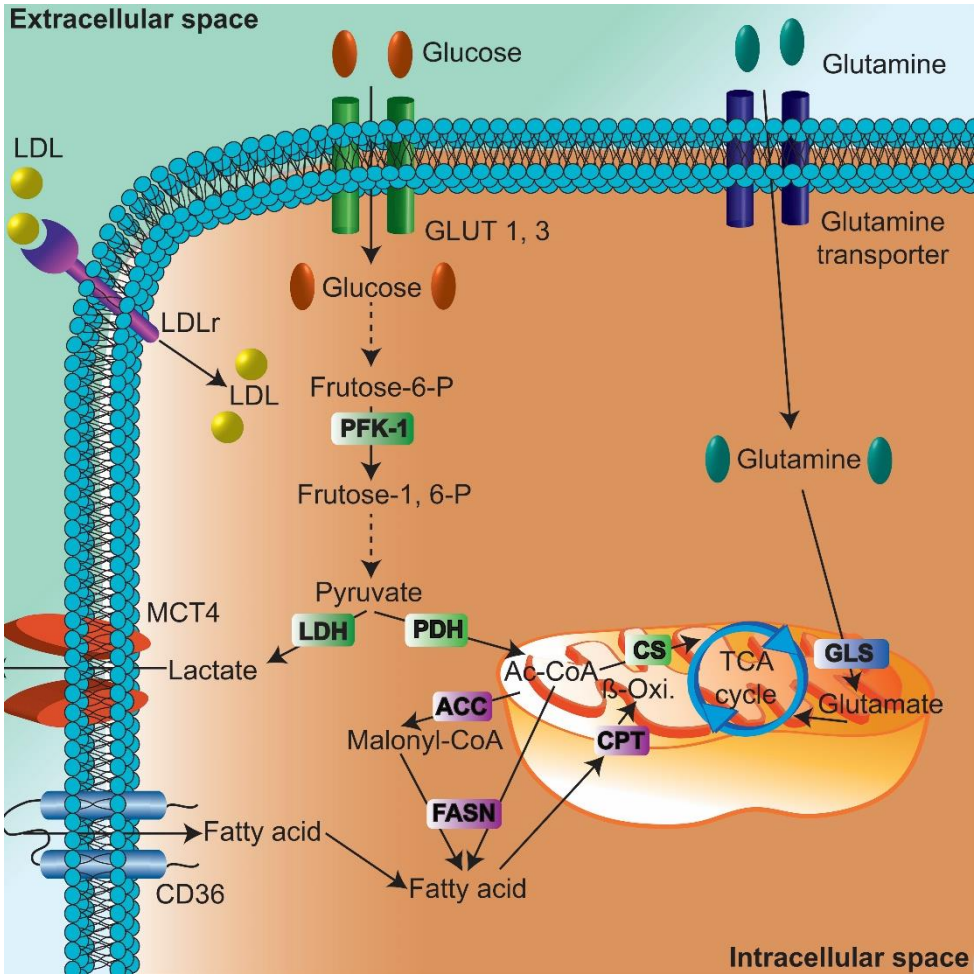

Figure 3. Metabolic pathways in cancer cells. Proliferating cancer cells display increased glucose uptake, a task mediated via glucose transporters (GLUTs, GLUT1 and GLUT3). Then, glucose enters glycolysis being metabolized through the activity of phosphofructokinase 1 (PFK1) and other enzymes (not shown) originating pyruvate. Lactate dehydrogenase (LDH) converts pyruvate into lactate, which is transported to the extracellular space by the monocarboxylate transporters (MCTs), namely MCT4. Pyruvate can also be converted to acetyl CoA (Ac-CoA), by pyruvate dehydrogenase (PDH), which in turn can be converted to citrate by citrate synthase (CS). Citrate fuels the tricarbocylic acid (TCA) cycle or is involve in fatty acid (FA) synthesis. Ac-CoA can be metabolized to malonyl-CoA by acetyl-CoA carboxylase (ACC) enzyme. Both citrate and Ac-CoA are used in *de novo* FA synthesis through FA synthase (FASN) enzyme. ß-oxidation of FA relies on the rate limiting step of FA transport to the mitochondria by carnitine palmitoyl transferase (CPT). Another source of FA are the exogenous FA, which are uptaken from the environment, by FA transporters, as, for example, CD36. Glutamine can be transported to the intracellular space and converted into glutamate, via glutaminase (GLS), and then converted to α-ketoglutarate acid that enters the TCA cycle.

Briefly, due to the lack of permeability and hydrophobicity of lipid bilayer of the eukaryotic plasma membrane, glucose transport into the cell happens with help of membrane associated carrier proteins – the glucose transporters: the Na^+-coupled glucose transporters (SGLT) and the glucose transporter facilitators (GLUTs) (Scheepers and Schurmann 2004). They are ubiquitous transporters, displaying different expression pattern concerning tissue specificities (Medina et al. 2003). In a proliferating cancer cell, glucose uptake is increased by the activity of GLUTs namely, GLUT1 and GLUT3, starting the glycolytic pathway (Krzeslak et al. 2012; Younes et al. 1997).

The enzymes involved in glycolysis are, in order, hexokinase (HK), phosphoglucoisomerase (PGI), phosphofructokinase (PFK), aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase (GAPD), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase (En) and pyruvate kinase (PK). Among them, three catalyse irreversible reactions, which are key regulating points of glycolysis: HK, PFK and PK (Akram 2013).

Inside the cell, glucose is phosphorylated and metabolized to glucose-6-phosphate (G-6-P) by HK (Wilson 2003). G-6-P is converted into fructose-6-phosphate (F-6-P), which is then phosphorylated to fructose 1,6-biphosphate (F-1,6-P) by PFK1 (Fig. 3), a rate limiting step of glycolysis (Clem et al. 2008). G-6-P is also an essential substrate for the pentose phosphate pathway where nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate are produced (Lima et al. 2016; Wilson 2003). NADPH is important for glutathione synthesis and defence against reactive oxygen species (ROS) and ribose-5-phosphate interplays in nucleotide synthesis (Denicola and Cantley 2015).

The end product of glycolysis is pyruvate (Fig. 3), which in non-cancer cells is a substrate for the tricarboxylic acid (TCA) cycle (Fig. 3). In contrast, cancer cells actively drive the pyruvate to be converted into lactate via lactate dehydrogenase (LDH), despite it can also be converted to acetyl coenzyme A (Ac-CoA) in the mitochondria by the pyruvate dehydrogenase (PDH) (Fig. 3). Ac-CoA is converted into citrate by citrate synthase (CS), which will fuel the TCA cycle to generate energy in the form of ATP (Gray, Tompkins, and Taylor 2014; Teicher et al. 2012), or alternatively, will be involved in FA synthesis (Martinez-Outschoorn et al. 2016) (Fig. 3). Ac-CoA can also be a precursor in ketone bodies synthesis (Rogers et al. 2014), and can also be derived from acetate in hypoxia conditions (Denicola and Cantley 2015).

De novo FA synthesis is almost exclusive of liver and adipose tissues (Deep and Schlaepfer 2016) with the cellular requirements normally being fulfilled by utilization of dietary FA (Wu, Daniels, and Lee 2014). However, in tumour cells almost all FA are produced via *de novo* synthesis, because lipogenic enzymes are overexpressed (Benedettini et al. 2008) in response to oncogenic signalling pathways such as the phosphatidylinositide 3 kinase (PI3K)/protein kinase B (AKT) and androgens actions (Wu et al. 2014). Acetyl coenzyme A carboxylase (ACC) metabolize Ac-CoA to malonyl-CoA, a substrate for FA synthesis through the action of fatty acid synthase (FASN) enzyme (Deep and Schlaepfer 2016; Flavin, Zadra, and Loda 2011). Increased expression of FASN is a common phenotype in several human carcinomas, like PCa (Swinnen et

al. 2002), and it is regulated by the mitogen-activated protein kinase (MAPK) and PI3K pathways (Kuhajda 2006). FA synthesis is essential for cell proliferation but ß-oxidation of FA is an important source of energy generating ATP in conditions of hypoxia, stress nutrient and autophagy (Denicola and Cantley 2015; Martinez-Outschoorn et al. 2016). This relies on the rate limiting step of FA transport to the mitochondria by the carnitine palmitoyl transferase (CPT) (Kuhajda 2006). CPT is important because some of FA synthesized are large molecules and cannot cross the inner mitochondrial membrane. Therefore, CPT will convert long-chain FA into acylcarnitine derivatives in order to enter the mitochondria and be oxidized, which will not happen with short-chain FA (Rogers et al. 2014). CPT is overexpressed due to adenosine monophosphate-activated protein kinase (AMPK) and p53 (Deep and Schlaepfer 2016) and its inhibition can induce cellular death in PCa (Schlaepfer et al. 2014). Moreover, cancer cell may acquire exogenous FA from the environment in order to fuel ß-oxidation (Fig. 3). This process is mediated by FA transporters present in the plasma membrane, as is the case of CD36, which is an uptake channel overexpressed in tumours with great progression capability and in metastasis (Lengyel et al. 2018; Zafirovic et al. 2017).

Glutamine is, besides glucose, a critical nutrient for metabolic pathways (Teicher et al. 2012). It is metabolized by glutaminase (GLS) into glutamate (Fig. 3) and then to an intermediary precursor of the TCA cycle, α-ketoglutarate, and various nonessential amino acids (Denicola and Cantley 2015; Zheng 2012). Under hypoxic conditions, glutamine generates citrate for lipid synthesis (Deep and Schlaepfer 2016).

The normal prostate has a unique metabolic profile, being more glycolytic than other non-pathological tissues (Gonzalez-Menendez et al. 2018). In prostate cells an increased glycolytic rate is necessary to sustain the citrate production, which is counterbalanced by the inhibited TCA cycle and low oxidative phosphorylation (Cutruzzolà et al. 2017). Prostate epithelial cells in the PZ of the prostate are programmed to favour citrate synthesis and accumulation in the seminal fluid over citrate utilization in the TCA cycle (Eidelman et al. 2017; Gonzalez-Menendez et al. 2018). Instead of degrading citrate to ATP production, citrate is accumulated because of the inhibition of mitochondrial aconitase, the enzyme responsible for oxidation of citrate. This happens due to the high accumulation of intracellular concentrations of zinc, which is another feature of prostate epithelial cells (Eidelman et al. 2017; Lima et al. 2016). In normal prostate cells, citrate concentration is 12-fold higher than blood plasma levels and zinc concentration 200-fold higher, confirming the unique profile of the prostate (Cutruzzolà et al. 2017).

In malignant prostate cells, there is a shift in this energy metabolism and citrate is oxidized in response to low zinc levels, providing a more efficient energy production (Lima et al. 2016). This occurs unlike most of cancer cells, because accumulation of zinc within prostate cell can be toxic by inducing the release of cytochrome c from mitochondria, activating the caspase cascade and leading to mitochondrial apoptosis (Cutruzzolà et al. 2017; Eidelman et al. 2017). Therefore, prostate malignant cells decrease the amount of stored zinc in order to avoid cell

death (Eidelman et al. 2017). This alteration is believed to be an early event in the malignant progression (Cutruzzolà et al. 2017).

Once normal prostate epithelial cells rely on aerobic glycolysis by inhibiting TCA cycle and oxidative phosphorylation due to citrate accumulation, PCa cells lose their ability to accumulate zinc (Elia et al. 2016) and corrupt tumour microenvironment fibroblasts to activate Warburg effect and inducing oxidative phosphorylation (Cutruzzolà et al. 2017). Fibroblasts are a source of lactate to PCa cells, which shuttle between fibroblasts and tumour tissue by the MCTs, and it is used in anabolic process (Fu et al. 2017). In case of metastatic PCa, generally to the bone, cancer cells interact with adipocytes stimulating lipolysis and in turn, adipocytes stimulate the Warburg effect on cancer cells (Cutruzzolà et al. 2017). These metabolic alterations are summarized on Table 2.

Table 2: Metabolic alterations in PCa cells (adapted from (Cutruzzolà et al. 2017)).

	Normal Cells	**Cancer Cells**	**Metastatic Cells**
Oxidative Phosphorylation	Inactive	Active	Inactive
TCA cycle	Inactive	Active	Inactive
Glucose	Consumed	Consumed	Consumed
Lactate	-	Consumed	Accumulated
Citrate	Secreted	Consumed	-

It has been also described that prostate cells can also reprogram metabolism, activating the Warburg effect, in response to inflammation, which speeds up the malignant transformation (Cutruzzolà et al. 2017).

Hence, deepening the knowledge of the unique specificities of the metabolic reprogramming of PCa cells will make possible to adapt treatment strategies to the different stages of disease counteracting the tumour requirements for progression.

There are several signalling pathways disrupted in cancer cells that are responsible for the metabolic alterations mentioned before (Jose, Bellance, and Rossignol 2011; Kim and Kim 2017). It is the case of PI3K pathway, hypoxia inducible factor 1 (HIF1) and MYC-dependent signalling, as well as the AMPK pathway and p53 signalling.

Aberrant activation of **PI3K pathway** is one of the most usually altered signalling pathway in human cancers (Luo, Manning, and Cantley 2003). Once activated, PI3K phosphorylates the lipid second messenger phosphatidylinositol-4,5-bisphosphate (PIP2) converting it to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 can recruit and activate downstream serine-threonine kinases as AKT and PI3K-dependent kinase-1 (PDPK1) (Luo et al. 2003; Robey and Hay 2010). AKT increases ATP production by accelerating metabolism, being crucial for cell survival and growth (Cairns, Harris, and Mak 2011; Robey and Hay 2010). So, it stimulates

glycolysis by regulating glycolytic enzymes (Fan, Dickman, and Zong 2010; Robey and Hay 2010) and activates kinase mammalian target of rapamycin (mTOR), which promotes protein and lipid biosynthesis (Zheng 2012). The phospholipid phosphatase and tensin homolog (PTEN) inhibits PI3K signalling by converting PIP3 back to PIP2, and inactivating mutations in the *PTEN* gene are common in many cancers, including PCa (Luo et al. 2003).

Other signalling pathways influencing metabolism are **HIF-1 and MYC-dependent pathway**. They are associated with the tumorigenic phenotype leading to tumour aggressiveness, progression and resistance to treatment (Arbeit et al. 2006). HIF-1 is a heterodimeric transcription factor expressed in all cells that activate genes related with increased oxygen delivery during oxygen deprivation and oxygen homeostasis (Zheng 2012). It has been shown that HIF-1 amplifies the glycolytic phenotype by increasing the transcription of genes encoding GLUT1 and GLUT3, as well as glycolytic enzymes (Cairns et al. 2011). HIF-1 also activates pyruvate dehydrogenase kinase 1 (PDK1) that inhibit mitochondrial PDH and all the oxidative dependent pathway (Papandreou et al. 2006). This help the cells to adapt to a stressful environment and resist to apoptosis (Kim et al. 2006; Arbeit et al. 2006). **MYC** is an oncogenic transcription factor linked to an impairment of metabolic phenotype (Kim and Kim 2017) and it correlates with HIF because it is also responsible for stimulation of glycolysis by activation of GLUTs and glycolytic enzymes (Cairns et al. 2011). Besides that, MYC is also a central regulator of glutaminolysis by upregulating glutamine catabolism (Gao, Ping 2009; Liu et al. 2012).

AMPK is a cell energy sensor capable of detecting unbalances in adenosine monophosphate (AMP)/ATP ratio and regulating energy availability (Shackelford and Shaw 2010). In case of caloric restriction, AMPK stimulates anabolic and energy producing pathways leading cells to an oxidative metabolic phenotype (Waldman et al. 2018). Therefore, ATP-consuming functions are supressed, like FA, sterols, glycogen and proteins synthesis (Flavin et al. 2011; Kuhajda 2008) and AMPK acts like a tumour suppressor (Kim and Kim 2017). ATP-producing activities are stimulated in order to restore energy homeostasis and enhance cell's ability to survive, like activation of p53 to arrest cell cycle and conserve energy, glucose uptake, FA oxidation and mitochondrial biogenesis (Flavin et al. 2011; Jones et al. 2005). AMPK is regulated by the upstream liver kinase B1 (LKB1), which phosphorylates it in cases of metabolic stress: nutrient starvation, hypoxia and lower levels of ATP (Kuhajda 2008). In cancer cells, this checkpoint needs to be overcome and needs to be suppressed in order to promote cell growth through mTOR signalling (Cairns et al. 2011). In that case, AKT pathway is no longer inhibited, HK activity is increased and there is an activation of glycolytic metabolism (Kuhajda 2008).

P53 is a tumour suppressor protein that acts as a transcription factor (Bensaad et al. 2006) responsible for inducing apoptosis, controlling cell cycle and proliferation, DNA damage and repair and cell metabolism (Gray et al. 2014). P53's activity is maintained at low levels in normal cells through the activity of Mdm2, which decreases its function and targets it for degradation in the proteasome (Jones et al. 2005). P53 is responsible for creating a balance between the glycolytic phenotype and oxidative phosphorylation phenotype since it activates

the expression of HK, which converts glucose into G-6-P (Cairns et al. 2011). However, p53 inhibits the glycolytic phenotype by promoting the expression of p53-induced glycolysis and apoptosis regulator (TIGAR). This enzyme can protect cell against oxidative stress, lowering ROS levels (Bensaad et al. 2006) and can lower fructose-2,6-biphosphate (F-2,6-P) levels in consequence of inhibition of PFK1 (Cairns et al. 2011; Gray et al. 2014). On the other side, p53 promotes oxidative phosphorylation by activating the expression of synthesis of cytochrome c oxidase 2 (SCO2) or by activating PTEN, inhibiting the PI3K pathway (Vousden and Ryan 2009). In cancer cells, p53 is silenced and the checkpoints of control are lost, TIGAR is inhibited and glycolysis can proceed (Jose et al. 2011). Therefore, p53 loss might be related with alterations on glycolytic metabolism in cancer cells.

4. The Biological Role of Estrogens

4.1 Estrogens Circulating Levels in Men

Several different modalities are used in literature to measure steroid levels. In this particular case, estrogen can be measure essentially by radioimmunoassays (RIA), electrochemiluminescence imunoassays and liquid chromatography tandem mass spectrometry (LC-MS/MS), but also gas chromatography-mass spectrometry (GC-MS) (Table 3). Between these modalities, LC-MS/MS can detect small amounts of steroids and reach a lower limit of quantification. Hence, it is the best modality to use (Snaterse et al. 2017) as it is described by Wang *et al*. (Wang et al. 2015).

Different studies use distinct units to measure serum steroid concentrations, and data obtained from independent studies were converted to molar concentrations to facilitate comparisons.

Concerning the reference 17β-estradiol (E_2) serum concentrations in men, a wide range can be found, all of them obtained from healthy individuals between 30 to 90 years old. This range is relevant because it includes the critical age when men, normally, develop PCa. Thus, E_2 levels are very low in men with a mean concentration ranging from 0,028 to 0,235 nM (Table 3).

E_2 levels remain fairly constant through lifetime (Carruba 2007). Yet, males are exposed to high estrogen/androgen (E/T) ratio twice in their lifetime: as a foetus with maternal E_2 and during old age when serum T decrease due to reduced Leydig cell function in the testis. Regarding age, E_2 serum remain essentially unchanged (Belanger et al. 1994; Hammond et al. 1978). However, with age several endocrine events occur such as decline in testicular function as already mentioned, increase in adiposity and extragonodal aromatization, which will influence the E_2 levels (Ho et al. 2011). Besides that, circulating estrogens levels can fluctuate

with metabolic diseases (Neuzillet et al. 2017), as well as due to environmental factors and ethnicity, manipulating the hormonal system (Ahluwalia et al. 1981).

The relationship between PCa and E_2 serum levels has been largely controversial and there are no statistically significant associations between serum concentrations of this hormone and the risk of PCa. However, Barrett-Conor *et al*. (Barrett-Connor et al. 1990) associated elevated serum levels of E_2 with an increased risk of PCa. Considering the progression of the disease and available literature, a slight increase in E_2 levels is observed in the majority of the existent studies ranging between 0,050 and 0,300 nM (Table 3). However, this is non-consensual matter and larger, rigorous and strictly controlled studies are needed to establish the relationship between E_2 levels and PCa.

Table 3: E_2 serum levels in healthy men and PCa patients.

E_2 serum levels (nM)		**Methodological approach**	**Reference**
Healthy men	*PCa patients*		
0,114 – 0,125	0,110 - 0,128	Dextran-coated charcoal method	(Ahluwalia et al. 1981)
0,110 - 0,160	0,120 – 0,160	RIA	(Barrett-Connor et al. 1990)
0,200	-	RIA	(Belanger et al. 1994)
0,093	-	Chemiluminescence immunoassay	(Cao et al. 2012)
0,200	-	RIA	(Chen et al. 2003)
0,066 - 0,221	0,066 - 0,233	-	(Endogenous Hormone and Prostate Cancer Collaborative 2008)
0,125	0,121	RIA	(Gann et al. 1996)
0,070	-	Chemiluminescence immunoassay	(Hagiuda, Ishikawa, and Marumo 2014)
0,100 – 0,150	0,050 - 0,300	Automated nonchromatographic radioimmunoassay	(Hammond et al. 1978)
0,235	0,247	-	(Hsing and Comstock 1993)
0,089 – 0,120	0,086 - 0,106	GC-MS	(Neuzillet et al. 2017)
0,062	0,066	RIA	(Nomura et al. 1988)

0,028 – 0,167	-	Chemiluminescence immunoassay	(Pellitero, Olaizola, and Alastrue 2012)
0,106	0,106	RIA	(Platz et al. 2005)
0,122	0,122	Heterogeneous competitive magnetic separation assay	(Salonia et al. 2013)
0,028 – 0,156	0,063 - 0,068	Electrochemiluminescence immunoassay	(Schnoeller et al. 2015)
0,107	0,101 - 0,105	Electrochemiluminescence immunoassay	(Severi et al. 2006)
0,035	-	LC-MS	(Wang et al. 2015)
0,093	-	LC-MS	(Hsu et al. 2015)
0,084	0,083	GC-MS	(Daniels et al. 2010)
0,108	0,159	Enzyme linked immunosorbent assay (ELISA)	(Usoro et al. 2015)

4.2 Estrogens Biosynthesis and Function

Steroid hormones are small lipophilic molecules that circulate on the blood stream (Chien, Rosal, and Chung 2017). They can be divided in three groups: (1) mineralocorticoids, (2) glucocorticoids and (3) sex steroids (estrogens, progestogens and androgens). This division is not only made by the chemical structure of steroids but because they act through specific steroid hormone receptors, which activate the transcription of set of specific genes, having, consequently, different physiological actions (Miller and Auchus 2011; Mindnich, Möller, and Adamski 2004). All steroids have cholesterol as the common ancestral precursor (Fig. 4). Cholesterol is synthesize from acetate in the endoplasmic reticulum, but the majority of its supply comes from plasma low-density lipoproteins (LDLs) that enter the cells through receptor-mediated endocytosis directing cholesterol to the endosomes (Miller and Auchus 2011). Then, free cholesterol inside cells need to be transfer into the inner mitochondria membrane where the steroidogenic pathway is initiated. Cholesterol transport needs assistant proteins because it is insoluble in the aqueous cytosol (Miller 1988). The transportation from the outer mitochondrial membrane to the inner mitochondrial membrane is possible by the steroidogenic acute regulatory protein (StAR) (Chien et al. 2017).

The overall steroidogenic pathway (Fig. 4) has fewer enzymes than reactions, which means that more than one reaction is catalyzed by the same enzyme (Miller and Auchus 2011). The steroidogenic enzymes are secreted from gonads, adrenals and placenta during pregnancy, but peripheral tissues including skin, bone, adipose tissue, breast, lung, endometrium, prostate, liver, gut, kidney, epididymis and brain have also been shown to display steroidogenic activity (Bouguen et al. 2015; Cooper et al. 2001; Labrie et al. 2005; Luu-The 2001). There are two big groups of enzymes in steroidogenesis: cytochrome P450 enzymes and hydroxysteroid

dehydrogenases (HSD). Cytochrome P450 enzymes can be at mitochondria (type1) or at endoplasmic reticulum (type 2) and catalyse irreversible reactions (Miller and Auchus 2011). For their activity they require NADPH as the electron donor, which can be generated from the pentose phosphate pathway or via mitochondria (Chien et al. 2017). HSDs also can be divided in two groups based on their structures: the short-chain dehydrogenase/reductase (SDR) family or the aldo-keto reductase (AKR) family. The first group use nicotinamide adenine dinucleotide (NAD+) as cofactor and the second uses NADPH, catalysing reversible reactions with the prevalence for oxidative or reductive mode (Miller 1988; Miller and Auchus 2011). These enzymes bind steroid substrates, like cholesterol and lipophilic steroids, in the hydrophobic environment (Chien et al. 2017).

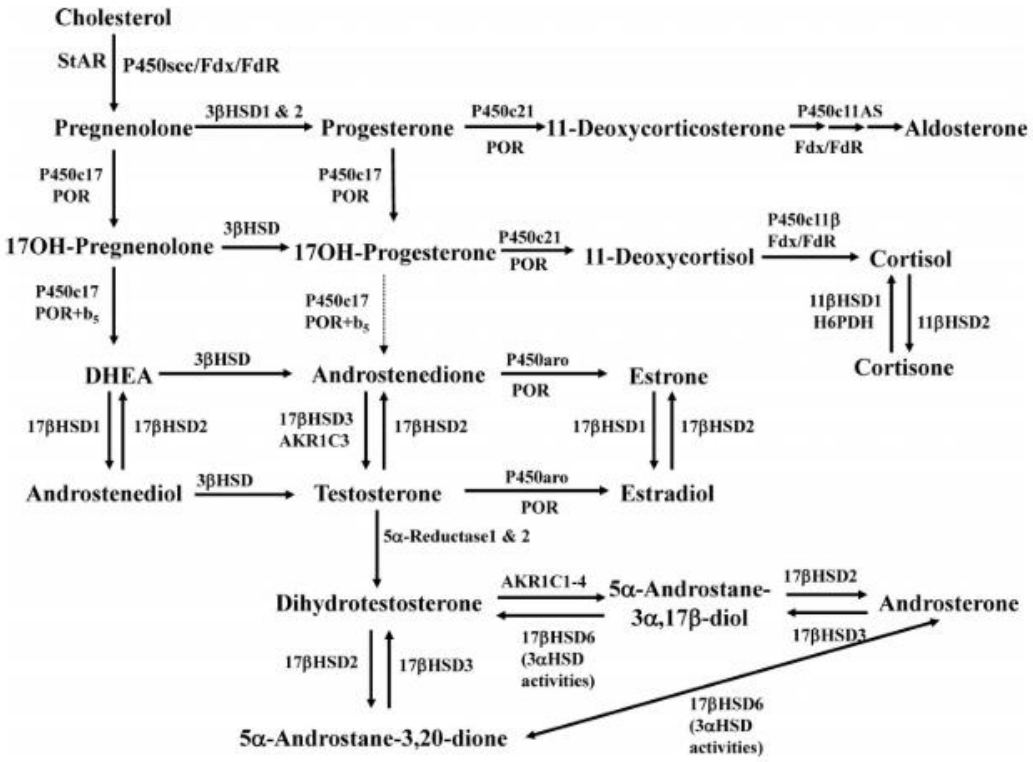

Figure 4. Human steroidogenic pathway. Key enzymes and cofactors are shown beside arrows. Two arrows with opposite directions represent a reversible reaction. The dashed arrow indicates poor flux of that reaction. The three small arrows indicate intermediate steps. Not all intermediate steroids, pathways, and enzymes are shown. (in (Miller and Auchus 2011)).

Estrogens synthesis is the last reaction on the steroidogenic pathway and it happens due to the activity of aromatase/P450aro/CYP19A1 enzyme (Cooke et al. 2017), a heme protein (Simpson and Davis 2001), which role is the aromatization of the A ring of the androgens precursors to estrogens in the endoplasmic reticulum (Miller 1988) or in the mitochondria (Królik and Milnerowicz 2012). In other words, it is a reaction where the conversion of C19 steroids (androgens) to C18 steroid (estrogens) occurs (Matzkin and Soloway 1992; Zhou, Pompon, and Chen 1991). For that, three molecules of molecular oxygen and six NADPH are needed (Zhou et al. 1991). Aromatization of androgens is the body's principal source of estrogen (Williams 2012). It occurs mainly in the Sertoli cells of the testis but also in others estrogen-sensitive tissues

such as placenta, ovary, adrenal, adipocytes, osteoblasts, Leydig cells, breast cells, prostate cells and endothelial cells (Matzkin and Soloway 1992; Takase et al. 2006; Williams 2012). The T produced in the testis is the main source that is delivery to the body tissues through blood circulation (Matzkin and Soloway 1992). In extragonadal tissues aromatase only can do its role using external androgenic precursors, like DHEA and DHEA sulfate (DHEA-S) and circulating T, because outside the gonadal tissues it is not possible converting cholesterol to C19 steroids (Simpson and Davis 2001).

As mentioned before, natural estrogens are steroids with 18-carbon, and contain an aromatic ring with the CH_3 group in 13 carbon. Three natural biological active estrogens are identified (Fig. 4): estrone (E_1), estriol (E_3) and E_2 the most biologically active.

All the natural estrogens, E_1, E_2 and E_3 are produced locally within the prostate via aromatization of androstenedione and T (Fig. 4).

The estrogens biosynthesis can be contradicted by its inactivation by enzymes present in the liver, testes, epididymis and ductus deferens (Cooke et al. 2017). This metabolic process is responsible for creating an inactive hormone and water-soluble make possible its excretion in the urine or feces (Zhu and Conney 1998). First estrogens are metabolized to form catechol estrogens (Królik and Milnerowicz 2012; Rogan and Cavalieri 2004). Secondly, catechol estrogens undergo an inactivation process called conjugation via phase II enzymes. Inactivation occurs by sulphate conjugation or glucuronidation forming estrogen conjugates (Królik and Milnerowicz 2012; Rogan and Cavalieri 2004). The metabolism of estrogens can also occur in extrahepatic tissues and, in that case, conjugation of catechol estrogen occurs by O-methylation (Cavalieri and Rogan 2016). Failure in estrogen metabolism makes impossible the succeed inactivation of catechol estrogens, which can induce carcinogenesis dependent of estrogens. The role of these compounds in PCa is explained in a separate section.

Despite E_2 is widely recognized as the major endocrine regulator of female physiology, other functions for this hormone have been described. In fact, estrogens actions are know in the brain (Cooke et al. 2017; Rubinow 2017; Schulster, Bernie, and Ramasamy 2016), skin (Cooke et al. 2017), thyroid (Królik and Milnerowicz 2012), adipose tissue (Chen, Brown, and Russo 2009; Rubinow 2017), urinary system (Cooke et al. 2017), immune system (Boibessot and Toren 2018; Cooke et al. 2017), and also at the vascular level (Cooke et al. 2017; Schulster et al. 2016), in bone mineralization (Cooke et al. 2017; Faustini-fustini, Rochira, and Carani 1999; Simpson and Davis 2001), and as regulators of spermatogenesis (Schulster et al. 2016).

In the **brain**, estrogens are responsible for regulating libido and sexual desire, cognitive function (Cooke et al. 2017; Schulster et al. 2016), appetite and energy expenditure (Rubinow 2017). Besides that, estrogens have an important role in the regulation of gonadotrophin secretion, because they act on the hypothalamus to decrease gonadotropin releasing hormone (GnRH) secretion, diminishing luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels (Faustini-fustini et al. 1999). Studies prove this interaction when after administration of aromatase inhibitors, there are a decrease of serum E_2 and an increase in T, LH and FSH (Mauras

et al. 2000; Rochira et al. 2005). Therefore, estrogens have a role in mediate changes in energy balance through effects on mood and motivation in men (Rubinow 2017).

E_2 on **adipose tissue** can influence synthesis and oxidation of FA and lipolysis (Chen et al. 2009; Rubinow 2017). It also has effects on adaptive and innate **immunity**, and regulatory immune functions as cellular differentiation (Boibessot and Toren 2018; Cooke et al. 2017; Rubinow 2017). Moreover, estrogens may increase effects on macrophages by augmenting the expression of inflammatory cytokines (Boibessot and Toren 2018). In **vascular smooth muscle** cells, E_2 is responsible for vasodilation. Thus, it is related with incidence of erectile dysfunction in case of inhibiting hypothalamus-pituitary axis and consequently FSH and LH, thus reducing circulating T necessary for normal erectile function (Cooke et al. 2017; Schulster et al. 2016). In the **bone**, it is responsible for mineralization and remodelling (Cooke et al. 2017; Faustini-fustini et al. 1999; Simpson and Davis 2001) as confirmed with aromatase inhibitor treatment where it was seen a decreased bone strength (Bajpai et al. 2006). In the **skin**, estrogen is responsible for wound healing by increasing keratinocytes proliferation (Cooke et al. 2017). Raised E_2 levels stimulate the developing and proliferation of **epithelial cells** of the prostate and the prostate stroma (Rahman, Hofland, and Foster 2016). Lastly, relatively to **spermatogenesis**, Leydig, Sertoli and germ cells produce estrogen at various states of testes development leading to T modulation (Correia et al. 2015). Aromatase is largely presenting Leydig cells, producing high amounts of E_2 in the testis that can inhibit LH and T levels (Beurden et al. 1978; Genissel, Levallet, and Carreau 2001). Excessively abnormal E_2 levels can lead to a reduction in the number of germ cells and viable sperm, likely by increasing apoptosis in the testis (Correia et al. 2014, 2015). In Sertoli cells the production of estrogens mostly occurs in early stages of development of sperm in immature testes and it is important to forming cell-to-cell adhesions with N-cadherins between germ cells (MacCalman et al. 1997). In germ cells, aromatase is found in elongated spermatids and spermatozoa and the E_2 produced is important to sperm maturation and capacitation as well as acrosome reaction and a successful fertilization (Schulster et al. 2016).

Estrogens also have been pointed out as metabolic regulators and their roles in the regulation the glycolytic and lipid metabolism will be explained in the following chapters.

4.3 Evidence of Estrogens as Metabolic Regulators

Energy metabolism pathways maintain homeostasis balancing the energy expenditure and energy storage processes. The first one starts with glucose transport, follow with glycolysis, TCA cycle, oxidative phosphorylation and ATP utilization. The storage process includes FA biosynthesis, FA disposition and cholesterol and triglycerides synthesis/storage (Chen et al. 2009). Moreover, another storage process is glycogenesis, where glucose can be transformed into glucose storage molecule, the glycogen, which is a typical event in liver and muscle cells (Dashty 2013), that has been shown not to be exclusive of these cell types.

Different types of evidence have been establishing that hormones can influence the metabolic pathways. To exert their actions steroid hormones bound to specific nuclear receptors, that act as transcription factors regulating the expression of target genes (Goffart and Wiesner 2002). In the specific case of estrogens, they can stimulate or inhibit the activity of key enzymes in these metabolic pathways, which will be discussed next.

First, considering the glucose transport, it has been demonstrated that it can be regulated by several peptides and steroid hormones, which is the case of EGF, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin and also estrogens (Welch and Gorski 2015). According to Shinkarenko *et al.* (Shinkarenko, Kayet, and Degani 1994), estrogens administration can enhance glucose consumption in immature rat uterus. Moreover, studies showed that E_2 stimulates GLUT1 in immature rat uterus (Welch and Gorski 2015) and in MCF7 human breast cancer cells (Rivenzon-Segal et al. 2003), GLUT3 in primate cerebral cortex (Cheng et al. 2016), and GLUT4 expression in primate cerebral cortex (Cheng et al. 2016) and in MCF7 cells (Cuesta et al. 2013), improving glucose cell uptake. Nevertheless, the expression of GLUT2 is not significantly affected by hormonal treatments in human breast cancer cells (Medina et al. 2003) and endometrial cancer cells (Medina et al. 2004).

Once glucose is inside the cells, glycolysis can proceed. In similarity with the effects reported on GLUTs, evidence shows that E_2 enhances the expression and activity of several key enzymes of glycolysis, namely, HK (Kostanyan and Nazaryan 1992), PFK (Kostanyan and Nazaryan 1992), PGK (Reiss 1988), En (Pastorelli et al. 2005; Reiss 1988) and PK (Kostanyan and Nazaryan 1992; Pastorelli et al. 2005; Reiss 1988) in rat brain, uterus and bone.

The end product of glycolysis, pyruvate, can be convert into lactate by LDH, which is used by cancer cells as a metabolic advantage (Martinez-Outschoorn et al. 2016). Previous studies reported that LDH is increased in response to E_2 treatment in breast cancer MCF-7 cells (Burke, Harris, and Mcguire 1978; Nagai, Sonohara, and Brentani 1988). The lactate can be taken up or export by malignant cells by a lactate shuttle, through MCTs (Oliveira et al. 2011). The effect of estrogens on the MCTs expression in cancer cells is unknown, but E_2-treatment of Sertoli cells, resulted in augmented MCT4 levels (Oliveira et al. 2011).

Next, in the TCA cycle a wide range of intermediary metabolites is produced. Once again, reports exists proving that E_2 is involved in the regulation of key enzymes for the TCA cycle. It is the case of CS, the enzyme responsible for the condensation between the acetyl group of acetyl-CoA and oxaloacetate to form citrate, where its activity is increased by E_2-treatment after ovariectomy (Beckett and Toth 2002). Another example is the study of Pastrorelli *et al.* (Pastorelli et al. 2005), which showed enhanced expression of mitochondrial aconitase 2, the enzyme that converts citrate to isocitrate, in the E_2-treated group (Pastorelli et al. 2005). A reports also showed that isocitrate dehydrogenase, the enzyme that catalyses the reaction between isocitrate and oxalosuccinate, displayed increased activity upon administration of exogenous E_2 (Yadav 1988). An effect observed in various tissues of ovariectomized rats, like brain, liver and kidney (Yadav 1988).

Mitochondria generates 95 % of the cellular energy in the form of ATP and NADH and FADH using molecular oxygen via electron transfer in oxidative phosphorylation (Goffart and Wiesner 2002). Mitochondrial respiration is enhanced by E_2 (Roberts and Szego 1953; Salmony 1955). Once ATP is produced, it has to shuttle across the inner mitochondrial membrane, providing mitochondrial energy to the cytosol. This is mediated by adenosine nucleotide translocators (ANTs). ANT1 and ANT2 are the two isoforms known with the expression of the first being upregulated by E_2 in female rat hearts (Too, Giles, and Wilkinson 1999).

In parallel, ß-oxidation is a cyclic process that occurs primarily in mitochondria, coupled to ATP production by the respiratory chain, and reduces FA in two carbons per cycle, producing, simultaneous, Ac-CoA, NADH and $FADH_2$. FA can be short-chain or long-chain, which determines their entrance in the mitochondria matrix, in order to let the oxidation happen. Long-chain FA can only enter the mitochondria with help of the CPT1 (Rogers et al. 2014). CPT1 activity was shown to be increased in PCa PC3 cell line in response to E_2 (Mas et al. 2018). Once inside mitochondria, the enzymes involved in the FA oxidation pathway - acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-L-hydroxyacyl-CoA dehydrogenase and ß-ketoacyl-CoA thiololase - act on CoA species (Rogers et al. 2014). Toda *et al*. (Toda et al. 2002) also studied the liver of aromatase-deficient (ArKO) mice, which are characterized by the total absence of estrogen production, and showed that the expression of hepatic ß-oxidation enzymes was diminished, implying estrogens in the ß-oxidation metabolism. A similar study conducted by Nemato *et al*. (Nemoto et al. 2000) using the ArKO mice, also demonstrated the decreased expression of ß-oxidation enzymes after treatment with an estrogen-antagonist, tamoxifen, which could be restored by supplementation with E_2 (Nemoto et al. 2000). Therefore, E_2 seems to potentiate FA ß-oxidation in the liver (Palmisano, Zhu, and Stafford 2017).

Acetyl-CoA, the end product of ß-oxidation, either can enter the TCA cycle to generate ATP or serve as a precursor for FA synthesis (Rogers et al. 2014). FA synthesis occurs in the liver or on adipose tissue and starts with the conversion of acetyl-CoA to malonyl-CoA by ACC (which has to be in the dephosphorylated/activated form (Zhu et al. 2014)), and then FASN catalyses the condensation of malonyl-CoA and acetyl-CoA into FA for storage (Wu et al. 2014). Both ACC and FASN expression and activity are modulated by E_2. Regarding ACC, reports showed the E_2-negative effects. Jacobs *et al*. (Jacobs, Vance, and Cole 2010), reported experiments showing that E_2-antagonist treatment maintain ACC in its dephosphorylated form, increasing FA synthesis. Also, Zhu *et al*. (Zhu et al. 2014) described that estrogen signalling decrease ACC-mediated FA synthesis. Moreover, FASN expression and activity can be supressed by E_2-treatment in estrogen receptor (ER) knockout mice via ERα (Bryzgalova et al. 2008; Gao et al. 2006). Therefore, E_2 promotes FA oxidation by inhibiting the synthesis (Palmisano et al. 2017). Exogenous FA are another source for the cells, which enter the cell by FA-specific membrane transporter, CD36 (Lengyel et al. 2018). E_2 diminish the expression of CD36 in heart tissue from high fat rats (Zafirovic et al. 2017), but their role regulating the expression levels of CD36 in cancer tissues and cells, in general, and particularly in PCa are entirely unknown.

5. Estrogens, Obesity and Prostate Cancer

5.1 Estrogens Actions as Carcinogens

It is well known the role of androgens maintaining growth and function of prostatic tissue, as well as, stimulating agents in PCa (Chen et al. 2003). More recently androgens were defined as modulators of PCa cells metabolism fuelling progression of disease (Massie et al. 2011; Vaz et al. 2012, 2016). Despite their less clear role in prostate physiology compared to androgens, estrogens have also been implicated in the onset and progression of PCa (Carruba 2007; Ho et al. 2011).

Indeed, the dual role of estrogens in prostate cells has been gaining consistency. If some studies defend that estrogens are potential causative agents of PCa, other indicate that these hormones may be protective against PCa, as will be discussed below.

An *in vivo* study using hypogonodal mouse model, showed that the administration of estrogens induced growth of the prostate with specific changes on both stroma and epithelium, initiating histological alterations like hyperplasia and dysplasia (Bianco et al. 2002). Another study using Noble rats combined androgens and estrogens treatment, which together are markedly induced cell division (Leav et al. 1989). A similar report using the same rats strain and treatment combination showed that tumours appear at an early age comparatively with androgen treatment alone (Noble 1977).

In vitro studies using the LNCaP cell line stimulated them with different concentrations of E_2 resulted the increase of cell proliferation (Castagnetta et al. 2015), and augmented expression of PSA and IGFs (Arnold et al. 2004). E_2-treatment also showed effects promoting invasion of prostate stromal cells by upregulating expression of metalloproteinase 2, a key enzyme involved in the degradation of extracellular matrix (ECM) (Yu et al. 2011). These findings correlate E_2 effects with prostate malignancy and PCa metastasis (Yu et al. 2011).

Another mechanisms underlying the carcinogenic actions of estrogens include exposure to estrogens doses that i) induce benign lesions derived from the basal-cell proliferation termed squamous metaplasia of prostatic epithelium; ii) upregulate growth factor signalling pathways; iii) activate MAPK; iv) increase cell-survival by overexpression of antiapoptotic mediators; v) increase oxidative stress-induced DNA damage; and vi) change gene expression (Ho et al. 2011). However, estrogen administration to men during long periods of time causes different degenerative changes according to the histological zone of the prostate (Huggins and Webster 1946). Usoro *et al.* (Usoro et al. 2015) performed a study comparing E_2 levels in patients with BPH or PCa. These authors showed that E_2 serum levels are raised in the patients and with lesser extent in the BPH than PCa. This is because of the differentiation of the prostatic epithelia and stroma and the increased activity of aromatase enzyme (Usoro et al. 2015).

Therefore, with the progression of the disease, a slight increase in E_2 levels tend to occur as also was systematized in (Table 3).

Another pathway involving estrogens in carcinogenesis is the failure in the process of conjugation of estrogens and their metabolites (Królik and Milnerowicz 2012). Endogenous estrogens are metabolized in the liver prior its possible excretion in the urine and feces in the form of catechol estrogens, as explained before. Failure in estrogen metabolism hampers their succeed inactivation (Królik and Milnerowicz 2012). However, catechol estrogen quinones can be inactivated with other protective body mechanisms: reactions with glutathione or reduction by quinone reductase, forming catechol quinones or semiquinones (Cavalieri and Rogan 2016). If all the inactivating process are insufficient, quinones can react with DNA, forming a covalent bond and creating depurinating adducts (Cavalieri and Rogan 2016). In this way, they can react with molecular oxygen and generate free radicals and promote strand breakage of DNA (Ho et al. 2011; Królik and Milnerowicz 2012). Those adducts leave apurinic DNA sites and, if the machinery of repair does not work (Cavalieri and Rogan 2010), leads to oxidative damage of genes and mutagenesis (Królik and Milnerowicz 2012; Rogan and Cavalieri 2004). This is a direct way by which estrogen can induced carcinogenesis and likely PCa (Rogan and Cavalieri 2004) because critical mutations are produced, leading to abnormal cell proliferation (Cavalieri and Rogan 2016).

Moreover, imbalance in estrogen homeostasis can also be caused by the overexpression of aromatase (Zhu and Conney 1998), and by exposure to exogenous chemical substances with estrogenic activity, the xenoestrogens, which can induce squamous metaplasia (Cavalieri and Rogan 2016) and polymorphisms in quinone reductase (Cavalieri and Rogan 2010). Thereby, any alteration on estrogen homeostasis will increase growth, inhibit anti-apoptotic processes and promote metaplastic and neoplastic changes in estrogen-sensitive tissues (Williams 2012), where estrogens act as epigenetic carcinogens (Cavalieri and Rogan 2010).

In general, several environmental chemicals acting as endocrine disruptors (EDs) can promote carcinogenesis. This group of chemicals include farming, pesticides, and environmental estrogens, which mimics the estrogen activity of endogenous hormones, through the activation of ERs. Also, it is believed that exposure to those compounds may interfere with the metabolism of steroid hormones, altering their availability and balance (Prins 2008). The predisposing for developing PCa in adulthood was proposed to be determined by exposure in fetal life to natural or environmental estrogens, a process known as estrogen imprinting (Lobaccaro and Trousson 2014; Prins et al. 2007). However, phytoestrogens, a type of EDs commonly present on Asian diets, are known as estrogen-chemioprevent agents of PCa (Ho et al. 2011). They exert a negative feedback on hypothalamic-pituitary-gonadal (HPG) axis, inhibiting LH production and consequently reducing T and estrogens synthesis. This is in line with the view of estrogens as protective factors against PCa (Rahman et al. 2016).

There are other reports of the protective role of estrogens in prostate. Glantz (Glantz 1964) studied patients with hepatic cirrhosis, a state characterized with hyperestrogenism, and showed that those patients had lower incidence of PCa. In rodents, in spite of low doses of E_2

enhance prostatic growth and enlargement, high doses can be growth inhibitors and impair function of the adult prostate (Naslund and Coffey 1986; Saal et al. 1997). Also in rats, neonatal estrogen administration reduced prostate weight and development and decreased androgen responsiveness in adulthood, which may have implications in benign and neoplastic growth aberrations (Prins 1992). *In vitro* assays using the PC3 cell line showed an inhibitory effect on growth after E_2-treatment (Carruba et al. 1994) and treatment with high doses induced apoptosis and diminished the aggressive metastatic properties of these cells (Kanagaraj et al. 2007). Marília *et al*. (Figueira et al. 2016) studied the relation between E_2 with the expression of SCF/c-kit system, and showed that high doses of E_2 have antiproliferative and proapoptotic effects in human PCa cell lines and in rat prostate.

Independently of the observed response of prostate cells, all E_2 effects are mediated by ERs (Carruba 2007). E_2 can diffuses across the cell membrane into target tissues (Palmisano et al. 2017) and its physiological effects can be seen through activation of the steroid nuclear hormone receptors: ER alpha (ERα) and ER beta (ERβ); alternatively E_2 can activate a membrane-bound G protein-coupled estrogen receptor (GPR30 or GPER) (Boese et al. 2017).

ERα e ERβ are transcribed from *ESR1* and *ESR2* genes respectively (Dey et al. 2013), and are located in cytosol or in plasma membrane of the cell of reproductive and non reproductive (liver, muscle and kidney) organs (Cooke et al. 2017). The affinity of receptors is specific to different ligands, with E_2 having higher affinity for ERα than ERβ (Królik and Milnerowicz 2012). The receptor is bound to heat-shock proteins (Hsp) or other proteins in order to prevent DNA binding and to prevent the degradation of the receptor (Joshua et al. 2008). When estrogens bind the receptor, a conformational change occurs promoting dissociation of Hsp90, phosphorylation of the receptor, dimerization and then the translocation into the nucleus (Feldman and Feldman 2001; Królik and Milnerowicz 2012). ERs interact directly with the genome, binding to sequences recognized by the DNA-binding domain, promoting long term genomic effects (Palmisano et al. 2017).

The GPER is associated with the cell membrane and endoplasmic reticulum and binds estrogens with less affinity (Cooke et al. 2017). Its mechanism of action is characterized by i) not involving transcription and protein synthesis; ii) activation by steroids coupled to high-molecular-weight molecular incapable of crossing the plasma membrane; iii) promotion of rapid cell responses; iv) activation of ion channels; v) increase of cyclic AMP and augmented intracellular Ca^{2+} levels; vi) interaction with other membrane receptors, like IGF and EGF receptor, nuclear steroid receptors, like ERs, and glucocorticoid, mineralocorticoid and vitamin D receptors; and vii) activation of effector molecules (PI3K, Akt, MAPK and protein kinases A and C). All these aspects of GPER activity were recently revised in a book chapter by Figueira *et al*. (Figueira, Cardoso, and Socorro 2018). The GPER has been identified in normal human prostate, BPH and neoplastic prostate tissue, however its role in PCa is yet to be clarified (Figueira et al. 2018).

The ability of estrogens to regulate proliferation and cell death in the human prostate is mostly reported to be due to ERα and ERß dependent mechanisms, which act as pro- or anti-tumorigenic agents, respectively (Takizawa et al. 2014). ERα is responsible for the development of the prostate and for its growth and proliferation (Tsurusaki et al. 2003), which is directly regulated by growth factors, the mediators of prostate stromal-epithelial interactions (Wong and Wang 2000). On the other hand, ERß is associated with anti-proliferation, anti-invasion and apoptotic activity (Cheng et al. 2004) and inhibits epithelial to mesenchymal transition (Dey et al. 2013). Interestingly, phytoestrogen and synthetic antiestrogens, which are associated to prevention of development of PCa, bind with higher affinity to ERß, proving its functions explained before (Carruba 2007).

In normal prostate, ERα expression is restricted to the stromal compartment and to the androgen-independent basal cell layer, which harbours prostate stem cells capable of proliferation. ERß is predominantly expressed in luminal cells and in basal epithelial cells with limited proliferation capacity, but it has lower or none expression in stromal cells (Bonkhoff 2017; Bonkhoff and Berges 2009; Rahman et al. 2016). Concerning histological prostate zones and according to Tsurusaki *et al*. (Tsurusaki et al. 2003), ERα expression occurs restrictedly in stroma of PZ and ERß expression in both, but higher in the stroma of PZ than TZ. Since BPH develops preferentially in TZ and PCa in PZ, this expression pattern also is supportive that ERß may be involved in the pathogenesis of BPH and ERα associated with the origin of PCa (Tsurusaki et al. 2003).

The balance created between both receptors and ECM can be disrupted by alterations between a healthy state and during PCa development, as it is possible to see in figure 5 (Bonkhoff and Berges 2009).

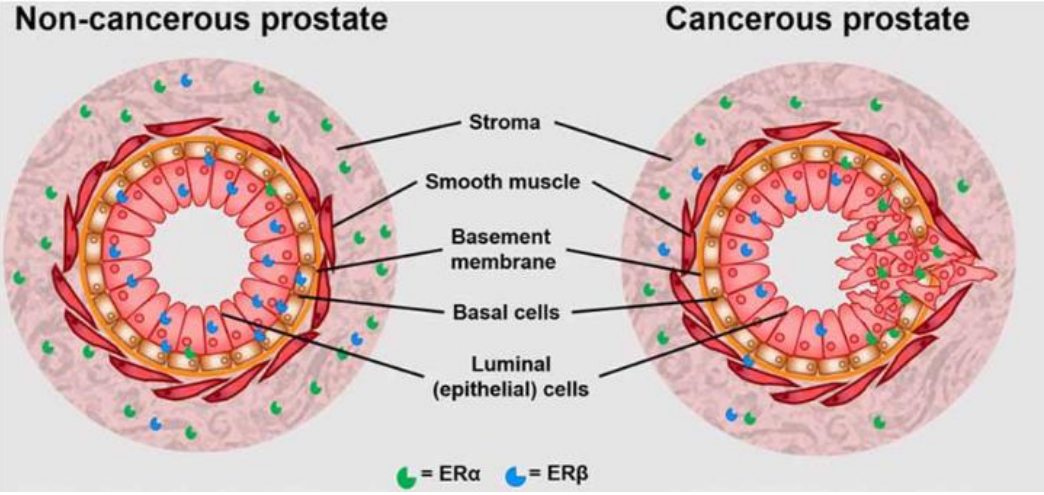

Figure 5. Expression of ERα and ERß in normal and cancerous prostate. During the development of the PCa, ERß (in blue) is downregulated and ERα (in green) is upregulated on the cells as well as in the surrounding environment. (in (Rahman et al. 2016))

During the progression of malignant prostatic epithelium occurs a swift in ER expression pattern (Fig. 5). The ERα expression is extended from basal cells to epithelial/luminal cells (Rahman et al. 2016), giving it oncogenic properties due to its overexpression (Bonkhoff 2017). ERα potentiates the carcinogenic effects on the prostatic epithelium promoting changes in dysplastic phenotype (Bonkhoff and Berges 2009). Contrary, ERβ expression implicates antitumoral activity through the activation of apoptotic pathways (Bonkhoff 2017). This ER is downregulated in epithelial cells during malignant progression, losing its tumour suppressor activity (Rahman et al. 2016). Thereby, the loss of ERβ creates an estrogen-sensitive condition where growth of cancer cells is stimulated by estrogen that acts through ERα (Carruba 2007).

Thereby, any alteration in estrogen homeostasis will increase growth, inhibit anti-apoptotic processes and promote metaplastic and neoplastic changes in estrogen-sensitive tissues (Williams 2012), where estrogens act as epigenetic carcinogens (Cavalieri and Rogan 2010). Hence, it is necessary to have regulatory mechanism of modulation of levels of estrogen. As it is the case of intermolecular steroid transformation between estrone and estradiol catalysed by 17β-hydroxysteroid dehydrogenase (Zhu and Conney 1998) where an excess of estrogen can be stored as estrone (Cavalieri and Rogan 2016).

5.2 The Relationship of Obesity with Prostate Cancer: a Link with Estrogens?

Overweight and obesity are associated with the accumulation of excessive subcutaneous and visceral body fat and are defined by a body mass index (BMI) between 25-29,9 kg/m^2 and greater than 30 kg/m^2, respectively (Ferro et al. 2017; Williams 2012). Obesity is a worldwide epidemic and by the year of 2030 more than a half of the world's adult population are expected to be overweight or obese.

A variety of obesity-related disorders have been widely recognized as a consequence of weight excess like hypertension, coronary artery disease, diabetes and subfertility (Ferro et al. 2017). The worst of all is the reported increased risk for several types of cancer in adults with increased BMI (Renehan et al. 2008).

Adipose tissue is responsible for energy storage in the form of lipids in adipocytes, which are released when a physiologic energy demand is imposed. Adipocytes work as an endocrine cells releasing growth factors, proinflammatory chemokines, free FA and hormones (Facchiano et al. 2013; Laurent et al. 2016). When the normal balance of adipose tissue is perturbed, changes like i) infiltration of macrophages, ii) localised hypoxia, iii) cellular death, and iv) extracellular matrix remodelling occur, causing an aberrant adipose tissue expansion, also known as obesity (Laurent et al. 2016; Taylor et al. 2015). Moreover, increased secretion of steroid hormones, chronic high insulin levels, insulin resistance, alterations on IGF-1 axis, altered lipid metabolism and secretion of adipokines, and persistence inflammation might be the mechanisms that associates cancer and obesity (Ferro et al. 2017; Taylor et al. 2015).

In the case of PCa, obesity has not been considered a risk factor, but obese patients have poor prognosis and reduced survival rates (Allott, Masko, and Freedland 2013). The prostate gland is surrounded by the periprostatic adipose tissue that might influence the progression of PCa. Laurent *et al.* (Laurent et al. 2016), showed that mature adipocytes secrete the chemokine CCL7, that was capable to diffuses through prostate capsule and interact to PCa cells, promoting their migration outside of the prostate gland. This relationship was exacerbated in case of obesity since it increases the odds for tumour invasion of the prostate surroundings (Laurent et al. 2016).

A typical unbalance of adipose tissue is related with a state of hyperestrogenism, which is thought to exacerbates the development of metabolic dysregulation in obesity (Rubinow 2017; Williams 2012). Hyperestrogenism happens due to the great peripheral conversion of T to E_2 in adipocytes, turning the adipose tissue particular rich in estrogens (Rahman et al. 2016; Rubinow 2017). Those elevated levels of E_2 create a hormonal imbalance and might influence PCa development and progression (Buschemeyer and Freedland 2007; Carruba 2007), which may explains the aggressiveness of disease in obese men (Buschemeyer and Freedland 2007). Another major endocrine change in obesity is the reduction of T levels by the E_2 negative feedback on the hypothalamus (Taylor et al. 2015), which is accompanied by lower PSA levels, turning PCa less likely to be diagnosed (Rhee, Vela, and Chung 2016). This might be another mechanism underling the association of obesity and PCa. Furthermore, adipose tissue exhibits high expression of ERα and ERß and fatty acyl esters (responsible for metabolism of estrogens) which regulate body weight, so it is reasonable to consider it a specific tissue for estrogen regulation (Rubinow 2017). However, ERß did not showed a clear effect on bodyweight with E_2 effects on adipose tissue being regulated predominantly through ERα (Chen et al. 2009; Rubinow 2017).

Regarding E_2 serum levels, obese men display a range concentration between 0,028 and 0,351 nM, which in general are close to the levels found in normal men (Table 4). However, there are studies showing increased E_2 serum levels in obese men (Schneider et al. 1979; Stanik et al. 1981).

Table 4: Concentration range of E_2 in obese men.

E_2 serum levels (nM)	Reference
0,028 – 0,156	(Schnoeller et al. 2015)
0,070	(Hagiuda et al. 2014)
0,081 – 0,351	(Ornstrup et al. 2015)
0,147	(Samavat et al. 2014)
0,149	(Facchiano et al. 2013)
0,106 – 0,128	(Migliaccio et al. 2013)
0,028 – 0,156	(Pellitero et al. 2012)
0,104	(Cao et al. 2012)
0,199	(Vermeulen et al. 1993)
0,136	(Zumoff et al. 1981)
0,123	(Loves, Ruinemans-koerts, and Boer 2008)
0,149	(Salonia et al. 2013)
0,298	(Schneider et al. 1979)
0,132	(Stanik et al. 1981)

Obesity implies a disruption of energy balance whereby the intake of energy by calorie uptake exceeds the expenditure. At the moment, it is known that this disruption can also be caused by combined forces of genetic and environmental origin, such as the endocrine disrupting chemicals with xenobiotic, or obesogenic activity which can mimic androgens and estrogen actions altering gene transcription and, consequently, inducing mitogenic processes and inhibiting anti-apoptotic ones (Williams 2012).

II. AIM

It is widely established that the prostate gland is a hormone target organ, highly dependent on the androgen actions, which rely on them to maintaining normal prostate development and function. Besides that, the role of androgens driving the changes that promote PCa development and progression is well known and, includes their recently reported actions as modulators of cell metabolism.

Considering estrogens, and in particularly E_2, it is recognized that they are synthesized in the prostate by the activity of aromatase over androgens, and their dual role as causative or protective agents in prostate carcinogenesis has been debated. However, little is known considering the mechanisms of E_2 effects towards prostate malignancy.

Despite the classical endocrine function of E_2 regulating mammary physiology, it was shown that this hormone could regulate the energy metabolism of breast cancer cells, which raises the curiosity about its action in prostate cells.

The present dissertation aims to:

1. Analyse the effects of E_2 in regulating the metabolism of neoplastic and non-neoplastic prostate cells;
2. Identify the metabolic pathways associated with the progression of PCa;
3. Infer the possible relationship between the metabolic alterations driven by E_2 in obese men and associated with PCa.

III. MATERIAL AND METHODS

1. Cell Lines

In the present study three human prostate cell lines models were used: non-neoplastic prostate cell line, PNT1A, and two neoplastic prostate cell lines, LNCaP and PC3. All were purchased from the European Collection of Cell Culture (ECACC, Salisbury, UK). PNT1A is a human post-pubertal prostate epithelial cell line. The LNCaP cell line was originated from lymph node metastasis of PCa, expresses the AR and is androgen-sensitive (Horoszewicz et al. 1983). PC3 cells had origin in bone metastasis of an undifferentiated grade IV adenocarcinoma of the prostate and are considered non-sensitive to androgens (Kaighn et al. 1979). Both LNCaP and PC3 are widely used as *in vitro* models of disease, representing an early androgen-sensitive stage of PCa, and a late and castration-resistant stage, respectively.

2. Cell Culture and 17β-Estradiol Treatment

Prostate cell lines were cultured and maintained in RPMI 1640 phenol red culture medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich) and 1 % penicillin/streptomycin (Sigma-Aldrich), in an air incubator at 37° C equilibrated with 5 % CO_2. At 60 % confluence, the culture medium was replaced by phenol red-free RPMI 1640 medium containing 5 % charcoal-stripped FBS (CS-FBS) (Sigma-Aldrich). This medium is steroid hormones-free and cells were maintained in this condition for additional 24 h. Thereafter, cells were cultured in the presence (or absence) of 0.1 nM, 1 nM and 100 nM of E_2 (E258, Sigma-Aldrich) for 24, 48 and 72 h through replacement culture medium by CB-FBS alone or containing E_2. This range of experimental concentrations for E_2 has already been tested in previous studies (Blitek et al. 2010; Fatima et al. 2017; Immonen et al. 2009) and fit (or are above) the serum E_2 levels reported in the literature (Table 3).

The E_2 stock solution was prepared by dissolving E_2 in ethanol, resulting in a 10^{-5} M E_2 solution. All other E_2 working solutions were prepared from the stock by serial dilutions. After treatment, cells were trypsinized, harvested, and stored at -80º C for RNA and protein extraction. Culture medium of E_2-treated and untreated cells was collected for measurement of extracellular metabolites.

3. Total Protein Extraction

Total proteins were isolated from human prostate cells using the radioimmunoprecipitation assay buffer (RIPA buffer) (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 nM Tris, 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail (Applichem, Darmstadt, Germany) and 10 % phenylmethtylsulfonyl fluoride (PMSF) (Fisher, Darmstadt, Germany). Samples were kept on ice for 20 min and occasionally mixed. Then, samples were centrifuged at 14000 rpm for 20 min at 4° C, and total proteins, present in

supernatant, were recovered to a new eppendorf tube. Total protein concentration was assessed using BCA Protein Assay Kit (Prod#23225, Lot#SA244529, Thermo Scientific). In a 96-well plate, 1 µL of protein sample was mixed with 80 µL of recently prepared working kit reagent and 19 µL of milli-Q water to reach a total volume of 100 µL. 1 µL of RIPA buffer was added instead of 1 µL of protein sample to be used as the blank. The absorbance was measured spectophotometrically (xMark™ Spectrophotometer, Bio-Rad, Hercules, CA, USA) at 562 nm. The calibration line for protein quantification was obtained in the same way using serial concentrations of bovine serum albumin (BSA).

4. Western Blot Analysis

Total protein (25 µg) of all cell lines were heat-denatured at 100° C for 5 min and resolved on 12,5 % (or 8 % in case of high molecular weight proteins) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed at 200 V for 55 min, approximately. Then, proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) at 750 mA for 1 h and 30 min. Membranes were blocked with 5 % skimmed dried milk for 1 h and then incubated overnight at 4° C with rabbit anti-GLUT1 (1:1000, CBL242, Millipore), anti-GLUT2 (1:1000, SC-9117. Santa Cruz Biotechnology), anti-GLUT3 (1:1000, H-50, SC-30107, Santa Cruz Biotechnology), anti-MCT4 (1:10000, A5441, Sigma-Aldrich), anti-LDH (1:10000, EP15664, Abcam), anti-PFK1 (1:1000, H-55, SC-67028, Santa Cruz Biotechnology), anti-CD36 (1:400, ab64014, Abcam), anti-FASN (1:1000, C20G5, #3180, Cell Signalling Technology), and anti-ACC (1:1000, #3662, Cell Signalling Technology) primary antibodies; a mouse anti-CPT1A (1:1000, [8F6AE9], ab128568, Abcam) antibody also was used. The anti-α-tubulin (1:1000, T9026, Sigma-Aldrich) antibody was used for protein loading control in all blots. After washing, the incubation of membranes with the goat anti-rabbit (1:40000, IgG-HRP, SC-2004, Santa Cruz Biotechnology) or goat anti-mouse (1:40000, IgG-HRP, SC-2005, Santa Cruz Biotechnology) secondary antibody proceeded for 1 h. At the end, membranes were washed, incubated with ECL substrate (Bio-Rad) for 5 min, and scanned with the Chemidoc™ MP Imaging System (Bio-Rad). Band densities were obtained by the volumetric analysis tool of Bio-Rad Image Lab 5.1 software and normalized with the respective α-tubulin band density.

5. Quantification of Extracellular Metabolites

The concentration of glucose and lactate in the culture medium of untreated and E_2-treated prostate cells was assessed by means of spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain).

For glucose quantification, 1 µL of cell culture medium was recovered at 0 h and 48 h after addition of E_2 and 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 min. Then the absorbance values

were measured at 505 nm (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad). Glucose assay consists in the oxidation of glucose present in the samples by the glucose oxidase present in the assay reagent. This reaction will form hydrogen peroxide, which will react with phenol and aminophenazone (also present in the prepared reagent) forming quinone. The quinone detected by the development of a red/violet colour was measured spectrophotometrically.

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 min. Absorbance values were read at 505 nm (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 results in the generation of pyruvate and peroxide that later will be transformed into quinone by the action of peroxidase. The development of red/violet colour due to the presence of quinone was measured spectrophotometrically.

The glucose consumption and lactate production in PNT1A, LNCaP and PC3 prostate cell lines in response to E_2 treatment were determined by comparison with the metabolite content in the culture medium samples at 0 h, and normalized for the total number of cells in each experimental group.

6. LDH Activity Assay

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

7. Statistical Analysis

Statistical significance of differences between experimental groups was assessed by unpaired T-test with Welch's correction or one-way ANOVA, followed by Tukey post-test, using GraphPad Prism v6.01 (GraphPad Software, San Diego, CA, USA). Significant differences were considered when p values <0.05. All experimental data are shown as mean ± SEM (n=3, x3 for each experimental condition).

IV. RESULTS

1. 17β-estradiol Treatment Increased Glucose Consumption and Lactate Production in Human Prostate Cells: Preliminary Analysis

Non-neoplastic (PNT1A) and neoplastic (LNCaP) human prostate cells were exposed to several concentrations of E_2 (0.1, 1 and 100 nM), and their behaviour consuming glucose and producing lactate (72 h) was evaluated by means of spectrophotometric assays that quantify glucose and lactate content in the culture medium.

Regarding glucose consumption, an increase was observed in E_2-treated PNT1A cells (Fig. 6A). Treatment with 0.1 nM or 1 nM for 24 h, 1 nM or 100 nM for 48 h and 100 nM for 72 h showed significant differences when compared to control (7.20 vs. 4.49 nmol/cell ($p<0.05$); 9.77 vs. 4.49 nmol/cell ($p<0.001$); 10.81 vs. 5.34 nmol/cell ($p<0.01$); 8.37 vs. 5.34 nmol/cell ($p<0.05$); 10.20 vs. 6.59 nmol/cell ($p<0.05$), respectively, Fig. 6A).

In the case of LNCaP cells (Fig. 6C), the results obtained for 24 h of treatment with 1 nM E_2 or for 48 h with 0.1 nM showed no significant differences when compared to control; though, augmented glucose consumption was observed in the groups treated with 0.1 nM or 100 nM for 24 h (19.57 vs. 13.19 nmol/cell; 19.97 vs. 13.19 nmol ($p<0.05$), respectively, Fig. 6C), 1 nM or 100 nM for 48 h (26.44 vs. 21.76 nmol/cell ($p<0.001$) and 24.59 vs. 21.76 nmol/cell ($p<0.05$), respectively, Fig. 6C), as well as in all the concentrations tested for 72 h (29.19 vs. 2.39 nmol/cell, 28.45 vs. 2.39 pmol/cell, and 22.00 vs. 2.39 nmol/cell ($p<0.001$), respectively, Fig. 6C).

Concerning lactate production, the E_2-treated non-neoplastic PNT1A cells showed no significant differences comparatively with the control group (Fig. 6B). On the other hand, an increase in lactate production was observed after exposure of the neoplastic LNCaP cells to E_2 (Fig. 6D). This effect was observed in the groups treated with 0.1 nM (131.84 vs. 69.14 nmol/cell ($p<0.05$), Fig. 6D), 1 nM (142.96 vs. 69.14 nmol/cell ($p<0.01$), Fig. 6D) and 100 nM (125.99 vs. 69.14 nmol/cell ($p<0.05$), Fig. 6D) E_2 for 24 h and 1 nM for 48 h (131.13 vs. 95.41 nmol/cell ($p<0.05$), Fig. 6D) relative to control.

Overall, E_2 increased glucose consumption and lactate production in both cell lines in a concentration and time-dependent manner (Fig. 6).

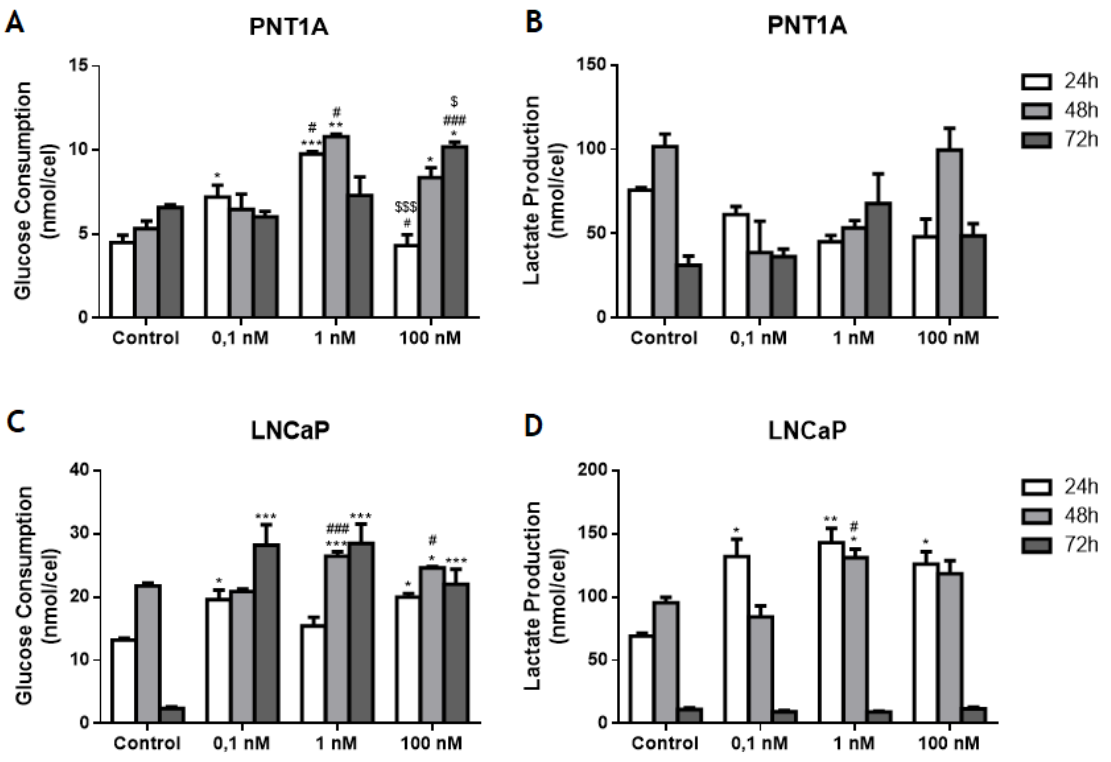

Figure 6. Glucose consumption and lactate production in non-neoplastic PNT1A epithelial cells (A, B) and neoplastic LNCaP human prostate cells (C, D) after treatment with several concentrations of E_2 (0.1, 1, and 100 nM) for 24, 48, and 72 h. Glucose and lactate content was determined by spectrophotometric assays. Errors bars indicate mean ± S.E.M. (n=6). * p<0.05, ** p<0.01, *** p<0.001 comparatively with the control group. # p<0.05, ### p<0.001 when compared with 0.1 nM-treated group. $ p<0.05, $$$ p<0.001 when compared with 1 nM-treated group.

After analysis of the results obtained, the 1 nM E_2 concentration and the incubation time for 48 h were selected for the subsequent experiments considering studying the influence of this hormone in the glycolytic and lipid metabolism of human prostate cells.

2. 17ß-estradiol Exposure Enhanced the Glycolytic Metabolism in Both Neoplastic and Non-neoplastic Human Prostate Cells

The non-neoplastic PNT1A cell line and two neoplastic cell line models (LNCaP and PC3) were used to further analyse the effect of E_2 modulating the glycolytic metabolism of human prostate cells. An increase in glucose consumption was observed in the E_2-treated groups when compared with the control non-treated cells PNT1A, LNCaP and PC3 (2.09 vs. 1.83 nmol/cell (p<0.05), 2.69 vs. 1.75 nmol/cell, (p<0.05), and 4.51 vs. 2.96 nmol/cell, (p<0.05), respectively) (Fig. 7A).

Lactate production in response to 1 nM E_2 for 48 h followed the pattern of glucose consumption displaying an augment in all cell lines (Fig. 7B). PNT1A cells showed an increased lactate production after E_2-treatment (237.29 vs. 154.28 nmol/cell in the control group ($p<0.01$)), and in LNCaP and PC3 cells, lactate production was also augmented relative to control group (73.36 vs. 41.58 nmol/cell ($p<0.001$), and 396.70 vs. 180.88 nmol/cell ($p<0.05$), respectively).

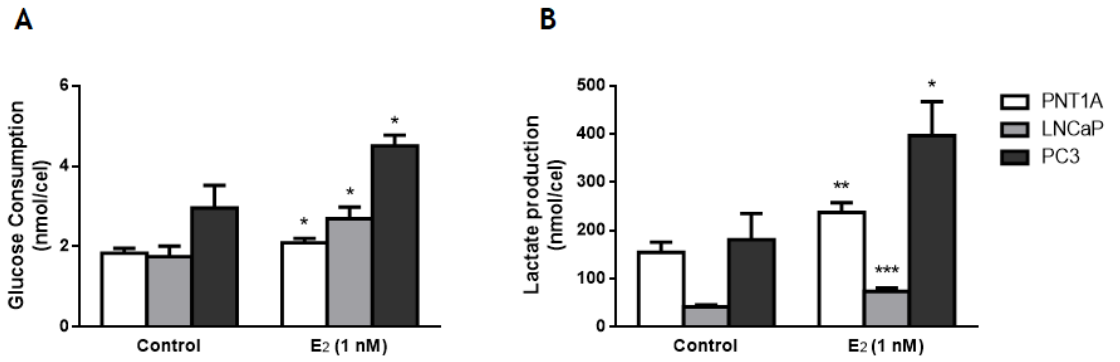

Figure 7. Glucose consumption (A) and lactate production (B) in non-neoplastic PNT1A epithelial cells and neoplastic LNCaP and PC3 human prostate cells after treatment with 1 nM of E_2 for 48 h, obtained by spectrophotometric assays. Errors bars indicate mean ± S.E.M. (n=3 for 3 independent experiments). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ comparatively with the respective control groups.

The results obtained on glucose consumption and lactate production were complemented by analysing the protein expression or activity of GLUTs and other key targets of glycolytic metabolism, namely, PFK, LDH and MCT4 (Fig. 8).

Underpinning the augment observed in glucose consumption, an altered expression of GLUT1, GLUT2 and GLUT3 was observed in all cell lines after stimulation with E_2 (Fig. 8). The expression of GLUT1 was increased in PC3 cells relatively to control (1.45-fold variation, $p<0.05$) (Fig. 8A). On the other hand, the expression of GLUT1 in E_2-treated PNT1A and LNCaP cells was not statistically significant altered ($p=0.5041$, and $p=0.5960$, respectively, Fig. 8A). Concerning GLUT2, the augmented expression in response to E_2 was seen in all cell lines; 1.94-fold variation ($p<0.05$) in PNT1A, 2.09-fold variation ($p<0.05$) in LNCaP, and 1.65-fold variation ($p<0.05$) in PC3 cells, comparatively with the control untreated group (Fig. 8B). The same response was observed for GLUT3. E_2 increased GLUT3 expression in both non-neoplastic PNT1A cells (1.78-fold variation, $p<0.01$) and neoplastic LNCaP and PC3 cells (1.97-fold and 2.07-fold variation, respectively, $p<0.05$) when compared to non-treated group (Fig. 8C).

The expression of PFK1 was analysed in order to evaluate the metabolization of the internalized glucose. After E_2 treatment, an augmented expression of PFK1 was only observed in the neoplastic LNCaP cells (1.45-fold variation comparatively with control, $p<0.05$). In the case of PNT1A and PC3-treated cells, no significant alterations were found ($p=0.5802$, and $p=0.9183$, respectively) (Fig. 8D).

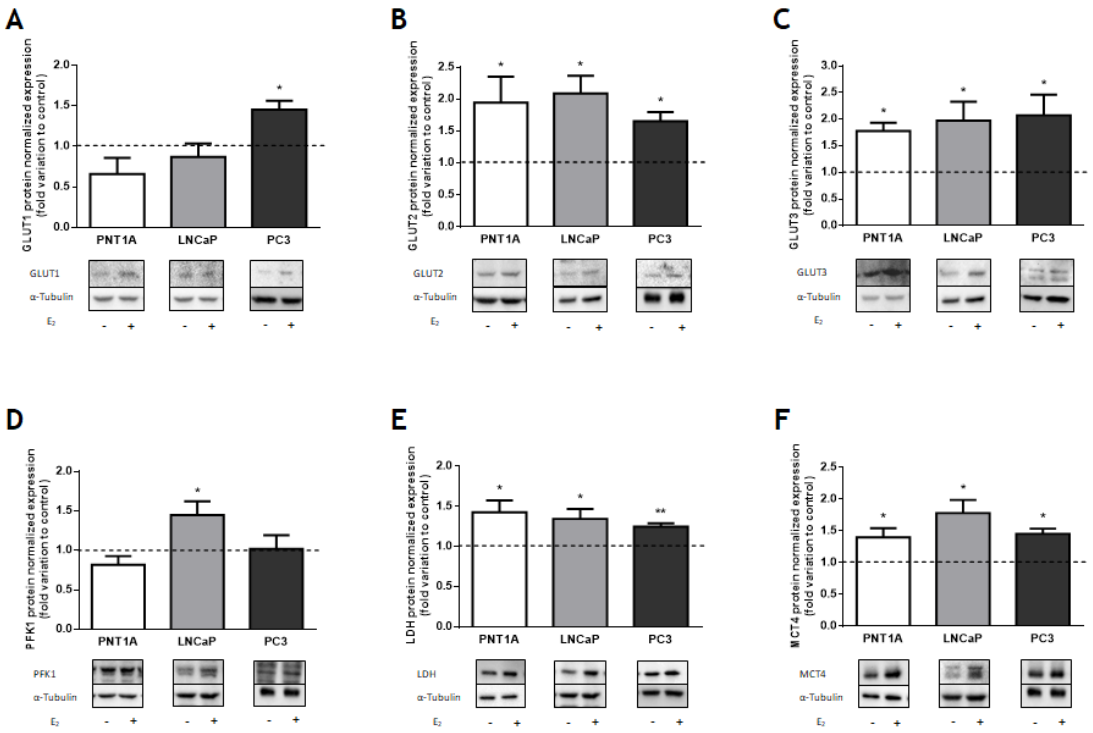

Figure 8. Expression of metabolism-associated proteins, glucose transporters GLUT1 (A), GLUT2 (B), GLUT3 (C), glycolytic-associated enzymes, PFK (D) and LDH (E), and lactate exporter MCT4 (F) in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after stimulation with 1 nM of E_2 for 48 h, obtained by Western blot analysis after normalization with α-tubulin. Results are expressed as fold-variation comparatively to control (dashed line). Errors bars indicate mean ± S.E.M. (n=3 for 3 independent experiments). * p<0.05, ** p<0.01. Representative blots are shown below the respective graph.

The final product of glycolysis, pyruvate, can be converted to lactate by the activity of LDH. The expression of this enzyme was significantly increased in PNT1A (approximately 1.43-fold variation, p<0.05), LNCaP (approximately 1.35-fold variation, p<0.05) and PC3 cells (approximately 1.25-fold variation, p<0.01) comparatively with the control untreated group (Fig. 8E). The activity of LDH in response to E_2 treatment accompanied the expression results since it was also increased in PNT1A (1.78-fold variation, p<0.05), LNCaP (3.10-fold variation, p<0.05) and PC3-treated cells (2.14-fold variation, p<0.05) relatively to control group (Fig. 9).

Lactate can be exported to the extracellular space by the MCT4. Its expression in non-neoplastic and neoplastic human prostate cell lines was also increased after E_2 stimulation. An augmented expression was found in PNT1A, LNCaP and PC3 (1.40-fold variation, p<0.05; 1.79-fold variation, p<0.05; and 1.46-fold-variation, p<0.05, respectively), relatively to control (Fig. 8F).

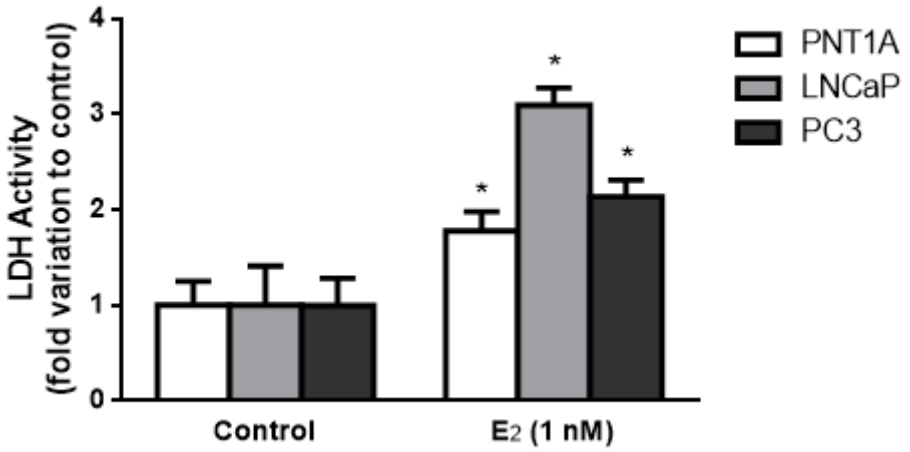

Figure 9. LDH enzymatic activity in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after treatment with 1 nM of E_2 for 48 h, determined by spectrophotometric assay. Results are expressed as fold-variation comparatively to control. Errors bars indicate mean ± S.E.M (n=3 for 3 independent experiments). * $p<0.05$.

3. 17β-estradiol Treatment Altered the Handling of Fatty Acids in Human Prostate Cells

Pyruvate, the product of glycolysis can be converted to Ac-CoA, a central player in lipid metabolism that can be metabolized to malonyl-CoA by the ACC enzyme (Fig. 3). The expression of ACC was increased in both non-neoplastic PNT1A cells (1.44-fold variation, $p<0.05$) and neoplastic PC3 cells (1.41-fold variation, $p<0.05$) treated with E_2 compared with non-treated cells (Fig. 10A). However, no significant alterations were found in LNCaP-treated cells compared with the control group ($p=0.2815$) (Fig. 10A).

Another crucial enzyme involved in lipid metabolism in cancer cells is the FASN enzyme (Fig. 3). The expression of FASN was augmented in the three human prostate cell lines under study in response to E_2 (Fig. 10B). That response was found in PNT1A, LNCaP and PC3 cells (1.53-fold variation, $p<0.05$; 1.74-fold variation, $p<0.01$; 1.34-fold variation, $p<0.05$, respectively), relatively to control (Fig. 10B).

B-oxidation of FA relies on the rate limiting step of FA transport into the mitochondria, a process depending on the function of CPT1A (Fig. 3). E_2 treatment increased CPT1A expression in all human prostate cell lines (Fig. 10C). PNT1A, LNCaP and PC3 cells displayed CPT1A expression increased by 1.98- ($p<0.01$), 1.54- ($p<0.05$), and 1.24- ($p<0.05$) –fold comparatively with the respective control groups (Fig. 10C).

Lastly, the extracellular FA uptake into the intracellular space is possible through the FA uptake channel CD36. The expression of CD36 was increased in both non-neoplastic PNT1A cells (2.53-fold variation, $p<0.01$) and neoplastic LNCaP cells (1.69-fold variation, $p<0.05$) treated with E_2. However, the expression of CD36 in E_2-treated PC3 cell was decreased compared with the respective control group (0.50-fold variation, $p<0.05$) (Fig. 10D).

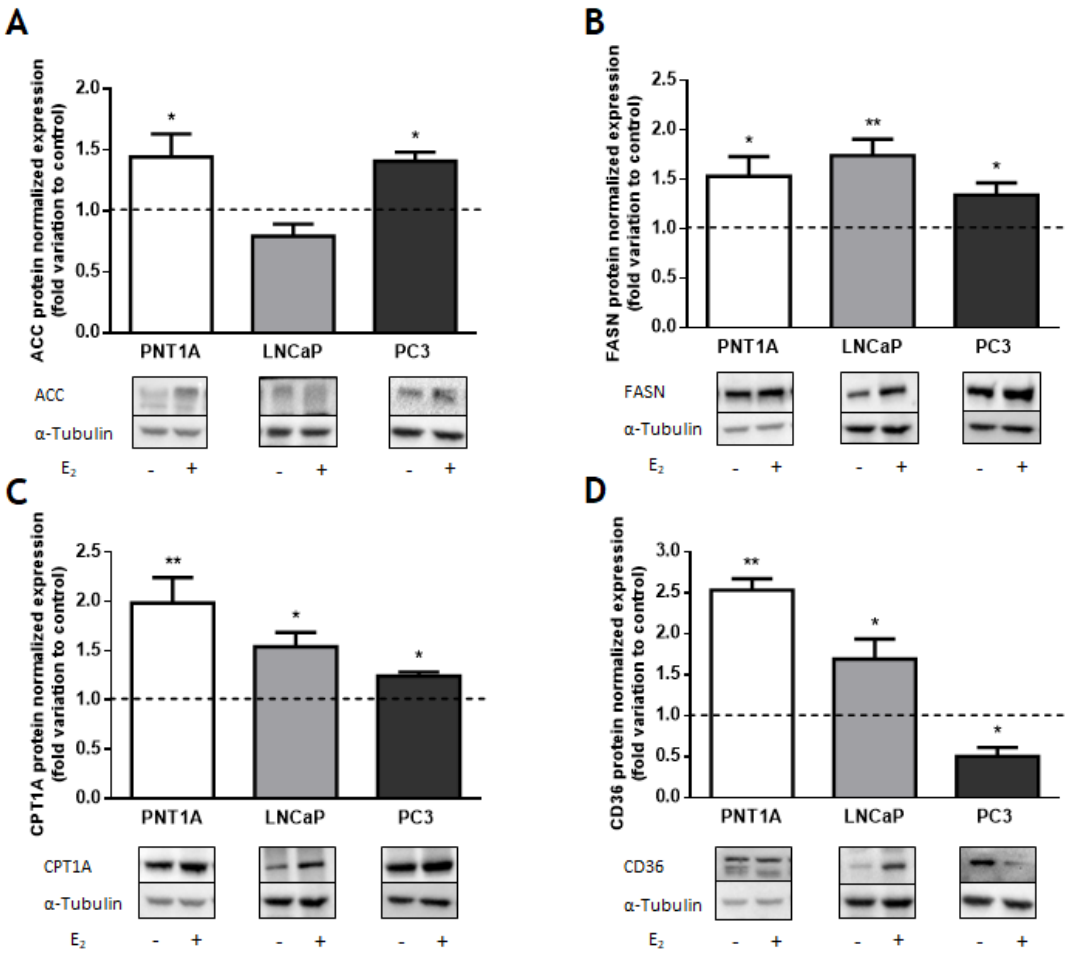

Figure 10. Expression of lipid metabolism-associated enzymes, ACC (A) and FASN (B), FA transporter CPT1A (C) and FA uptake channel CD36 (D) in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 human prostate cells after stimulation with 1 nM of E_2 for 48 h, obtained by Western blot analysis after normalization with α-tubulin. Results are expressed as fold-variation comparatively to control (dashed line). Errors bars indicate mean ± S.E.M (n=3 for 3 independent experiments). * $p<0.05$, ** $p<0.01$. Representative blots are shown below the respective graph.

V. DISCUSSION AND CONCLUSION

The present dissertation investigated the role of E_2 in modulating the glycolytic metabolism and lipid handling in non-neoplastic, PNT1A, and neoplastic, LNCaP and PC3 human prostate cell lines, as a strategy to evaluate the action of this sex hormone stimulating the development and progression of PCa.

Firstly, glucose consumption and lactate production by prostate cells, in response to different concentration of E_2 and for different time points, were analysed. E_2-treatment increased glucose consumption and lactate production both in neoplastic (LNCaP) and non-neoplastic (PNT1A) prostate cells (Fig. 6), though effects were more notorious in the LNCaP cell line. This preliminary assay allowed to select the 1 nM of E_2 concentration and the exposure time of 48 h for analysis of metabolism in different cell line models of PCa (LNCaP – androgen sensitive and PC3 – androgen-insensitive cells) comparatively with the non-neoplastic PNT1A cells.

Concerning glycolytic metabolism, 1 nM E_2 for 48 h (Fig. 7A) significantly increased glucose consumption both in non-neoplastic PNT1A cells and neoplastic LNCaP and PC3 cells. The more notorious augments on glucose consumption were observed in neoplastic cell lines, and followed the aggressiveness of PCa (PNT1A < LNCaP < PC3, Fig. 7A). Therefore, it was established that E_2 stimulates the glycolytic metabolism of prostate cells maintaining the phenotype previously described, which followed cell lines aggressiveness (Vaz et al. 2012). Neoplastic prostate cell lines were more glycolytic, with the PC3 cell line being the most glycolytic compared with androgen-sensitive cell lines.

In all cell lines, the augmented glucose consumption in response to E_2 was accompanied by the increased expression of GLUTs (Fig. 8A, B and C), though with slightly different responses considering the GLUTs isoforms. Non-neoplastic PNT1A and neoplastic LNCaP and PC3 cell lines showed an augmented expression of both GLUT2 and GLUT3, whereas GLUT1 was only increased in the PC3 cell line. Nevertheless, these findings showed that GLUTs' expression is regulated by estrogens, which at least for our knowledge is the first report in human prostate cells. However, the effect of sex steroid hormones, namely androgens, regulating the expression of GLUT1 and GLUT3 in LNCaP cells was previous described (Massie et al. 2011; Vaz et al. 2012). Altogether, these findings demonstrate that GLUTs levels in prostate is under hormonal control. GLUT1 and GLUT3 have been shown to be overexpressed in PCa and related with the aggressiveness of the disease, being more expressed in androgen-independent cells (Gonzalez-Menendez et al. 2018; Macheda, Rogers, and Best 2005), which is the case o PC3 cells. The results obtained herein followed the report expression pattern for GLUT1 and GLUT3 (Fig. 8A and 8C). GLUT2 is another GLUT family member that is more associated with high glycolytic cells and glucose uptake in the liver, intestine and kidney cells (Macheda et al. 2005; Thorens et al. 1988). However, GLUT2 increased expression has been reported in cancer cells (Giatromanolaki et al. 2017; Hamann et al. 2018; Lin et al. 2016). This suggests that besides GLUT1 and/or GLUT3, also GLUT2 might have contributed to the enhanced glucose uptake observed in prostate cell lines. This is a novel result since no previous studies has assessed the expression of GLUT2 in prostate cells. Therefore, the higher expression of GLUT1, GLUT2 and

GLUT3 in prostate cell lines might imply that E_2 supports PCa cell growth by accelerating glucose intake and utilization.

Once inside the cells, glucose enters the process of glycolysis, which culminates with the production of pyruvate. PFK1 is an important enzyme that catalyses a rate limiting step of glycolysis, the conversion of F-6-P into F-1,6-P. LNCaP-treated cells (Fig. 8D) displayed increased expression of PFK1, which is in concordance with the augmented glucose consumption and lactate production (Fig. 7). Indeed, the augmented expression of PFK1 indicates a higher activity of the glycolytic pathway in order to produce more pyruvate, which in turn would be converted to lactate. In PNT1A and PC3 cells, despite the augmented glucose consumption and lactate production, no significant alterations on the PFK1 expression were perceived (Fig. 8D). This might suggest that other sources of pyruvate from other metabolic routes can be fuelling the production of lactate. Pyruvate can also be generated from the reversible reaction catalysed by alanine transaminase (ALT), which converts the amino acid alanine to pyruvate in the cytosol (Gray et al. 2014). Besides that, pyruvate can also be produced via the TCA cycle by the glutaminolysis pathway (Lunt and Vander Heiden 2011). Expression analysis of ALT or GLS (Fig. 3), as well as measurement of the enzymatic activity will be necessary to ascertain this hypothesis.

The end-product of glycolysis, pyruvate, can be transported to the mitochondria and enter the TCA cycle or, alternatively, can be converted to lactate by the activity of LDH (Fig. 3). Lactate generation is highly important for cancer cells because it regenerates NAD+, necessary for continued flux through glycolysis. This potentiates faster growth over carbon utilization to produce ATP and enhances the ability of cancer cells to survive, growth and invasion through the establishment of an acidic microenvironment (Lunt and Vander Heiden 2011). Lactate production was increased in response to administration of E_2, an effect observed in all cell lines (Fig. 7B). These findings are in agreement with the augmented glucose consumption, and are supported by the increased expression (Fig. 8E) and activity (Fig. 9) of LDH detected in E_2-treated.

The produced lactate is exported to the extracellular space by the activity of MCTs. MCT4 has been associated with highly glycolytic cells and has been shown to be upregulated in PCa cells (as well as in other cancers (Pinheiro, Longatto-Filho, Ferreira, et al. 2008; Pinheiro, Longatto-Filho, Scapulatempo, et al. 2008)). Moreover, MCT4 expression has been correlated with the more aggressive stages of the disease and poor prognosis (Choi et al. 2016; Pértega-Gomes et al. 2011; Vaz et al. 2012). MCT4 expression levels were increased after E_2-treatment in all cell lines, mainly in the neoplastic ones (Fig. 8F). Therefore, the higher expression of MCT4 in E_2-treated cells might imply a higher lactate export, which also is supportive of the enhanced production of this metabolite in response to E_2.

Overall, E_2 stimulated the glycolytic pathway in non-neoplastic, PNT1A, and neoplastic, LNCaP and PC3 cells, but with more pronounced effects in malignant cells, which typically displayed increased activity of glycolytic enzymes and transporters.

Glycolysis and glutamine metabolism, are directly coupled to lipid metabolism. Cancer cells due to their high proliferative rate, require a substantial amount of metabolic energy needed for synthesis of cell membranes, which are mainly composed by FA (Zadra, Photopoulos, and Loda 2014). FA synthesis is known to affect growth, survival and invasion of cancer cells mainly by activating signalling proteins (Zadra et al. 2014). The lipogenic enzymes, such as ACC and FASN, are up-regulated in malignant phenotype and, unlike normal cells, synthesize large amounts of *de novo* FA (Benedettini et al. 2008; Zadra et al. 2014).

Lipid synthesis use Ac-CoA and NADPH generated from glucose and glutamine oxidation and converted Ac-CoA to malonyl-CoA by the rate-limiting step catalysed by ACC (Deep and Schlaepfer 2016). The expression levels of ACC after E_2-treatment (1 nM for 48 h) were significantly augmented in PNT1A and PC3 cells (Fig. 10A). These findings indicate that FA synthesis might be increased in response to E_2.

Continuing with lipid synthesis, malonyl-CoA can be converted into various forms of FA via FASN. FASN catalyses the terminal steps of *de novo* biosynthesis of long-chain FA and, under normal conditions, its levels are expected to be low (except in the liver, adipose tissue and lactating breast) because the majority of FA are derived from food intake instead of endogenous synthesis (Benedettini et al. 2008; Flavin et al. 2011; Zadra et al. 2014). FASN is found in earliest stages of tumour development, and its overexpression is associated with poor prognosis and reduced survival (Brusselmans et al. 2005; Swinnen et al. 2000). In addition, FASN expression is related with enhanced expression of key proto-oncogenes proteins (O'Malley et al. 2016). In the present study, E_2 stimulation increased the expression of FASN in PNT1A, LNCaP and PC3 cells (Fig. 10B). However, FASN had already been reported to be highly expressed not only in proliferating cells and those with high lipid metabolism, but also in hormone-sensitive cells, acting like an oncogene even in normal conditions (Kusakabe et al. 2000; Menendez and Lupu 2007). Indeed, steroid hormones, such as androgens (Galbraith, Leung, and Ahmad 2018; Swinnen et al. 1997) and E_2 (Santolla et al. 2012) was shown to regulate FASN expression. These findings are corroborated by the enhanced ACC expression and the co-activation of both enzymes predicts an overall augmented FA synthesis, and may explains the importance of upregulation of lipogenic enzymes in PCa progression.

In case of energy stress, FA can be consumed trough ß-oxidation in order to generate ATP and provide energy for cancer cell survival. CPT1A modulates ß-oxidation by controlling the entrance of long-chain FA into the mitochondria to be oxidized (Deep and Schlaepfer 2016). CPT1A expression is increased in high-grade PCa and it is needed to maintain viability and invasion of PCa cell lines, being responsible for anti-androgen resistance (Flaig et al. 2017). In response to administration of E_2, PNT1A, LNCaP and PC3 cell lines displayed increased expression of CPT1A (Fig. 10C). This seems to indicate that E_2-treatment augments the rate of FA oxidation on prostate cells.

As previously mentioned, cancer tissues can generate FA through *de novo* lipogenesis, but FA availability in the cell can also rely on the uptake of exogenous FA from the environment. This uptake is made by the FA scavenger receptor, CD36, which is associated with tumour

progression and metastatic process (Lengyel et al. 2018). In the case of PNT1A and LNCaP cells, CD36 expression was increased in response to E_2 stimulation (Fig. 10D), which may represent a mechanism to fuel FA oxidation since CPT1A expression also was increased in response to E_2 (Fig. 10C). The higher expression of CD36 may ensures the sufficient uptake of FA needed to satisfy cancer cell energy requirements (Enciu et al. 2018). On the other hand, an opposite effect was observed in PC3-stimulated cells, which displayed diminished expression of CD36 in response to E_2 (Fig. 10D), despite the likely augment in FA oxidation after CPT1A increased levels (Fig. 10C). This might be explained by an eventually lipid accumulation or by an metabolic dysfunction under excessive supply (Enciu et al. 2018). Previous studies showed that in cardiac muscle of high-fat rats, E_2 stimulation diminished CD36 expression because it caused a disruption in the translocation of CD36 to the plasma membrane resulting in impaired uptake of exogenous FA (Zafirovic et al. 2017).

The estrogenic effects reported in the different cell line models might depend on the activation of distinct ERs. Prostate cell lines have different expression pattern of ERs and their responses will depend on the ER subtype expressed. ERα is associated with pro-tumorigenic effects, whereas ERβ is associated with anti-proliferation, anti-invasion and apoptotic activity (Cheng et al. 2004; Tsurusaki et al. 2003). In low grade carcinomas there are an infrequent expression of ERα, due to gene hypermethylation (Lau et al. 2000), but with the progression of the disease to high-grade and metastatic cancer, ERα become more common and ERβ expression is progressively lost (Horvath et al. 2001; Lau et al. 2000). Therefore, PNT1A cells and LNCaP cells express ERβ, but not ERα, whereas PC3 cells express both ERs (Lau et al. 2000; Mak et al. 2013). However, since the PC3 cell line represents a more aggressive stage of the disease, it is possible that they express lower levels of ERβ than the other cell lines. The GPER has been shown to be expressed in all PCa cells lines used in the present study (Figueira et al. 2018). Nonetheless, GPER is another estrogen receptor which mediates E_2 biological responses, so it might also regulate metabolism in prostate cells (Boese et al. 2017; Figueira et al. 2018)

Herein, no experiments were included to determine which ERs is involved in the estrogenic response. However, it is possible to assume that up-regulation of glycolytic and lipid metabolism in response to E_2 in PC3 cells would be an effect mediated by ERα, whereas in PNT1A and LNCaP cells would be mediated by the only ER expressed, ERβ.

Estrogens are well known regulators of fat distribution and deposition (Rubinow 2017). On the other hand, in obesity, the accumulation of adipose tissue increases E_2 production, since adipocytes highly express aromatase, inducing a state of hyperestrogenism (Colleluori et al. 2018; Taylor et al. 2015). Adipocyte hypertrophy results in an increase lipogenesis or extracellular FA uptake and consequently, a progressive accumulation of lipid inside adipocytes (Rubinow 2017). Hence, hyperestrogenism associated with obesity exacerbates the progression of obesity and metabolic dysregulation (Rubinow 2017). As demonstrated in the literature, E_2-treatment exert regulatory control on several key steps of metabolism (Palmisano et al. 2017).

Consequently, E_2 abnormalities resultant of obesity might disrupt prostate cells' metabolism, as is supported by the results obtained in the present thesis. Moreover, since the up-regulation of glycolytic and lipid metabolism driven by E_2 was associated with PCa progression and development, it is likely that obesity is associated with the increased risk of PCa and its aggressiveness because of the deregulation in hormone balance.

In conclusion, the results obtained in the present dissertation showed that E_2-exposure, even with the low concentration of 1 nM, mimetizing men serum levels, disrupts the metabolism of both neoplastic and non-neoplastic prostate cells towards a glycolytic phenotypes. Moreover, E_2 seems to augment fatty acid uptake, biosynthesis and oxidation in prostate cells and it is possible that E_2 induce the activation of both lipogenesis and FA degradation in a coordinated way. These metabolic alterations may sustain the proliferative potential of growing cells ensuring the energy needs. A relationship between PCa, obesity and estrogens might be possible to establish since hyperestrogenism may potentiate the same metabolic alterations showed this *in vitro* approach and, therefore, increase the odds for tumour development and invasion.

The outcomes of this dissertation are in line with the studies that claim E_2 as the causative agent in prostate carcinogenesis and, as suggested here by driving the metabolic alterations associated with the progression of PCa.

Future work, disclosing the mechanisms underlying the estrogenic effects in prostate will be crucial to identify possible targets for PCa treatment in particular in PCa associated to obesity. It is pertinent to enlighten the relationship between obesity and PCa by evaluating the E_2 effects on key obesity metabolic points. In addition, it would be of paramount importance to define which ER is involved in E_2 response in prostate cells. Finally, transpose the experimental strategy of this dissertation to an *in vivo* model will be an interesting approach to confirm the impact of the E_2 effects for prostate tumour onset and progression.

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