Unravelling the effect of STEAP1 knockdown in prostate cancer cells: from protein expression profile to clinical applications

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A todas as pessoas que diariamente lutam contra o cancro,
e a todos os investigadores que batalham para o tentar travar.

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List of publications

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Resumo Alargado

O cancro da próstata (PCa, *"prostate cancer"*) é um dos cancros mais frequentes na população masculina e continua a ser uma considerável fonte de morbidade e mortalidade para os homens no mundo inteiro. Numa fase inicial da doença, o cancro está confinado à glândula prostática dependendo da ação estimuladora dos androgénios. Esta dependência é a característica que permite a utilização da terapêutica de privação androgénica (ADT, *"androgen-deprivation therapy"*), a qual tem por base a redução dos níveis circulantes de androgénios ou o bloqueio das suas ações. A continua administração desta terapia leva a que as células neoplásicas da próstata se tornem resistentes ao tratamento, sendo capazes de sobreviver, extravasar a glândula prostática e originar metástases. Esta fase da doença designa-se PCa resistente à castração (CRPC, *"castrate-resistant PCa"*) e implica o uso de agentes quimioterapêuticos. Pelas limitações que as terapêuticas existentes apresentam, o uso de terapias combinadas direcionadas aos reguladores de sobrevivência e às proteínas envolvidas na regulação do crescimento celular prostático tem surgido como uma opção promissora para o tratamento do PCa. O desenvolvimento e progressão do PCa resulta de diversas alterações genéticas e metabólicas, que conferem às células tumorais resistência ao tratamento, capacidade de proliferação exacerbada, e características invasivas. Curiosamente, muitas proteínas associadas ao cancro têm sido referidas como tendo um papel multifuncional no controlo das alterações biológicas que as células do PCa adquirem. Entre elas, está a STEAP1 (*"Six-Transmembrane Epithelial Antigen of the Prostate"*).

A STEAP1 é uma proteína transmembranar identificada como sobre-expressa no PCa e em vários outros tipos de cancros. Em tecidos não tumorais, a expressão da STEAP1 é baixa e apenas restrita à glândula prostática, o que sugere que o aumento da sua expressão poderá favorecer e acompanhar o desenvolvimento tumoral. Tem sido sugerida a função da STEAP1 como um oncogene, dada a sua capacidade de regular a proliferação celular, apoptose, invasão e metastização conduzindo à progressão do PCa. Para além disso, vários estudos têm demonstrado que a STEAP1 está envolvida na comunicação intercelular podendo funcionar como um canal iónico ou proteína transportadora. Assim, tendo em consideração os vários processos biológicos que podem estar associados ao papel da STEAP1, a sua localização e sobre-expressão à superfície da célula, e à sua reduzida expressão em tecido não tumorais, torna-se previsível que esta proteína exerça um papel crucial na manutenção da homeostase celular e possa ser usada como um possível alvo terapêutico para o PCa. No entanto, o significado clínico da expressão da STEAP1 no PCa e a sua utilidade como um biomarcador necessitam ainda de alguma clarificação. Para além

disso, existem poucos estudos sobre a regulação da expressão da STEAP1 no PCa e sobre os mecanismos celulares subjacentes às funções biológicas no PCa.

A presente tese teve como primeiro objetivo estabelecer o significado clínico da proteína STEAP1 no PCa e explorar a sua utilidade como um biomarcador. Também foi investigada a sua relação com as outras proteínas da família da STEAP (STEAP2, STEAP3 e STEAP4). Através de bases de dados e ferramentas bioinformáticas, verificou-se que o *STEAP1* é um gene diferencialmente expresso no PCa, desde lesões benignas até adenocarcinoma metastático, sugerindo que desregulação deste gene pode estar envolvida nas fases iniciais do desenvolvimento da doença. Relativamente às outras proteínas da família da STEAP, os nossos resultados indicam que o *STEAP2* e *STEAP4* são também sobre-expressos no PCa, e que o *STEAP3* é sub-expresso. No entanto, apenas a elevada expressão do *STEAP1* foi associada a um mau prognóstico em pacientes com PCa, comparativamente a pacientes sem alterações na expressão das proteínas da família da STEAP. Para além disso, comparando apenas pacientes com alterações na expressão destas proteínas, verificamos que a sobre-expressão do *STEAP1* continua a ser associada a pior prognóstico, enquanto que a sobre-expressão do *STEAP4* indica um bom prognóstico para pacientes com PCa estando associado a um maior tempo de sobrevida.

A desregulação da STEAP1 pode exercer alto impacto no desenvolvimento do PCa, e no trabalho desenvolvido nesta tese demostramos que o *STEAP1* pode ser regulado por mecanismos epigenéticos. Para além de se confirmar a alta expressão do *STEAP1* em linhas celulares neoplásicas da próstata (LNCaP) e a baixa expressão deste gene em linhas celular não neoplásicas da próstata (PNT1A), verificou-se que a ilha CpG localizada junto do local de início da transcrição está metilada em células PNT1A e desmetilada em células LNCaP. Este resultado sugeriu que a desmetilação do promotor do gene *STEAP1* poderá contribuir para a sua sobre-expressão no PCa. O recrutamento de enzimas como as DNA metiltransferases e histonas desacetilases são essenciais para que ocorram estas alterações epigenéticas. O tratamento das células PNT1A com inibidores destas enzimas aumentou a expressão do gene *STEAP1*, sugerindo mais uma vez, que a hipometilação do promotor do gene *STEAP1* poderá estar associado à sobre-expressão no PCa.

A caracterização global do proteoma de células LNCaP silenciadas para o *STEAP1* de forma a aprofundar as funções moleculares e celulares da proteína STEAP1 foi outro dos focos desta dissertação. Para isso, células LNCaP silenciadas, ou não silenciadas para o *STEAP1* foram sujeitas a técnicas de identificação e quantificação proteica. Comparando os dois grupos experimentais (células LNCaP silenciadas *versus* células LNCaP não silenciadas), foram encontradas 526 proteínas diferencialmente expressas. Os processos celulares de endocitose, transporte de RNA, apoptose, biossíntese de aminoácidos e vias metabólicas foram os mais significativos, sugerindo que a STEAP1 pode modelar estes

processos biológicos. De facto, o silenciamento do gene *STEAP1* promove a apoptose das células do PCa atenuando a proliferação e o metabolismo celular, estimula a endocitose e enfraquece a comunicação intercelular através de vesiculas. Estes resultados reforçam o papel oncogénico da STEAP1 no PCa.

Por último, verificou-se em células LNCaP, que a ação de fármacos anti-androgénicos, nomeadamente bicalutamida, enzalutamida e apalutamida parece ser independente dos níveis de expressão da STEAP1. Por outro lado, o tratamento de células LNCaP com fármacos quimiterapêuticos à base de taxanos, paclitaxel, docetaxel e cabazitaxel, parece depender dos níveis de STEAP1. A aumentada expressão da STEAP1 parece promover uma ação mais eficiente dos fármacos quimiterapêuticos à base de taxanos, verificando-se estimulação da apoptose e diminuição da proliferação descontrolada das células da próstata.

Em conclusão, os principais resultados desta tese confirmaram assim a ação crucial da STEAP1 no desenvolvimento e progressão do PCa. Esta dissertação demonstrou que a sobre-expressão do *STEAP1* poderá ser usada como um biomarcador de prognóstico, indicando que pacientes com PCa sobre-expressando este gene têm um menor tempo de sobrevida; também mostrou que a redução da expressão da STEAP1 poderá ser uma estratégia terapêutica para diminuir o desenvolvimento e progressão do PCa; e ainda, de forma inovadora, esta tese demonstrou que a ação de fármacos quimioterapêuticos à base de taxanos são mais eficazes no tratamento do PCa sobre-expressando STEAP1. Investigação adicional sobre a relação da STEAP1 no PCa poderá vir a ser uma base fundamental para o desenvolvimento de novas abordagens terapêuticas para o PCa, assim como para a sua utilização como biomarcador.

Palavras-chave

STEAP1; cancro da próstata; prognóstico; alterações epigenéticas; proteómica; fármacos anti-andrógenos; quimioterapêuticos.

Abstract

Prostate cancer (PCa) is one of the most common cancers in the male population and continues to be a considerable source of morbidity and mortality for men worldwide. In an early stage of the disease, the cancer is confined to the prostate gland depending on the stimulating action of androgens. This dependence is the characteristic that allows the use of androgen-deprivation therapy (ADT), which is based on reducing circulating levels of androgens or blocking their actions. The continuous administration of this therapy makes the PCa cells resistant to treatment, being able to survive, extravasate the prostate gland and cause metastases. This stage of the disease is the so-called castrate-resistant PCa (CRPC) and involves the use of chemotherapeutic agents. The limitations of the existent therapeutic options have been stimulating the use of combined therapies targeting survival regulators and proteins involved in the regulation of prostate cell growth and its emergence as a promising option for the treatment of PCa. The development and progression of PCa results from several genetic and metabolic changes, which give tumor cells treatment resistance, exacerbated proliferation capacity and invasive features. Interestingly, many cancer-associated proteins have been reported to have a multifunctional role in controlling the biological changes acquired by PCa cells. Among them is STEAP1 ("Six-Transmembrane Epithelial Antigen of the Prostate").

STEAP1 is a transmembrane protein identified as overexpressed in PCa and several other types of cancer. In non-tumor tissues, STEAP1 expression is low and only restricted to the prostate gland, suggesting that the increase in its expression may favor and accompany tumor development. STEAP1 has been suggested to act as an oncogene, as this protein appears to have the ability to regulate cell proliferation, apoptosis, invasion and metastasis leading to PCa progression. Furthermore, several studies have shown that STEAP1 is involved in intercellular communication and can function as an ion channel or transporter protein. Thus, taking into account the various biological processes that may be controlled by STEAP1, its location and overexpression at the cell surface, and its low expression in non-tumor tissues, it is predictable that this protein plays a crucial role in the maintenance of cell homeostasis and might be used as a possible therapeutic target for PCa. However, the clinical significance of STEAP1 expression in PCa and its usefulness as a biomarker still needs clarification. Furthermore, there are few studies on the regulation of STEAP1 expression in PCa and on cellular mechanisms underlying biological functions in PCa.

The present thesis aimed to first establish the clinical significance of the STEAP1 protein in PCa and explore its usefulness as a biomarker. Its relationship with the other

proteins of the STEAP family (STEAP2, STEAP3 and STEAP4) was also investigated. Using databases and bioinformatics tools, it was found that the *STEAP1* gene is differentially expressed in PCa, from benign lesions to metastatic adenocarcinoma, suggesting that deregulation of this gene may be involved in the early stages of the development of the disease. Concerning to the other STEAP family proteins, our results indicate that *STEAP2* and *STEAP4* are also overexpressed in PCa, but *STEAP3* is underexpressed. However, only the high expression of *STEAP1* was associated with a poor prognosis of patients with PCa, compared to patients without alterations in the expression of STEAP family proteins. Furthermore, comparing only patients with alterations in the expression of these proteins, we found that the overexpression of *STEAP1* continues to be associated with a worse prognosis, while the overexpression of *STEAP4* indicates a good prognosis for patients with PCa, meaning a longer survival time.

The dysregulation of STEAP1 can have a high impact on the development of PCa, and the work developed in this thesis demonstrated that *STEAP1* can be regulated by epigenetic mechanisms. In addition to confirming the high expression of *STEAP1* in neoplastic prostate cell lines (LNCaP) and the low expression of this gene in non-neoplastic prostate cell lines (PNT1A), it was found that the CpG island located at near the transcription start site is methylated in PNT1A cells and demethylated in LNCaP cells. This result suggested that demethylation of the *STEAP1* gene promoter may contribute to its overexpression in PCa. The recruitment of enzymes, such as DNA methyltransferases and histone deacetylases, are essential for epigenetic modifications to occur. The treatment of PNT1A cells with inhibitors of these enzymes increased the expression of the *STEAP1* gene, suggesting once again that the hypomethylation of the *STEAP1* gene promoter may be associated with its overexpression in PCa.

The global characterization of the proteome of LNCaP cells silenced for *STEAP*1 in order to unravel new molecular and cellular functions associated with STEAP1 was another focus of this dissertation. For this, LNCaP cells silenced, or not, for *STEAP1* were subjected to protein identification and quantification techniques. Comparing the two experimental groups (STEAP1 siRNA LNCaP cells *versus* scramble siRNA-LNCaP cells), 526 proteins were found to be differentially expressed. The cellular processes of endocytosis, RNA transport, apoptosis, amino acid biosynthesis and metabolic pathways were the most significant, suggesting that STEAP1 can modulate these biological processes. In fact, STEAP1 silencing promotes apoptosis of PCa cells, attenuating cell proliferation and metabolism, stimulates endocytosis and diminishes intercellular communication by vesicles. These results emphasized the oncogenic role of STEAP1 in PCa.

Finally, it was found that the action of antiandrogenic drugs on LNCaP cells, namely bicalutamide, enzalutamide and apalutamide, appears to be independent of STEAP1

expression levels. On the other hand, treatment of LNCaP cells with taxane-based chemotherapeutic drugs, paclitaxel, docetaxel and cabazitaxel, appears to depend on STEAP1 levels. High STEAP1 expression levels seem to promote a more efficient action of chemotherapeutic drugs based on taxanes, with stimulation of apoptosis and a decrease in uncontrolled proliferation of prostate cells.

In conclusion, the main results of this thesis confirmed the crucial action of STEAP1 in development and progression of PCa. This dissertation showed that STEAP1 overexpression may be used as a prognostic biomarker, indicating that patients with PCa overexpressing this gene have a shorter survival time; also it showed that the reduction of STEAP1 expression might be a therapeutic strategy to decrease the development and progression of PCa; and yet, in an innovative way, this thesis demonstrated that the action of chemotherapeutic drugs based on taxanes are more effective in the treatment of PCa overexpressing STEAP1. Additional investigation into the relationship between STEAP1 and PCa will be a fundamental basis for the development of new therapeutic approaches for PCa, as well as its usefulness as biomarker.

Keywords

STEAP1; prostate cancer; prognosis; epigenetic alterations; proteomics; anti-androgen drugs; chemotherapeutics.

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List of Abbreviations

5-aza-dC	DNMT inhibitor
ADT	Androgen-deprivation therapy
AFZ	Anterior fibromuscular zone
AKT	Serine/threonine protein kinase
ANOVA	Analysis of variance
AR	Androgen receptor
ARE	Androgen response elements
Bax	Bcl-2 associated-X protein
Bcl-2	B-cell lymphoma 2 protein
BPH	Benign prostatic hyperplasia
CBP	CREB binding protein
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CRPC	Castration-resistant PCa
CT	Computerized tomography
CTLs	Cytotoxic T lymphocytes
CYK	Cytokeratin
CZ	Central zone
DEPC	Diethylpyrocarbonate
DHT	5α-dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyltransferases
DTT	Dithiothreitol
E2	17β-estradiol
ECL	Enhanced chemiluminescence substrate
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
ER	Oestrogen receptor
ERG	ETS related gene
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FDA	Food and drug administration
FDR	False discovery rate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene expression omnibus
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HDMs	Histone demethylases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGPIN	High-grade PIN
HMTs	Histone methyltransferases
HPG	Hypothalamic-pituitary-gonadal
HPSE	heparanase
HRP	Horseradish Peroxidase

JAK2/STAT3	Janus kinase 2/signal transducer and activator of transcription 3
KEGG	Kyoto encyclopedia of genes and genomes
KLK3	kallikrein related peptidase 3
LBD	C-terminal ligand binding domain
LFQ	Label-free quantification
LGPIN	Low-grade PIN
LNCaP	human prostate adenocarcinoma cell line
mAbs	Monoclonal antibodies
MAPK	Mitogen activated protein kinase
MEM	Minimal Essential Medium
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MTT	1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan
NTD	N-terminal transcription activation domain
OS	Oxidative stress
PBS	Phosphate buffer saline
PBS-T	PBS containing 0.1% (w/v) Tween-20
PCa	Prostate cancer
PCR	Polymerase Chain Reaction
PET	Positron-emission tomography
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PKB	Protein kinase B
PMSF	PhenylMethylSulfonyl Fluoride
pNA	p-Nitro-Aniline
PNT1A	human non-neoplastic prostate cell line
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
PVDF	PolyVinylidene DiFluoride
PZ	Peripheral zone
qPCR	Quantitative real-time polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-qPCR	Reverse transcription qPCR
SEM	Standard error of the mean
siRNA	small interfering RNA
SRD5A2	Steroid 5-α-reductase type 2
STEAP	Six-Transmembrane Epithelial Antigen of the Prostate
TCGA	The cancer genome atlas
TET	Ten-eleven translocation
TMPRSS2	Type II transmembrane serine protease
TRAMP	Transgenic Adenocarcinoma of the Mouse Prostate
TSA	HDAC inhibitor
TZ	Transition zone

uPA	Urokinase-type plasminogen activator
WB	Western Blot
β2M	Beta-2-microglobulin

Chapter 1

General Introduction

General Introduction

Prostate cancer: from aetiology to therapy

1.1. Onset and development of prostate cancer

The human prostate gland is the major accessory gland of the male reproductive system, having a shape and size of a walnut. Anatomically, this gland is located frontal to the rectum and immediately below the urinary bladder, surrounding prostatic urethra and the ejaculatory ducts (Figure I.1.A) [1, 2]. The prostate gland is composed of glandular and stromal elements, tightly fused within a pseudocapsule [1]. McNeal's anatomic concepts describe four anatomic regions in the prostate, namely, the peripheral zone (PZ), the central zone (CZ), the transition zone (TZ) and the anterior fibromuscular zone (AFZ) (Figure I.1.A) [3, 4]. The McNeal classification is based on the different embryologic origins and can be distinguished by histologic, anatomic landmarks, biological functions, and susceptibility to pathologic disorders [1, 4]. The PZ is enfolded around the outer portion distally and constitutes over 70% of the glandular prostate, being recognized as the most susceptible zone for the developing prostate cancer (PCa) [3, 5–7]. The TZ is located near the prostatic urethra constituting about 5% of the prostate gland in young adult. In older men, the TZ is commonly augmented and is the main site where benign prostatic hyperplasia (BPH) primarily originates [1, 6, 7]. The CZ surrounds the ejaculatory duct and accounts about 20% of the glandular prostate [7]. Less than 5% of prostate cancers begin here, but are considered more aggressive than PZ or TZ cancers with a far greater risk of extracapsular extension and seminal vesicle invasion [8].

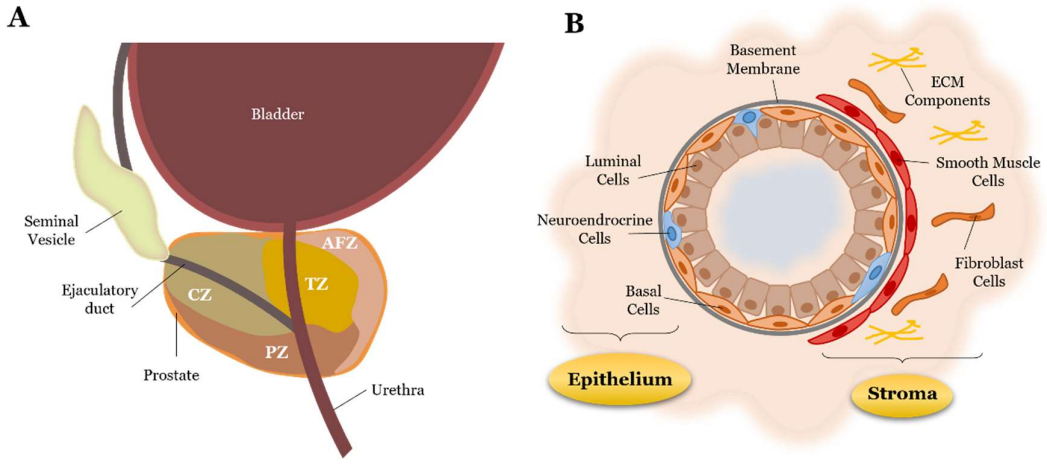

Figure I.1. Schematic representation of prostate anatomy and detail of prostatic epithelium. (A) The prostate gland surrounds the bladder neck, the initial portion of the urethra and the ejaculatory ducts. It can be divided in three glandular zones - central zone (CZ), peripheral zone (PZ) and transition zone (TZ), and the anterior fibromuscular stroma (AFZ) highlighted in different colours. **(B)** Detail of prostate epithelium composed by the luminal cells responsible for the production of prostatic secretions. Basal cells are on the base of epithelium in contact with the basement membrane. Located among the epithelial cells also exist neuroendocrine cells that are involved in the regulation of secretory activity and prostate cell growth. Prostate epithelial cells maintain contact with the stroma, including smooth muscle cells, fibroblast cells and components of the extracellular matrix (ECM).

The AFZ is a unique non-glandular zone that bounds the anterior and apical surface of the prostate grand and, according to McNeal, has no importance for the prostatic function and prostate pathology [3]. An alternative anatomical classification divides the prostate gland in an anterior lobe, a dorsal lobe, a lateral lobe and a ventral lobe [9].

Normal prostate tissue has a structured organization consisting of prostatic ducts lined with epithelial cells surrounded by a fibromuscular stroma [10]. Homeostasis of normal prostate tissue is maintained by the crosstalk between epithelial cells and the surrounding stromal components [2, 11, 12]. The glandular prostatic epithelium is well-organized tissue composed of acini and ducts constituted by three types of cells, luminal, basal and neuroendrocrine cells (Figure I.1.B). Luminal cells are columnar epithelial cells specialized in the production of prostatic secretions and responsible for the main prostate function. Luminal cells produce the prostate specific antigen (PSA), which has been used over the years in PCa screening and disease monitoring [9]. Luminal cells express typical markers such as the androgen receptor (AR), and cytokeratins 8 and 18 (CYK8, CYK18) [13, 14]. In the absence of androgens, luminal cells undergo apoptosis, leading to the regression of the prostate tissue [15]. Basal cells adhere to the basement membrane and have the ability to produce keratin, p63 and cytokeratins 5 and 14 (CYK5, CYK14), and selectively express the insulin-like growth factor receptor, epidermal growth factor receptor and estrogen receptor (ER), essential in the maintenance of cell-growth [13, 14]. Neuroendocrine cells comprise less than 1% of the prostatic epithelium and express chromogranin A, synaptophysin, enolase 2, and CD56, which promote the growth of prostate [13, 16]. It was described that the basal and luminal layers contain multipotent stem cells capable of generating basal, luminal, and neuroendocrine cells [17, 18]. Interactions between the epithelium and basement membrane are fundamental to maintain epithelial cell polarity involving apical and basal surfaces, which represent the well-differentiated cell state [10]. The nonepithelial tissue of the prostate, referred to as stroma, is composed essentially, by fibroblasts, smooth muscle cells and extracellular matrix (ECM) proteins (Figure I.1.B) [11]. Fibroblasts express typical markers such as the vimentin, whereas smooth muscle cells are marked by the expression of smooth muscle alpha-actin (α-SMA) and calponin [19]. The ECM forms a dynamic and structured mixture of collagens, proteoglycans, thrombospondin, and hyaluronic acid, that respond to tissue injuries and allow its regeneration [12].

The main function of the prostate gland is to secret several factors, including PSA, prostatic acid phosphate, prostaglandins and citrate, which strongly collaborate in fertilization, and sperm motility, survival and delivery to the female reproductive system. The prostatic secretions represent 30% of the semen volume and have a basic pH that contributes for neutralizing the acid environment of the duct deferent and female vagina [20]. The maintenance of the secretory functions of the prostate are dependent on androgens and their receptor. In several tissues, including prostate, testosterone is metabolized to 5α-dihydrotestosterone (DHT) by the activity of 5α-reductase enzyme [21]. Androgens mainly act through binding to its receptor, the AR, which is a transcription factor, belonging to the nuclear receptor superfamily. AR actions regulate the transcription of target genes involved in prostate cell proliferation and differentiation from infancy to adulthood. Moreover, AR action also contributes to the development and progression of PCa [22].

Considering the onset of PCa, there is a good agreement that this cancer develops from prostate epithelial cells [10]. However, conflicting evidence exists regarding if the oncogenic transformation in PCa arises from basal [23, 24] or luminal epithelial cells [18, 25]. In addition, also it has been hypothesized that PCa arising from luminal cells will be more aggressive than those arising from basal cells [23]. The prostatic epithelium can be damaged and driven the carcinogenesis of prostate due to several factors, such as, inflammation, infections, genetic/epigenetic changes, persistent activation by androgens, exposure to carcinogens and/or genetic factors [10, 13]. The first identifiable histologic alteration in prostate malignant transformation is so-called prostatic intraepithelial neoplasia (PIN) (Figure I.2.) [26]. PIN lesions can be divided into two grades, low-grade PIN (LGPIN) and high-grade PIN (HGPIN). HGPIN lesions are considered the most likely precursors of PCa [27, 28], however, they do not appear to raise serum PSA concentration [29]. HGPIN has been characterized by an enhanced of cellular proliferation within the prostatic acini and duct, with basal and luminal cells presenting dysplastic features including cytoplasmic

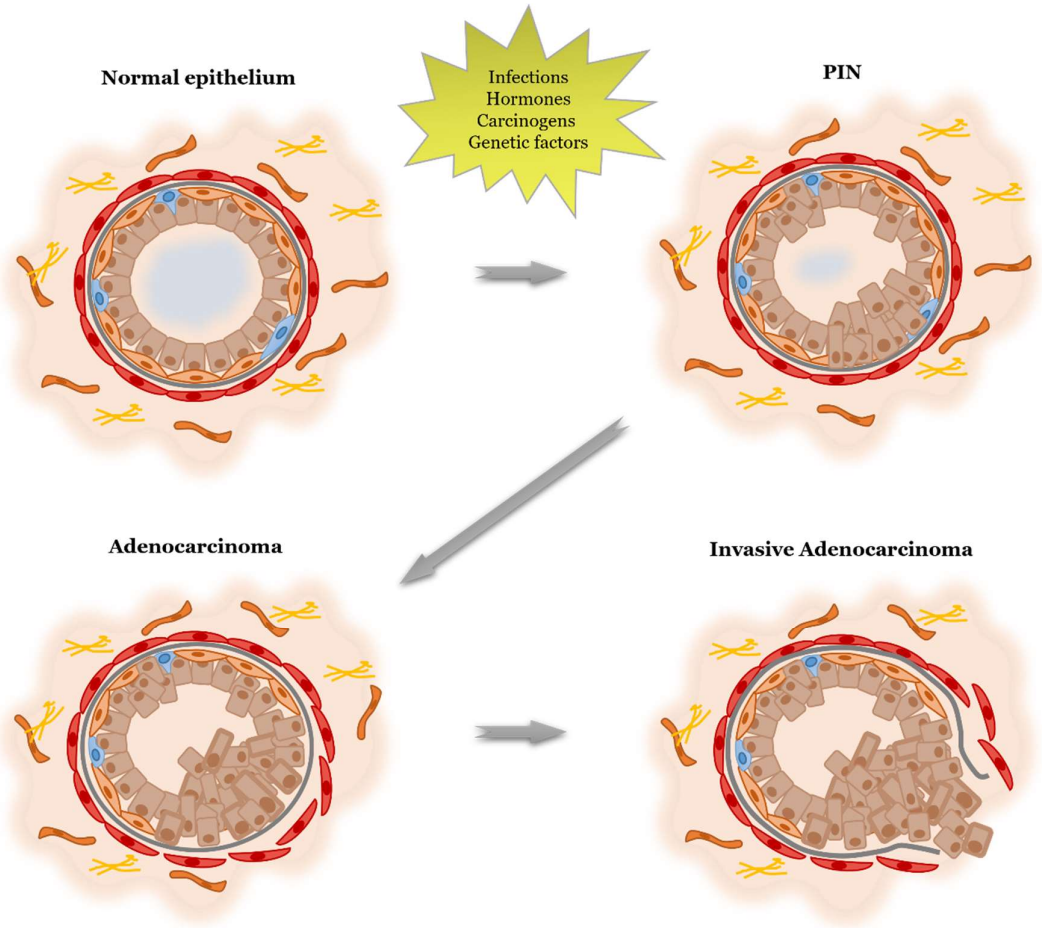

Figure I.2. Schematic representation of the proposed model of the cellular events associated with the development and progression of PCa. Damage in the prostate normal epithelium induces the development of pre-neoplastic lesions called prostatic intraepithelial neoplasia (PIN). This stage progresses to localized prostate adenocarcinoma where the basal cell layer is lost, which then becomes invasive adenocarcinoma when the basement membrane is degraded, and neoplastic cells can invade other organs.

hyperchromasia, nuclear crowding ad prominent nucleoli [13, 30]. Characteristically, HGPIN lesion contain basal cell layer around their periphery, although it is thin and often discontinuous. This is an important diagnostic feature because preservation of the basal cell layer can help to differentiate PIN from prostatic adenocarcinoma in which the basal cells are absent [13, 31].

An alternative precursor of PCa is the proliferative inflammatory atrophy (PIA). PIA lesion is characterized by the increased proliferative index and reduced apoptotic rate of the granular epithelium associated with inflammation [32]. The induction of prostate inflammatory process causes a decrease in the number of basal cells and an accumulation of luminal cells leading an aberrant atrophy of tissue, hyperplasia, and somatic genome mutation, which may lead to the development of PIA and PIN lesions. Both lesions are accepted as precancerous lesions anticipating the establishment of PCa [33, 34]. Prostatic adenocarcinoma mostly arises in the PZ of the prostate and initially is represented as a small foci of intraductal dysplasia, that with time differentiates and progresses into an invasive adenocarcinoma (Figure I.2.) [35, 36]. The tumour foci leads to a disruption of prostate tissue and a decrease on glandular activity and prostatic fluid production [35]. Histologically, PCa is characterized by the destruction of the basal cell layer, derangement of the basement membrane, decreased epithelial cell polarity, and lack of connection of the glandular acini formed by the prostate epithelial cells [37]. As the tumour progresses, neoplastic cells increase the production of proteolytic enzymes, which cause degradation of the basement membrane, allowing the spread to adjacent tissues and the development of a metastatic disease [38]. Firstly to lymph nodes and then to distant organs, including the bones, liver, and lungs, with bone as the most common site of metastasis [39]. In fact, in the context of epithelial neoplasia, the prostate stroma induces alterations in the tumour microenvironment, it is the so-called the reactive stroma. This phenotypic histological change leads to a loss of well-differentiated smooth muscle cells, increase of fibroblast population and increase of secretion and deposition of ECM components, such as matrix metalloproteinase (MMP). All these changes can lead to epithelial cell depolarization and formation of conduits favouring neoplastic cell migration [12, 19]. All these histological changes cause a thousand-fold increased release of PSA from prostate neoplastic cells into the blood [37].

The histological patterns and aggressiveness of PCa are reflected in the degree of histologic differentiation. This is graded using the Gleason Score grading system, a standard method entirely based on the architectural arrangements of prostatic carcinoma, which has been modified over the years [40, 41]. This score is categorized into five basic grade based on morphology/histology/pathology comparing the extent of cancer progression to normal tissue, where 1 looks like normal prostate tissue with well differentiated cellular architecture and 5 represents an aggressive and un-differentiated cellular architecture [42]. These five basic grade are used to generate a histologic score that ranges from 2 (more differentiated, less aggressive) to 10 (less differentiated, more aggressive) [42]. The Gleason score remains the most reliable indicator of the potential that PCa has to grow and spread, and, consequently, it is an important determinant of disease prognosis and treatment options available for patients.

1.2. Epidemiology and risk factors

PCa is the second most common cancer in men, and represents the fifth leading cause of cancer-related mortality. In 2020, 1.4 million new cases of PCa were diagnosed worldwide and, approximately 375,000 associated deaths were reported by World Health Organization [43]. In Portugal, PCa is the most frequent cancer in men and the fourth cause of cancer deaths [44].

In the last years, epidemiologic evidence has identified several biological and genetic factors, but also environmental and lifestyle factors have been shown to contribute to the appearance and progression of PCa (Figure I.3.). Overall, the risk factors for a high prevalence of PCa can be divided in two main classes: endogenous (age, family history, ethnicity, hormones, genetic factors, inflammation and oxidative stress) and exogenous (dietary factors, physical inactivity, obesity, environmental factors, occupation and smoking) [45–48]. Nevertheless, age, race, and a positive family history of PCa have been intimately associated as the major incidence of PCa. Moreover, age is considered the highest risk factor for the development of PCa. The peak of incidence is found in older men of approximately 70-74 years old [47]. Increasing age leads

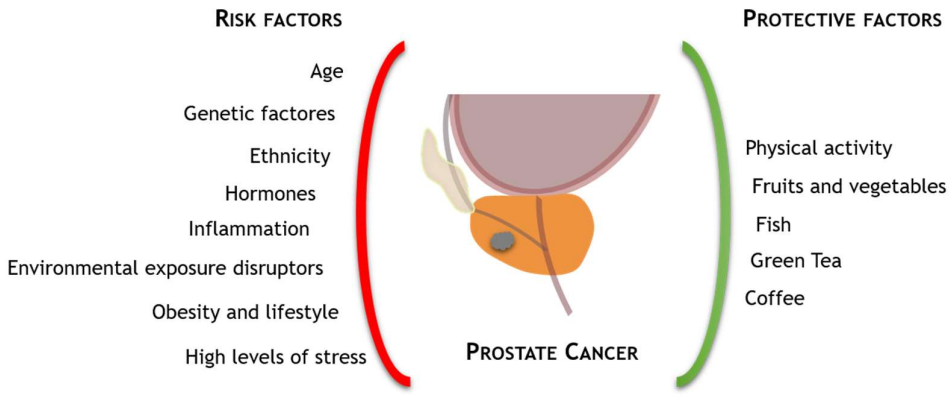

Figure I.3. The diverse risk factors contributing to prostate carcinogenesis (left side) and protective factors against prostate malignancy (right side).

invariably to a physiological decline, diminishing the ability of the organism to resist stress, damage, and disease. An enhanced molecular damage in DNA and consequently, an increase of oxidative stress, are enormously associated to a great susceptibility for the onset of aged-associated disease, as is the case of PCa [45, 49]. Race and family history seem to contribute to the development of PCa and, in fact, African American men have the highest rates of PCa in the world [48]. The cause of this disparity appears to be linked to polymorphisms in genes involved in hormonal regulation and external factors as lifestyle and environment factors [47, 50]. Considering the family history, the risk of developing PCa doubles for men who have a father or brother affected by PCa, and risk increases further when multiple first-degree relatives are affected [51]. There is genetic heterogeneity in the familial form of PCa and several studies have been revealing various susceptibility genes [52–57]. Using linkage analysis based on a genome-wide search, *HPC1* (hereditary prostate cancer 1) [52] and

PcaP (predisposing for prostate cancer) [53] were discovered as hereditary PCa-susceptibility genes. The 2′,5′-oligoisoadenylate synthetase–dependent ribonuclease L (*RNASEL*) gene [54], macrophage scavenger receptor 1 (MSR1) gene [55] and elaC homolog-2 (ELAC2)/HPC2 gene [57] also were related as susceptible genes to PCa risk. Another candidate gene is steroid 5-α-reductase type 2 (*SRD5A2*), which encodes the 5α-reductase type 2 that catalyses the conversion of testosterone to the more active metabolite DHT. Polymorphisms in *SRD5A2* gene increase the catalytic activity of the enzyme and consequently, increase PCa risk [56].

Deregulation of hormones metabolism, particularly of androgens, is other risk factor for the development of PCa. Androgens and AR signalling have a central role in PCa development and progression. Analysis of some studies revealed that high serum and intraprostatic androgens concentrations have a positive association with larger prostate volume increasing PCa risk [58–60].

Some epidemiologic studies have suggested that chronic inflammation and/or sexually transmitted infectious agents also have a role on the pathogenesis of PCa [33, 61–65]. In fact, a persistent inflammation or prostatitis or chronic inflammation disease episode are significantly associated with PCa development [33, 61, 62]. Among the sexually transmitted disease, gonorrhoea and syphilis, or infectious pathogens, like human papillomaviruses, are the principal simulators of the inflammatory process and intimately related with PCa [63–65].

Among the exogenous risk factors for the development of PCa, the environmental exposure to chemical disruptors must be considered. Exposure to phthalate [66] and bisphenol A [67, 68], and the occupational exposure to pesticides [69, 70] have been associated with an increased risk of PCa, as these compounds mimic and interfere with the hormone receptors, deregulating hormone response.

Lastly, obesity and lifestyle factors have been studied with respect to PCa risk. Obesity lead to metabolic disorders related with the deregulation of hormones synthesis and action, and inflammation [71]. Diets highly enriched in animal fat, processed meats and saturated fat have been positively associated with PCa [72, 73]. Also, studies have shown a positive correlation between high calcium intake [74] and low vitamin D levels [75] with risk of developing PCa.

Regarding protective factors for PCa, several other diet products and regular physical activity seem to be identified as having a role minimizing the risk of developing this disease (Figure I.3.). Cohort and case-control studies have shown that vitamin D [76] and E [77] reduce the risk for PCa, and vegetables, fruits, and legumes [78] have antioxidant and antiproliferative properties on PCa cells. Fish consumption low-temperature cooked has good impact protecting against the development of PCa [79]. Green tea contains potent antioxidant and antiproliferative polyphenols that are reported to reduce PCa cell growth in cell cultures [80], and the consumption of coffee also contributes to inhibit PCa progression [81]. Finally, physical activity is one of the several factors known to lower the risk of developing any type of cancer, as well as improving outcomes in patients already diagnosed [82, 83].

1.3. Molecular pathways of carcinogenesis

There are several pathways involved in androgen-dependent and -independent progression of PCa, and many antiapoptotic gene products, growth factors and their receptors that are differently expressed in the different stages of disease progression, contributing to tumour growth and cell survival, migration and invasiveness. The signalling pathways responsible for PCa carcinogenesis do not only include AR, but a number of other genomic and nongenomic pathways are involved on prostate pathogenesis and eventually a crosstalk between them exists.

1.3.1. AR signalling

Androgens play a central role in the control of normal prostate as well as PCa cell growth and proliferation [10]. Androgens are the primary regulators of the proliferation/apoptosis ratio, stimulating proliferation and inhibiting apoptosis of prostate cells, and, thus, inducing the development of PCa [10, 84]. The major circulating androgen, testosterone, is produced in the testes by Leydig cells under regulation of the hypothalamic-pituitary-gonadal (HPG) axis [28]. Once produced, testosterone mostly circulates bound to serum sex hormone-binding globulin (SHBG) (Figure I.4.) and albumin but only the free form enters prostate cells by diffusion. As mentioned before, testosterone can be converted into DHT by the activity of 5α-reductase. Both testosterone and DHT exert their actions through binding to the AR. ARs are essential transcription factors in the regulation of male sexual development and maintenance of accessory sexual glands [22, 28, 84]. PCa growth and disease progression is initially dependent on AR activation, via testosterone and DHT. The main mechanism of action leads to the nuclear translocation of the ligand-receptor complex and subsequent binding to the androgen response elements (AREs), which initiates the transcription of genes that regulate cellular differentiation, proliferation and apoptosis [28, 84, 85] (Figure I.4.).
AR also known as, nuclear receptor subfamily 3 group C member 4 (NR3C4), belongs to the nuclear receptor group of the steroid hormone-binding and consists of three major functional domains: an amino-N-terminal transcription activation domain (NTD), followed by the DNA binding domain (DBD), and the C-terminal ligand binding domain (LBD) that binds androgens [22, 86, 87]. Testosterone binds to the ligand-binding pocket and promotes the dissociation of HSPs from the AR. Also, it causes the LBD undergoing a conformational change that allows the recruitment of co-factors, and recognition and binding to AREs in the promoter regions of target genes involved in growth and survival [86, 88]. In primary PCa, the action of AR keeps the same role as in normal prostate, for example, synthesis of PSA and modulating lipid metabolism [21]. However, it also triggers other events that promote epithelial cell growth, as the induction of the type II transmembrane serine protease (TMPRSS2):ETS fusion [27], [58]. The TMPRSS2 is an androgen-regulated gene overexpressed in PCa, which encodes a protein belonging to the serine protease family that functions in prostate carcinogenesis and relies on gene fusion with ETS transcription factors, such as ETS related gene (ERG) and ETV1. The TMPRSS2:ETS fusion is considered the most common chromosomal rearrangement in PCa and drives the overexpression of ETS oncogenes, previously identified as the most expressed proto-oncogenes present on malignant epithelial prostate cells [58, 89, 90].

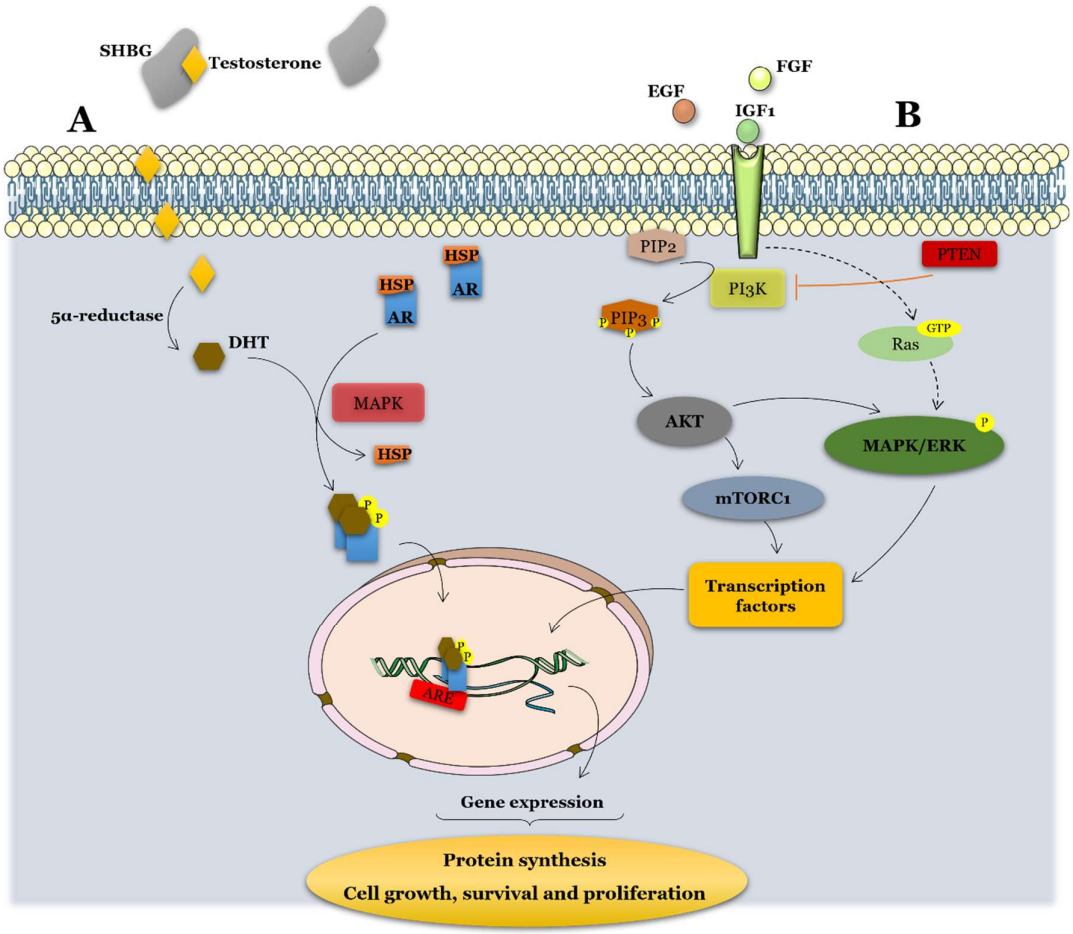

Figure I.4. Overview of the androgenic regulation (pathway A) and PI3K/AKT signalling (pathway B) in PCa. In the cytoplasm, activity of AR is regulated by ligand-binding and heat shock proteins (HSP). Testosterone is transported into the cytoplasm of androgen-receptive cells and is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT binding leads to dissociation of AR from HSP and its phosphorylation by the mitogen-activated protein kinase (MAPK), which is followed by receptor dimerization and translocation into the nucleus where it binds to the androgen response elements (AREs) in the DNA activating transcription of genes essential for cell growth, survival and proliferation. On the other hand, PCa cell fate is controlled by receptor tyrosine kinases (RTK) activated by several growth factors, such as insulin-like growth factor (IGF1), fibroblast growth factor (FGF) and epidermal growth factor (EGF). RTK activation leads to the stimulation of phosphatidylinositol 3-kinase (PI3K) that phosphorylates phosphatidylinositol 4,5-bisphosphonate (PIP2) into phosphatidylinositol 3–5-triphosphate (PIP3). This process is inhibited by the tumour suppressor phosphatase and tensin homolog (PTEN). PIP3 activates, which subsequently removes the inhibition on the mTOR/Raptor complex (also known as mTORC1), thus leading to mTORC1 activation. mTORC1 is pivotal in the translation of proteins for protein synthesis and activation of transcription factors that translocate to the nucleus inducing the expression of pro-proliferation and anti-apoptotic genes. Other intracellular pathways also converge on the mTORC1 complex is constituted by the Ras-dependent pathway. Activated Ras (a small GTPase) phosphorylates and activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) cascade, regulating the activity of several transcription factors that are important for the cell cycle and proliferation. The activation of these signalling pathways inhibits apoptosis and induce the proliferation, invasion, and migration of PCa cells, being also implicated in tumour metastization.

ARs also have two active functional domains (AFs) that initiate transcription when activated. AF-1 is present in the NTD and its activation is androgen-independent. AF-2 is located in the LBD and is ligand-dependent [87]. AF-1 may enable cross-coupling between androgenic and growth factor signalling pathways [84, 88]. Therefore, these AFs are deemed clinically important as they could provide the key to understand the development of castration-resistant PCa (CRPC). At early stages of disease, PCa growth is androgen-dependent, the so-called androgen-sensitive PCa. However, with the continuous tumour development, PCa cells became androgen-insensitive and the disease progresses to the so-called CRPC [84].

Androgenic blockade, through LBP, or androgens and 5α-reductase antagonists, as well as HPG overstimulation via luteinizing hormone (LH)/gonadotropin-releasing hormone (GnRH) analogues, leads to prostate epithelial cell apoptosis and a transient response in preventing the proliferation of PCa [28]. Patients that acquire resistance to the use of androgen-deprivation therapy (ADT) inevitably develop CRPC, a more lethal form of PCa. The role of AR in PCa progression and development of CRPC has been attributed to several factors, such as AR gene amplification, activating mutations and aberrant expression of co-activators [85, 86, 91]. These alterations lead to an increased AR expression, activation of AR by non-androgenic ligands, broadened ligand specificity and sensitivity and increased AR transactivation, which ultimately contribute to tumour cell growth in low androgen environment [84, 91, 92]. AR mutations in primary PCa are rare, however these mutations are prevalent in about 50% of CRPC [93, 94]. These mutations lead to alterations that improve the functional activity of the receptor, such as increased AR sensitivity to low levels of ligand, non-androgen ligand binding, ligand-independent activation as well as AR-independent pathways [87, 93, 94]. Furthermore, recent data indicate that an increased expression of constitutively active AR splice variants follows castration and are associated with poor prognostic and a rapid recurrence of PCa [95, 96].

1.3.2. PI3K/AKT signal transduction

The reduction in AR activation by endogenous androgen ligands leads to hypersensitization of other pathways of AR activation through ligand-independent mechanisms [91, 97]. Various growth factors, cytokines, kinases and other proteins have been shown to interact with and activate AR in a ligand-independent manner, including insulin-like growth factor (IGF1), fibroblast growth factor (FGF) and epidermal growth factor (EGF) [98, 99]. This growth factors activate tyrosine receptor kinases, which results in the activation of phosphatidylinositol 3-kinase (PI3K) and subsequently the PI3K/AKT pathway [100] (Figure I.4.). The serine/threonine protein kinase (AKT), also known as protein kinase B (PKB), is one of the major downstream effectors of PI3K. Binding of ligands to the membrane growth factor receptors initiates a cascade of events that activate PI3K, which converts phosphatidylinositol 4,5-bisphosphonate (PIP2) to phosphatidylinositol 3–5-triphosphate (PIP3). PI3K activation stimulates AKT, which recruits proteins to the luminal cell cytoplasm [100, 101]. Downstream targets of AKT, namely, the mammalian target of rapamycin complex 1 (mTORC1), forkhead box protein O1 and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) cascade, activate several transcription factors, such as c-myc, which induces

the expression of proteins associated with cell survival and proliferation, cell cycle progression, migration and angiogenesis, and, thus, contributing to the progression of PCa [91, 100–102].

The PI3K/AKT signalling pathway is frequently activated in PCa cases as a consequence of protein phosphatase and tensin homolog (PTEN) gene loss [84, 101, 103]. Genetic analysis has identified genomic deletions or mutations in about 70% of primary PCa samples and 20% of the CRPC tumours cases [103]. PTEN is a tumour suppressor gene that acts by negatively regulating the PI3K/AKT pathway and halting the cell-cycle at the G1 stage therefore counteracting cellular proliferation. Loss of PTEN thus results in an increase in the PI3K/AKT pathway as well as impairing normal AR regulation, resulting in increased cellular proliferation, AR expression and reduced apoptosis [101, 103]. PTEN's expression is inversely correlated with Gleason Score and serum PSA levels and therefore is significantly associated with aggressive PCa, with up to 85% of advanced prostate carcinoma being negative for PTEN expression [104].

1.3.3. Epigenetics mechanisms

In addition to genomic alterations, epigenetic modifications have been reported to regulate various cellular responses associated with cancer progression, including cell proliferation, apoptosis, invasion, and senescence. Epigenetic changes, including aberrant DNA methylation, histone modifications and non-coding ribonucleic acids, also contribute to the initiation and progression of PCa. Globally, DNA methylation and histone modifications are the most studied epigenetic alterations affecting gene expression (Figure I.5.).

1.3.3.1. DNA methylation

The major epigenetic mechanism described in cancer is DNA methylation. This process refers to the addition of a methyl group at the five position of the cytosine within CpG-enriched islands, preferentially located at the 5′ promoter region of more than 60% of human genes [105–107]. This reaction is catalysed by a family of DNA methyltransferases (DNMTs), namely DNMT1, DNMT3a, and DNMT3b [108]. DNMT1, often associated with DNA methylation maintenance, has a higher catalytic activity to preferentially methylate hemimethylated DNA during replication and is mostly responsible for maintaining the DNA methylation status [108]. DNMT3a and DNMT3b are responsible for a new methylation pattern to unmodified DNA and are thus known as *de novo* DNMTs [108]. In contrast, DNA demethylation is a reverse action catalysed by a Ten-eleven translocation (TET) enzymes, TET1, TET2 and TET3 [109]. Homeostasis between demethylation and methylation of the genome is a dynamic mechanism essential for mammalian development, differentiation and cellular preservation.

Aberrant DNA hypermethylation results in inactivation of crucial tumour suppressor gene involved with DNA repair, cell cycle, apoptosis or cell adhesion, while DNA hypomethylation could lead to activation of oncogenes [108]. In PCa, DNA hypermethylation is the most characterized alteration among all the epigenetic modifications identified [110]. Numerous key genes have been implicated in DNA methylation changes. Among the most well-described alterations is glutathione S-transferase Pi1 (GSTP1) promoter hypermethylation and subsequent silencing that occurs in early

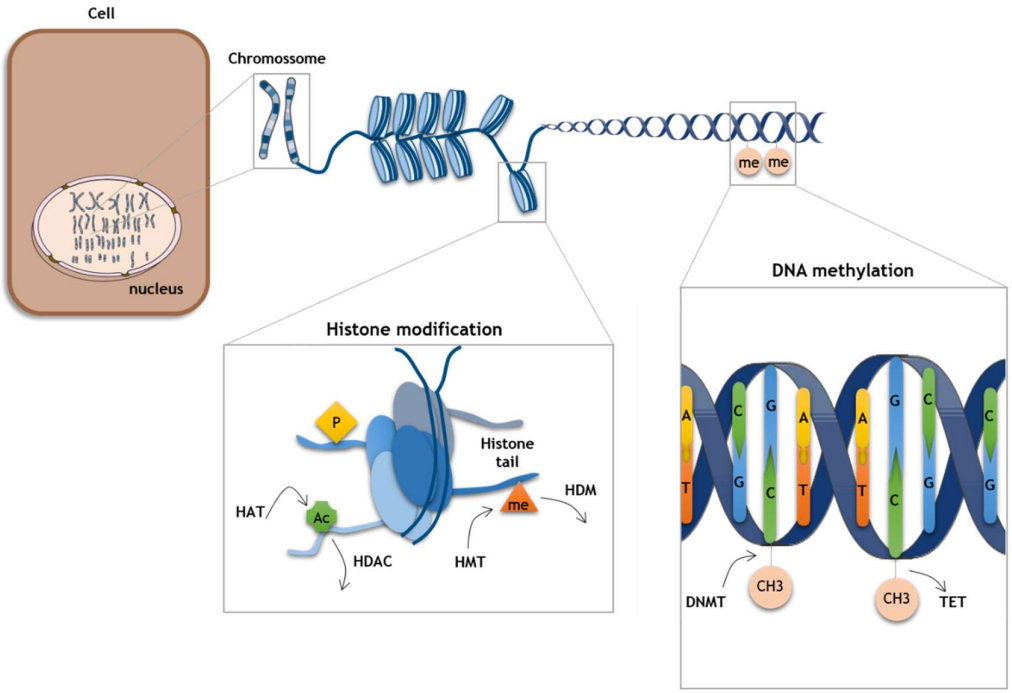

Figure I.5. Schematic model of epigenetic regulation. The expression of most human genes is regulated by epigenetic modifications. DNA methylation and histone modifications are two different epigenetic processes that control gene transcription and expression. DNA methylation always exists in CG-rich areas of the human genome called CpG islands, which can be methylated by DNA methyltransferases (DNMTs) resulting in repression of genes transcription. DNA demethylation is catalysed by a family of Ten-eleven translocation (TET) enzymes. Histone acetyltransferases (HATs), histone deacetylases (HDACs), histone lysine methyltransferases (HMTs) and lysine demethylases (HDMs) are the responsible for inducing histone modifications, which involves modification of amino acids on four different histone tails, resulting in the regulation of gene expression contributing to gene activation or gene silencing.

stages of prostate carcinogenesis, and so has been proposed as a potential prognostic biomarker [111]. Also, the AR promoter itself appears to be hypermethylated in some of CRPC cases, resulting in the loss of AR expression [112]. Additionally, others important regulatory genes, such as PTEN, E-cadherin, the tumour suppressors cyclin dependent kinase inhibitor 2A (CDKN2), IGF-I and Ras association domain family member1A (RASSF1A), are commonly inactivated by promoter hypermethylation in PCa contributing to carcinogenesis and progression of disease [113, 114].

Conversely, a global DNA hypomethylation has also been widely associated with development and progression of PCa [115, 116]. However, DNA hypomethylation is more frequently observed in a late phase, such as metastatic PCa, rather than in the early stages of the carcinogenesis process [117]. DNA hypomethylation refers to the demethylation of normally methylated CpG sites and contributes to upregulation of gene expression. The expression of urokinase-type plasminogen activator (uPA), gene with a role in tumour invasion and development of metastasis through the degradation of ECM, is normally repressed by DNA methylation. But the promoter region of uPA was shown to be hypomethylated and associated with high levels of uPA expression leading to oncogenic effect [118, 119]. Other gene involved in the degradation of ECM is heparanase (HPSE). And in PCa, increased HPSE expression was confirmed as a result of promoter hypomethylation [120]. Also,

cytochrome P450 family 1 subfamily B member 1 (CYP1B1), a major member of the cytochrome P450 superfamily, was reported to be highly expressed in PCa tissues compared to non-malignant tissue, and these high expression levels were caused by aberrant promoter hypomethylation [121]. Thus, DNA hypomethylation could contribute to oncogenesis by the activation of tumour-promoting genes.

1.3.3.2. Histone modifications

Histone post-translational modifications, such as acetylation, phosphorylation and methylation also play an important role in the progression of many tumour types including PCa (Figure I.5.). Histones are responsible for chromatin organization and compaction by folding DNA in the nucleus and, as with DNA methylation, histone modifications could alter gene expression without changing nucleotide DNA sequences. These modifications are caused by the enzymes referred as *written*, histone methyltransferases (HMTs) and histone acetyltransferases (HATs), and/or *erased*, histone demethylases (HDMs) and histone deacetylases (HDACs) [106, 107]. Both processes where these enzymes are responsible, histone methylation and histone acetylation, are implicated in the control of gene transcriptional regulation as well as in non-histone protein post-translational modifications [122].

HMTs promote the addition of one, two or three methyl groups from S-adenosylmethionine (SAM) to certain lysine or arginine residues in the N-terminal domains of core histone, and HDMs are responsible for removing these methyl groups. Deregulation of KMTs and KDMs has been observed in PCa cells and has been associated with cancer cell proliferation [106, 123]. The strong overexpression of a HMT enhancer of zeste homolog 2 (EZH2) observed in metastatic PCa was linked to transcriptional repression through trimethylation of lysine 27 on histone 3 (H3K27me3) [124, 125]. On the other hand, the activity of KMD6 also was upregulated mediating transcriptional activation of specific target genes involved in key PCa carcinogenic pathways, including AR signaling [125]. Overexpression of others KDMs including lysine-specific demethylase 1 (LSD1/KDM1A), and Jumonji C-domain (JmjC) 2-oxoglutarate-dependent dioxygenase KDM superfamily (JmjC-KDMs) as JMJD1A/KDM3A, JMJD1B/KDM3B, JMJD2A/KDM4A, JMJD2C/KDM4C, JARID1B/KDM5B, and PHF8/KDM7B, were also reported in PCa, resulting in higher cell proliferation, migration, and invasion [126].

Unlike histone methylation, histone acetylation is usually associated with activating transcription due to DNA molecule relaxing by histone charge neutralization, whereas histone deacetylation is correlated with gene silencing [107]. HATs catalyse histone acetylation by the addition of an acetyl group, while HDACs carry out deacetylation by the removal of an acetyl group, and deregulation of HATs and HDACs is often observed in PCa. Co-activator complex proteins with a conserved HAT domain, such as p300/CREB binding protein (CBP), play an important role as coactivators of AR leading for progression of AR-dependent PCa cells [127]. Hyperacetylation of histone 3 lysine residues 9, 14, and 18 (H3K9ac, H3K14ac, H3K18ac) induces CRPC development via p300 activity [128]. Importantly, curcumin, a specific inhibitor of CBP and p300 acetyltransferase activity, has been reported to possess antiproliferative and proapoptotic properties suppressing PCa growth and metastasis [129, 130]. Furthermore, higher levels of acetylated histone 4 lysine 16

(H4K16ac) in PCa cells was reported to induce transcriptional activation of pro-inflammatory genes, such as tumour necrosis factor alpha (TNF-α) nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [131].

HDACs are a large family of chromatin remodelers comprising four major classes (I, II, III, and IV) regulating both transcription factors and histone deacetylation. Class I includes HDAC1, HDAC2, HDAC3, and HDAC8, class II members are HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10, class III includes HDACs belonging to class of sirtuins (SIRTs), SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7, and class IV representative is HDAC11 [132]. Overexpression of HDACs, including HDAC1, HDAC2, and HDAC3, have been tightly associated with PCa progression and aggressiveness [133]. HDAC1 activity induced Yan Yang 1(YY1)- mediated repression of XIAP-associated factor 1 (XAF1) in PCa cells [134]. In addition, HDAC3 activity augments AKT phosphorylation in PCa cells and its overexpression correlates with AKT phosphorylation in human PCa samples [135]. Interestingly, HDAC6 regulates AR protein stabilization in CRPC, through heat shock protein 90 (Hsp90) transcription factor substrate controlling AR nuclear localization and activation through AR deacetylation [136]. Increased HDAC activity was associated with ERG expression, which inhibits HATs activity in PCa cells [137]. Modulation of the role of HDAC enzymes can alter the cellular regulation of proto-oncogenes and tumour suppressor genes thereby influencing potential neoplastic proliferation. Recently, several studies have explored the use of HDAC inhibitors as a potential therapeutic option to PCa, due to their effects on reduction of the proliferation and tumour growth, metastasis and re-sensitize PCa to androgens [132, 137–140].

1.4. Prostate cancer diagnosis

Early detection and treatment possibilities for PCa have increased success rates in the management and control the disease. The principal methods to detect the initial phases of PCa include the digital rectal examination, the serum levels of PSA and transrectal ultrasound guided biopsy followed by histologic examination [42, 141, 142]. Rectal examination is part of a routine medical inspection to screen dysfunction in male for checking the signs of enlargement or irregularity which leads to a biopsy [142]. However, the performance of digital rectal examination to detect initial stage of PCa is limited.

Historically, the human prostatic acid phosphatase (PAP) was the first serum biomarker used to monitor and assess progression of PCa until the introduction PSA [143]. PSA measurement has been widely used as a PCa biomarker, however it seems to have important limitations because elevated PSA levels also can be found in other prostate pathologies, such as BPH or prostatitis [37, 142, 143]. In the last years, to improve the use of PSA ceras screening method, several derivatives of its measurement were implemented, such as total *vs*. free-PSA, PSA density and age-adjusted PSA levels [144–146]. Therefore, other biomarkers have been searched in tissue samples or body fluids (e.g. blood, urine and semen). PCa antigen 3 (PCA3) gene, TMPRSS2:ERG fusion gene, PTEN expression, AR, circulating tumour cells, circular RNAs and extracellular vesicles, are among the most promising biomarkers for PCa diagnosis [147–151].

In doubtfulness PCa cases, magnetic resonance imaging (MRI) technique is usually used to determine the extent and stage of PCa, and also to check lymph nodes for the possibility of containing cancer cells. This method involves a standard MRI followed by an MRI detecting injected magnetic particles [42]. Other imagological techniques employed as important tools helping the diagnosis of PCa are positron-emission tomography (PET) and computerized tomography (CT) [42]. These imaging techniques based on the detection of cancer metabolites using different radiotracers has remarkably evolved in the past decades. PET/CT with 2-deoxy-2-[fluorine-18]fluoro-D-glucose (^{18}F-FDG), an analogue of glucose, provides valuable functional information based on the increased glucose uptake and glycolysis of cancer cells and depicts metabolic abnormalities before morphological alterations occur [153]. However, it showed modest results with limited sensitivity in PCa, which displays a high glycolytic profile only in advanced stages [154]. Subsequently, new tumour imaging PET/CT tracers, ^{11}C-/^{18}F-choline and ^{11}C-acetate, were developed, and their substantial uptake was depicted in PCa cells [153]. ^{11}C-choline and ^{18}F-choline are two of major radiotracers of choline, which is a precursor for the synthesis of phospholipids essential to cell membrane [155]. ^{11}C-acetate uptake may therefore serve as a biomarker of fatty acid synthase (FAS) activity and thus of an important metabolic process in cancer, namely the increased lipid synthesis required for maintaining cell membrane integrity [156]. However, ^{11}C- /^{18}F-choline has limited value for the detection of primary PCa because of limited sensitivity and specificity for the differentiation of benign from malignant prostatic changes [155], and ^{11}C -acetate uptake is not specific for PCa [156]. Later, small molecule prostate-specific membrane antigen (PSMA)-based PET/CT tracer was introduced for having higher sensitivity and specificity in detecting nodal and metastatic lesions [157].

The final diagnosis of PCa is based on the microscopic evaluation of prostate tissue obtained via needle biopsy. A pathologist examines these samples and issues a primary Gleason score grade for the predominant histological pattern and a secondary grade for the highest pattern [141].

1.5. Therapeutic options

Treatment approaches for PCa differ depending on the stage of the disease. Several types of therapeutic options are available such as surgery, cryosurgery, radiation therapy, hormone therapy, chemotherapy, vaccine treatment, immunotherapy and bone-directed treatment (Figure I.6.) [42]. Active surveillance is the recommended treatment option for low-risk PCa monitoring its progression while not undergoing definitive therapy [141, 152]. Active surveillance involves a series of PSA testing, physical examinations, prostate biopsies, or a combination of these to monitor for progression with an intent to cure those who develop significant disease. Medication is only given to relieve symptoms and improve quality of life [158]. Concerning PCa treatment, the first approach is radical prostatectomy and/or radiotherapy to destroy the cancerous cells confined within the prostate capsule [159]. Radical prostatectomy encompasses removing the prostate gland and if necessary the surrounding tissue, and radiotherapy has the objective of eradicating local PCa before it metastasizes [160]. However, frequently this treatment fails and the cancer recurs [84].

Prostate cryotherapy is also an available treatment option for localized PCa consisting freeze prostate tissue and cause the cancer cells to die [161]. These therapeutic approaches based on surgery

often are used in combination with therapeutic approaches based on drugs, namely hormone therapy and chemotherapy (Figure I.6.). Similarly, to the non-neoplastic prostate cells, PCa cells need androgens to growth and survive, making the ADT an effective first-line therapy. This therapy can involve two approaches: surgical castration (i.e., orchiectomy) or, more commonly, chemical castration with drugs targeting AR signalling regulated by the HPG axis (e.g., GnRH agonists, AR antagonists, and CYP17A1 inhibitors). This castration reduces tissue androgens levels and also reduced the expression of several androgen-regulated genes [39, 156]. However, several adverse effects of ADT are known, such as decreased bone mineral density, metabolic changes, hot flashes and sexual dysfunction [163]. Although most men show positive outcomes for 1 to 2 years with ADT, clinical progression occurs with the disease entering the stage of CRPC [84, 87]. When PCa is considered castrate resistant different treatments options are needed which includes chemotherapy [42, 141]. This aggressive and lethal form of PCa progresses and metastasizes, not existing currently an effective therapy, being done only palliative care [164]. Life-extending treatments encompass the use of new hormone therapies, chemotherapy, radioisotopes and immunotherapy [141, 159].

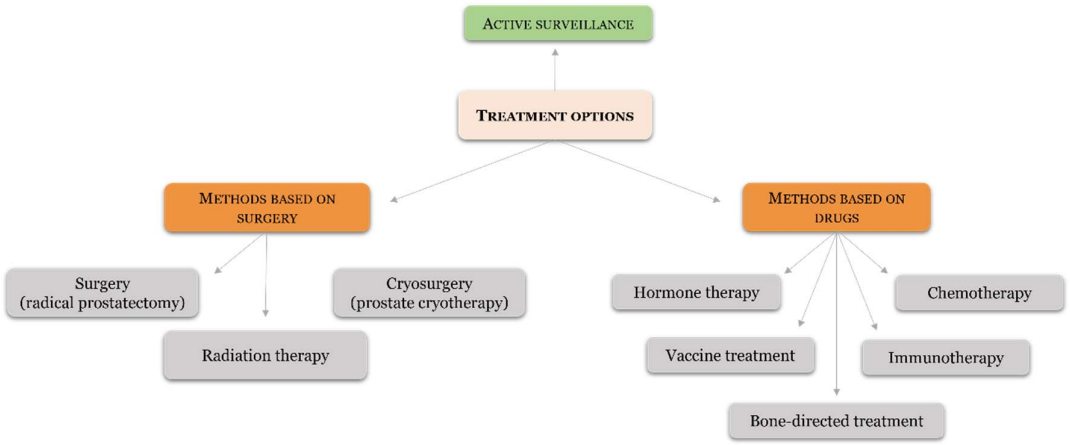

Figure I.6. Treatment options for PCa. Active surveillance for PCa is sometimes called expectant management or watchful waiting and involves closely watching a patient's condition but not giving any treatment. There are different types of treatment for patients with PCa based on surgery (left) and drugs (right). Depending on the stage of disease, a combination of different treatments is usually used.

1.4.1 Anti-androgenic drugs

Anti-androgens are classified as steroidal and nonsteroidal, and they differ in chemical structure, pharmacological effects and safety profiles. Steroidal anti-androgens can lower testosterone levels and also bind to other hormone receptors. Nonsteroidal anti-androgens used as monotherapy are more specific for the AR actions, and seem to be more safer than steroidal anti-androgens [166]. The first generation nonsteroidal anti-androgens used for several years was flutamide and nilutamide, and later in the 90's it was approved the second generation nonsteroidal anti-androgen bicalutamide [167]. These three compounds exert antagonistic activity to neutralize androgenic pathway and, consequently, inhibit gene expression and cell growth stimulated by androgens, having benefits in PCa therapy. However, bicalutamide is the one with the lowest

hepatotoxicity [160, 162]. According to the Early Prostate Cancer (EPC) trial program that evaluated bicalutamide in a localized or locally advanced disease, bicalutamide showed a benefit for overall survival in patients with locally advanced disease undergoing radiotherapy [169]. Furthermore, the affinity of these compounds for the AR is still relatively low [170]. Thus, to improve to efficacy and minimize adverse side-effects of PCa treatment, the third generation anti-androgens was development, such as enzalutamide and apalutamide, with a higher affinity to the AR, optimizes the androgen blockade effect [166]. Enzalutamide and apalutamide were approved by the food and drug administration (FDA) agency in 2012 and 2018, respectively, as a competitive AR antagonist, that are administered orally. Both have a similar mechanism of action, binding to the receptor ligand-binding domain and inhibiting AR translocation to the nucleus. Their affinity for AR is more than five-fold higher than that of bicalutamide [171]. Clinical efficiency of enzalutamide (also designed of MDV3100) was verified in metastatic CRPC showing 81% and 29% reduction in the risk of PCa radiographic progression and death, respectively [172]. Treatment of non-metastatic CRPC patients with enzalutamide was shown to improve metastasis-free survival [173]. Different studies also have reported that apalutamide (also known as ARN-509) could reduce the PSA levels between 46% to 89% in metastatic CRPC patients [174], prolong metastasis-free survival in patients with non-metastatic CRPC [173] and extend the time to symptomatic progression of adverse events [173]. Unlike enzalutamide, apalutamide does not cross the blood-brain barrier, however has a greater anti-tumour activity at a lower concentration [171].

1.4.2. Chemotherapeutic drugs

As the disease progresses to CRPC stage, treatment involves the use of chemotherapeutic drugs. Mitoxantrone was the first cytotoxic chemotherapy approved by FDA for metastatic PCa [175]. Next, other therapeutic agents for the treatment of CRPC were included, such as, the chemotherapeutic taxanes paclitaxel and docetaxel. After the discovery of the mechanism of action of paclitaxel, which is tubulin binding and enhanced microtubule polymerization resulting in mitotic arrest [176], other taxanes were explored and their synthetic and semisynthetic analogues with best properties and improved water solubility were produced [177]. The most successful semisynthetic analogue of paclitaxel is docetaxel. Docetaxel is a taxane derivative that induces microtubules stabilization, arresting cells in the G2M phase of the cell cycle, and it induces bcl-2 phosphorylation promoting a cascade of events that leads to apoptotic cell death [178]. Some studies using docetaxel as a single agent or in combination with other drugs showed objective response rates in up to 38% of patients, PSA declines in more than 50% of patients with hormone refractory PCa, and increased overall survival in metastatic PCa patients in approximately 24 months [173–175]. However, both paclitaxel and docetaxel drugs have a high affinity for multidrug resistance proteins [182]. Cabazitaxel is a novel third-generation semisynthetic analogue of docetaxel and it is a promising treatment for docetaxel-resistant CRPC [183]. Like paclitaxel and docetaxel, cabazitaxel binds to tubulin and promotes its assembly into microtubules while simultaneously inhibiting disassembly. This leads to the stabilization of microtubules, which results in the interference of mitotic and interphase cellular functions. The cell is then unable to progress further into the cell cycle, being

stalled at metaphase, thus triggering apoptosis of the cancer cell [177]. In the last years, several studies have been showed the cabazitaxel as more effective in improving the life-quality of metastatic CRPC patients. Cabazitaxel induced molecular changes in favour of killing PCa cells when compared with other taxanes [184], it showed a reduction of 30% in the serum PSA levels in patients with this pathology [185], and cabazitaxel markedly improved the prognostic outcomes of metastatic CRPC patients [185, 186].

Multiple prospective randomized clinical trials have been designed to evaluate the efficacy and toxicity of therapies and diverse combinations have been attempted [181, 187, 188]. The CHAARTED (Chemohormonal Therapy versus Androgen Ablation Randomized Trial for Extensive Disease in PCa) and STAMPEDE (Systemic Therapy in Advancing or Metastatic PCa: Evaluation of Drug Efficacy) trials showed a remarkable overall survival benefit when combining ADT with docetaxel, as well as increased time to progression to castration resistant status [189], [190]. In the FIRSTANA (Cabazitaxel Versus Docetaxel Both With Prednisone in Patients With Metastatic CRPC) trial, cabazitaxel showed no superiority versus docetaxel for overall survival of PCa patients as first-line treatment [191]. Although the taxanes docetaxel and cabazitaxel have similar efficacy, they have different safety profiles, favouring the lower dose tested of cabazitaxel [192]. However, in the CARD trial showed that high dose of cabazitaxel given with prophylactic granulocyte colony stimulating factor improves overall survival versus abiraterone or enzalutamide in metastatic CRPC patients previously treated with docetaxel and who progressed within 12 months with the alternative androgen-receptor axis inhibitor [193]. These results provide the evidence of a survival benefit with a taxanes treatment for CRPC patients. Furthermore, patient preference studies have increased in significance in recent years for evidence-based medicine [194]. Therefore, the most recent clinical trial aimed to evaluate patient preference between docetaxel and cabazitaxel, the CABADOC trial [195]. This study showed a significantly higher proportion of chemotherapy-naïve men with metastatic CRPC who received both taxanes preferred cabazitaxel over docetaxel. Less fatigue and better quality of life were the two main reasons driving patient choice [195].

STEAP1: from gene to effect on prostate cancer

2.1. *STEAP1* gene and protein structure

The human Six-Transmembrane Epithelial Antigen of the Prostate (STEAP) family comprises at least five homologous members, STEAP1, STEAP1B, STEAP2, STEAP3 and STEAP4 [181–183]. They share at least 60% of similarity and are able to form homo- or hetero-oligomers [198]. However, each family member seems to have different cellular location and function [183–185]. All members of the STEAP proteins family, unless STEAP1B, have six-transmembrane regions with an intracellular hydrophilic amino and carboxyl-terminal domains, indicating that the STEAP proteins may function as a channel and/or transporter [181, 184]. The first role attributed to this family of proteins was a contribution in metal homeostasis through metalloreductases capacity, based on their heme groups and F420H2:NADP+ oxidoreductase (FNO)-like structures [183, 186]. Thus, STEAP proteins family have been shown to have great importance in response to inflammatory, oxidative stress response, cell-cell communication, proliferation and tumour invasiveness, fatty-acids, and glucose metabolism as well as endoplasmic reticulum stress [181, 183, 186].

STEAP1 was the first member of the STEAP family to be discovered in 1999 by Hubert [197]. The *STEAP1* gene is located on chromosome 7q21.13. Its genomic organization consists of 5 exons and 4 introns with a total length of 10,359 bp, and several consensus regulatory elements that exist upstream of the 5'-flanking regions (Figure I.7.) [196]. The transcription mechanism

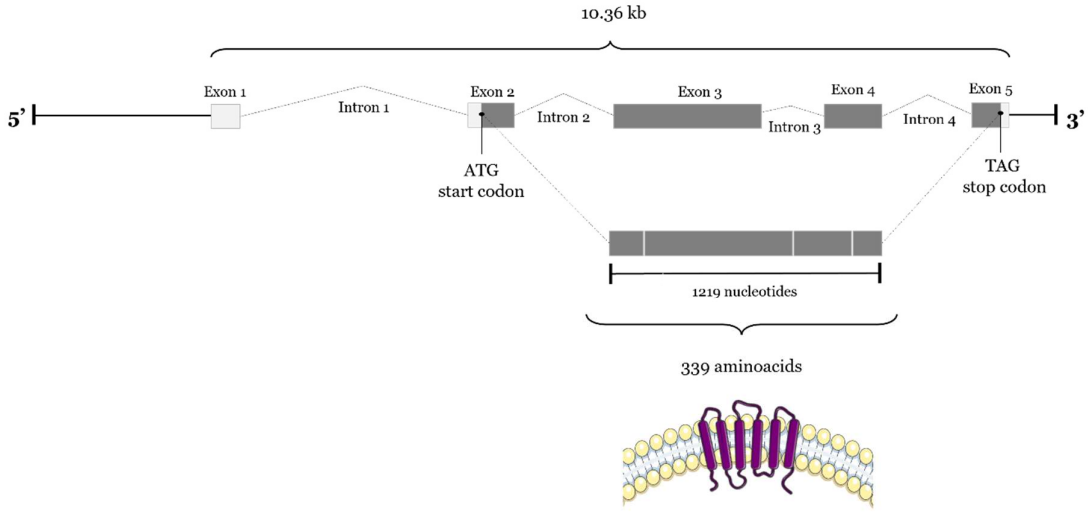

Figure I.7. Representation of the *STEAP1* gene organization and respective protein product. Human *STEAP1* gene includes 5 exons and 4 introns along 10.36 kb size. Light and dark grey boxes indicate noncoding and coding exons, respectively and fine lines correspond to introns. ATG corresponds to the translation initiation codon and TAG corresponds to a translation stop codon. The full-length *STEAP1* mRNA comprises 1,219 nucleotides and gives rise to a transmembrane protein with 339 aminoacids residues and 6 transmembrane domains.

of the *STEAP1* give rise to 2 different mRNA transcripts of 1.3 kb and 4.0 kb. However, only the first one is processed into the mature protein containing 339 aminoacids with a predicted molecular weight of 39.72 kDa (Figure I.7.) [199, 202]. STEAP1 is preferentially located at the plasma membrane of cells, since its structure is a composed by six-transmembrane domains with both N- and C-terminals located on the cytosolic domain, having three extracellular and two intracellular loops [196, 197]. In contrast with the other proteins, STEAP1 cannot reduce metals due to the loss of the N-terminal NADPH-oxidoreductase, FNO-like domain and Rossman-fold, which turns it the smallest of the classical STEAP proteins [203]. Nevertheless, it has been suggested that STEAP1 may be involved in iron metabolism, by its co-localization with transferrin, transferrin receptor, and lysosomes or endosomes [198].

Additionally, the structure of STEAP1 protein contains at least one heme-binding group, in the membrane, that may be related to the absorption of iron and copper [196, 204].

Concerning the ferric oxidoreductase activity of STEAP1, it contributes to the generation, metabolism and increased levels of intracellular reactive oxygen species (ROS), which induces the expression of redox-sensitive and pro-invasive genes, activating several signalling pathways involved in metastatic and proliferative cancer cells [196, 199, 201].

2.2. Tissue expression and their regulation

STEAP1 is highly expressed in a variety of tumours, with particle emphasis on PCa, as researchers have been shown that its expression is highly elevated in cancer cases compared with normal prostate tissue [197, 202, 205–208]. Among non-tumoral tissues, STEAP1 is almost restricted to the prostate gland, mainly at cell-cell junctions located in the plasma membrane of prostate epithelial cells or in basal layer, which may behave as a prostate stem cells reservoir [196, 209]. In addition, up-regulated STEAP1 expression has also been observed in other types of cancer, as breast cancer, colorectal cancer, Ewing's sarcoma, gastric cancer, ovarian cancer, lung cancer, liver cancer, glioblastoma, and also skin disorders [210–218]. However, STEAP1 protein expression levels in human PCa are 5- to 10- fold higher compared to other cancer types [197].

Moreover, the cellular localization and expression patterns of STEAP1 may depend on the histological stage of disease. Regarding BPH lesions, this protein is found in basal cells, while in PIN lesions and PCa cases it is located in luminal, basal and stroma cells [219]. Interestingly, high levels of STEAP1 are associated with low overall survival rates of PCa patients, being correlated with cancer recurrence and aggressiveness [219, 220]. This fact suggests the prognostic value of STEAP1 as a biomarker of PCa. In addition, another studies also evidenced the association of high STEAP1 levels with low overall survival of colorectal cancer patients, lung cancer, liver cancer, diffuse large B-cell lymphoma, acute myeloid leukaemia, multiple myeloma and glioblastoma, suggesting the importance of the prognostic value of STEAP1 as a biomarker of cancer [217, 218, 220, 221]. In contrast, some studies have indicated the prognosis of patients with high STEAP1 expression levels better than that of patients with low expression levels [212, 222].

At present, the carcinogenic effect of STEAP1 in tumour progression is being studied intensely. Certain investigations have shown that STEAP1 also plays a role in inhibiting cell invasion and proliferation [205, 223]. It is speculated that the carcinogenesis and anti-cancer effects of STEAP1 in several types of cancer may be related to different hormones levels and regulation of hormone receptor locally or throughout the body.

The mutual regulation has been reported between STEAP1 expression and sex hormones (Figure I.8). Initially, 17β-estradiol (E2) seemed to be the only known regulator of STEAP1 expression. Maia *et al.* (2008) demonstrated that treatment with E2 reduced the expression of STEAP1 in MCF-7 breast cancer cell line [210]. Later, Gomes *et al.* (2013) showed that DHT or E2 treatment suppressed the expression of STEAP1 in LNCaP PCa cells, and that this down-regulation is AR-dependent and ER-independent [208]. Recently, other approach of the regulation between STEAP1 expression and their promoter was reported. Markey *et al.* (2021) identified a novel partnership between EWS/FLI1 and NK2 homeobox 2 (NKX2.2) in the upregulation of STEAP1 in the Ewing's sarcoma cells [224]. They found that, in addition to EWSFL1, NKX2.2 also occupies the STEAP1 promoter and is equally important in regulating STEAP1 expression [224]. The binding of EWSFLI1 and NKX2.2 leads to very high expression of STEAP1 protein and causing a downstream increase in ROS and related genes resulting in increased tumour invasiveness [224].

2.3. Biological functions in cancer

Dysregulation of STEAP1 affects the occurrence and development of different types of cancers, advocating an oncogenic role for STEAP1 (Figure I.8.) [220]. Due to the location of STEAP1 protein at the cell surface membrane, it acts as an ion channel or transporter protein in both tight and gap junctions or even in cell adhesion process, playing an important role in intracellular communication between tumour cell and adjacent tumour stromal cell to augment tumour growth [197, 225]. Likewise, STEAP1 has the capacity to modulate the proliferation and invasion of cancer cells through the adjustment of intracellular concentration of few ions such as sodium (Na^+), calcium (Ca^{2+}), potassium (K^+), and small molecules also [196, 226]. Interestingly, it was described that higher levels of Na^+ promote an invasive phenotype and metastasis in PCa [227], as well as the modulation of Ca^{2+} and K^+ seems to have a prevalent role in PCa progression [228, 229].

Relating to the ferric oxidoreductase activity of STEAP1, it contributes to the generation, metabolism and consequent increase levels of intracellular ROS (Figure I.8.) [199, 212, 230]. Pan *et al.* (2008) showed that STEAP1 promotes cell growth by raising the intracellular levels of ROS [230], and later, Grunewald *et al.* (2012) based on transcriptome and proteome analyses, as well as functional studies revealed that STEAP1 expression correlates with oxidative stress responses and elevated levels of ROS, which induces the expression of redox- sensitive and pro-invasive genes [212]. It was also shown that knockout of STEAP1 reduces ROS levels leading to decline Ewing tumour proliferation, anchorage-independent colony formation, invasion *in vitro* and metastasis *in vivo* [212]. Thus, the increase of intracellular ROS- levels, as a consequence of STEAP1 overexpression, is a hallmark of several cancer, including PCa [199, 212, 230]. However, there is a contradictory study showing that STEAP1 silencing increased ROS production [211]. Nakamura *et al.* (2019) revealed

that inhibition of STEAP1 transcript was associated with decreased expression of antioxidant molecules regulated by the transcription factor, nuclear erythroid 2-related factor (NRF2), in colorectal cancer cells, leading to enhanced oxidative stress [211].

Decontrolled proliferation and resistance to apoptosis, cell migration and invasion are well-known and established hallmarks of cancer, contributing to tumour onset, progression, metastization process and aggressiveness [231]. A wide range of studies have reported the oncogenic role of STEAP1 protein in different tumours (Figure I.8.) [213–215, 218, 232–235], however a tumour suppressive function has also been observed by some studies [222, 223]. Our research group demonstrated that silencing the STEAP1 gene reduces androgen-dependent PCa cell viability and proliferation, while inducing apoptosis [232]. In addition, this work showed that the cellular and molecular effects of STEAP1 knockdown may be independent of DHT treatment, suggesting the blocking of STEAP1 as an appropriate strategy to activate apoptosis in PCa cells, as well as to prevent the proliferative and anti-apoptotic effects of DHT in PCa [232]. Based in microarray technology and functional analysis, STEAP1 was found overexpressed in lung cancer patients affecting endothelial cell migration and angiogenesis [233], and knockdown of STEAP1 suppressed the proliferation, migration, and invasion

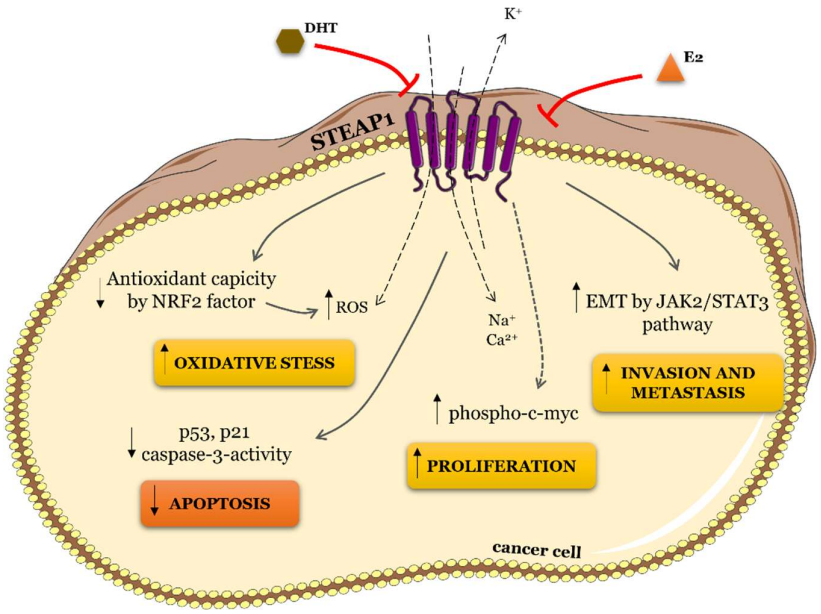

Figure I.8. Schematic representation of STEAP1 actions in cancer. Several factors have been identified in maintaining STEAP1 expression levels in cancer cells. Sex steroid hormones, such as 17β-estradiol (E2) and 5α-dihydrotestosterone (DHT) were shown to inhibit STEAP1 expression in cancer cells, an effect that may be mediated by genomic and/or non-genomic pathways. STEAP1 actively increases intra- and intercellular communication through the modulation of sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) concentrations. Regarding molecular pathways, STEAP1 inhibits cancer cells' apoptosis by suppressing the expression of tumour suppressor proteins (p21 and p53) and decreases caspase-3 activity. Additionally, STEAP1 induces tumour growth, invasion and metastasis by modulating the expression of the factor transcription c-myc and Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signalling pathway, respectively. STEAP1 also contributes to the generation of intracellular reactive oxygen species (ROS) and decreases antioxidant defence by nuclear erythroid 2-related factor (NRF2) transcription factor. Bar-ended red arrows indicate an inhibitory effect to STEAP1 expression. Grey arrows mean a STEAP1 stimulatory effect to cellular pathway.

of lung adenocarcinoma epithelial cells [215]. Additionally, this work also showed that STEAP1 regulated epithelial–mesenchymal transition (EMT) *via* Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signalling pathway [215]. In a tumour of the female reproductive system, a duality of outcomes was reported. While Sun *et al.* (2019) revealed a down-regulation of STEAP1 in endometrial carcinoma combined with increased cancer cell proliferation, colony formation, migration, invasion, and EMT progression [222], while Jiao *et al.* (2020) found a relationship between high STEAP1 levels and EMT-related genes, and demonstrated that STEAP1 promotes ovarian cancer metastasis by aiding EMT progression. Interestingly, down-regulation of STEAP1 in this study inhibited the invasion, migration, proliferation, clone formation, and EMT progression of human ovarian cancer cells and promoted apoptosis at the same time [214]. In breast cancer, Xie *et al.* (2019) showed that knockdown of STEAP1 expression enhances breast cancer cell invasiveness and migration, and is accompanied by increased expression of EMT-related genes, MMP2, MMP9, MMP13, vimentin, and cadherin (CDH)-2, as well as decreased CDH1 expression [223]. Regarding the gastrointestinal tract, up-regulation of STEAP1 in gastric cancer increased cell proliferation, migration, invasion, and consequence tumorigenicity, promoting peritoneal metastasis. These changes were achieved *via* the activation of the AKT/FoxO1 pathway, EMT and phosphorylation of eukaryotic initiation factor 4E (eIF4E) [213, 234, 235]. In liver cancer, STEAP1 silencing inhibited cell proliferation in hepatocarcinoma cell lines by G1 arrest induced by the suppression of cyclin D1 and the promotion of p27 activity [218]. In addition, knockdown of STEAP1 inhibited c-myc expression, which was identified as a component in STEAP1 signal transduction [218].

Inflammation predisposes to the development of cancer and promotes all stages of tumorigenesis, and there is evidence that STEAP1 could be relevant in the inflammatory responses. Liang *et al.* (2017) showed that *in vivo* STEAP1 is upregulated in patients with skin disorders, and co-expressed with proinflammatory cytokines interleukin (IL)-1, IL-36, neutrophil chemokines (C-X-C motif) ligand (CXCL)1, CXCL8, delineating the inflammatory milieu for this patients featuring dysregulated checkpoint signalling and excessive activation of inflammatory pathways [216]. Importantly, keratinocytes deficient in STEAP1 become less efficient in inducing neutrophil chemotaxis, strengthening that STEAP1 support neutrophil-rich proinflammatory responses in the skin disorders [216].

The tumour microenvironment is the ecosystem of cellular and molecular components that surrounds a tumour supporting their growth and eventually disperses through normal tissue. Recently it was demonstrated the relationship between STEAP1 protein and the tumour microenvironment in glioma datasets. Zhao *et al.* (2021) indicated that STEAP1 was positively correlated with multiple immune inhibitory cell types including neutrophils, regulatory T cells, macrophages and cancer-associated fibroblasts, promoting immune escape [217]. Results of this study showed that as STEAP1 expression increased, the immune phenotypes tended to be exacerbated, emphasising that the dysregulation of STEAP family could mediate an immunosuppressive microenvironment of glioma [217].

In sum, broad actions of STEAP1 targeting several cancer hallmarks have been reported in diverse human cancers, including PCa. Based on the accumulated knowledge, it is liable to speculate that STEAP1 can act as a oncoprotein favouring tumour development.

2.4. A potential therapeutic target and biomarker in prostate cancer

Considering the biological functions of STEAP1 highlighted in the previous section, and their location at the cell membrane associated to its low or absent expression in non-tumoral tissue, STEAP1 is currently considered as a promising therapeutic target in PCa. Moreover, it has been indicated as a candidate prognostic marker.

In the last years several strategies have been developed for targeting STEAP1, including antibody-drug conjugates, monoclonal antibodies (mAbs), DNA cancer-vaccines, and small-molecules therapy [236, 237]. It was described the use of mAbs with higher specificity to bind the STEAP1 extracellular loops leading to decrease of its oncogenic role [205, 238]. Challita-Eid *et al.* (2007) first generated two mAbs that bind to cell surface STEAP1 epitopes, and both of them inhibited STEAP1-induced intercellular communication in a dose-dependent manner to suppress tumour growth *in vivo* [205]. Subsequently, Esmaeili *et al.* (2018) developed a single- chain fragment variable antibody, against a STEAP1 epitope, that successfully inhibited intercellular communication between LNCaP PCa cells by blocking cellular gap junctions, demonstrating a high potential for mAbs or single-chain antibodies as effective agents for PCa immunotherapy. In addition, immunotherapy may be provided as an alternative treatment for PCa patients, and some studies have demonstrated that epitopes of STEAP1 are highly efficient in driving infiltration of cytotoxic T lymphocytes (CTLs) to inhibit tumour growth and its ablation [239–241], providing an adoptive immunotherapy for patients with PCa.

Producing an effective vaccine is one of the most important goals of tumour immunotherapy. It has been found that mouse STEAP-based vaccination can induce a specific CD8 T cell response to newly defined STEAP epitopes and prolong the survival rate of tumour-challenged mice, showing that vaccination against STEAP is a feasible option to delay tumour growth [242, 243]. Also, the vaccination with the specific STEAP1 (262-270) peptides encapsulated into PLGA microspheres in HLA-A*0201 transgenic mice could effectively cross-prime CTLs *in vivo*, revealing a new approach in PCa immunotherapy [244].

In terms of diagnostic value, STEAP1 mRNA is detectable in the serum of patients with different solid tumours whereas it is not amplifiable in non-malignant donors [245]. More recently, 89Zr-labeled MSTP2109A (89Zr-2109A), a radiolabelled antibody targeting STEAP1 for PET, detect acute changes in STEAP1 expression in PCa patients and locate metastatic CRPC sites including bone and soft tissue [246, 247]. Still, a novel contrast agent for ultrasound imaging by conjugating biotinylated STEAP1 monoclonal antibodies with streptavidin-coated SonoVue microbubbles, providing a prospective method to identify PCa [248]. Furthermore, DSTP3086S, a STEAP1–targeting antibody conjugated with an antimitotic agent monomethyl auristatin E, showed some antitumor activity in metastatic CRPC with an acceptable safety profile in a phase I study [249]. These

experiments suggest that STEAP1 targeting may be an attractive and selective way to deliver drugs to PCa cells.

Regarding the prognostic prediction value, STEAP1 is reportedly upregulated in PCa correlating with high Gleason scores, seminal vesicle invasion, biochemical recurrence and metastasis, all features of poor prognostic outcomes [220]. However, there is an opposite study showing that overexpression of STEAP1 is an independent biomarker for biochemical recurrence in PCa [206]. So, more studies are required to analyse the clinical significance of STEAP1 in PCa and to explore the role of STEAP1 in the occurrence and progression this type of cancer, and the potential mechanism underlying its regulatory functions.

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Chapter 2

AIM AND OUTLINE OF THE THESIS

Aim and outline of the thesis

The development and progression of PCa are driven by numerous factors, involving complex cellular and molecular mechanisms. The initial progression of PCa mainly depends on the androgen's actions, which enables the use of ADT with the aim of reducing androgens production and the AR-mediated effects. However, PCa can progress to the stage of CRPC, for which the usage of chemotherapeutic drugs is another option.

In the past few years, the multifunctional protein STEAP1 has been considered as a key target in development, diagnosis, and treatment of PCa. Our research group have demonstrated that STEAP1 is overexpressed in samples of prostate adenocarcinoma and PIN lesions compared with non-neoplastic cases. Furthermore, STEAP1 overexpression is associated with higher Gleason scores. However, it is not established if overexpression of STEAP1 contributes to prostate carcinogenesis, or if it is a consequence of cancer development itself. Previous work of our team also showed that STEAP1 silencing markedly inhibited the viability and proliferation of PCa cells, while inducing apoptosis. All these findings highly support the likely role of STEAP1 as an oncoprotein. Nevertheless, there are important knowledge gaps in the understanding of the molecular and cellular mechanisms associated with STEAP1 actions in PCa. Shedding light on these matters will be instrumental to clarify the usefulness of STEAP1 as a biomarker for management of PCa, as well as to disclose novel strategies for PCa treatment. Therefore, the present thesis aims to:

1. Explore the clinical significance of STEAP1 protein expression in PCa, compared with the expression of other STEAP family members, and unravelling its potential and usefulness in the prognostic of this disease;
2. Analyse the effect of epigenetic alterations in regulating STEAP1 expression and determining its overexpression in PCa;
3. Characterize the proteome of PCa cells silenced for STEAP1;
4. Determine the effect of anti-androgenic and chemotherapeutic drugs in regulating STEAP1 expression and how STEAP1 expression levels may influence the response of PCa cells to these drugs.

After a brief description of the prostate gland anatomy and physiology, characterization of the cellular and molecular basis underlying PCa development, and overview of the existent diagnosis and therapeutic approaches (**Chapter 1, Prostate cancer: from aetiology to therapy**), it was presented the expression pattern, function and role of STEAP1 in prostate tumours, highlighting its role as an oncoprotein (**Chapter 1, STEAP1: from gene to effect on prostate cancer**), which culminated in the establishment of the main objectives of this thesis described in **Chapter 2.**

The experimental approaches, results and the scientific outcomes of this thesis are described in **Chapters 3-6,** which are organized as follows:

Chapter 3 shows the differential expression pattern and clinical prognosis of STEAP1 protein in the onset and development of PCa compared with other STEAP family members, using the Oncomine database, CANCERTOOL and cBioPortal platform.

In **Chapter 4** are presented the results on the methylation levels of the *STEAP1* gene promoter in prostate cell lines and human samples of PCa. Also, the association between methylation levels and *STEAP1* gene expression using publicly available datasets is evaluated.

Chapter 5 investigates the proteome of PCa cells induced by STEAP1 knockdown using a label-free quantification combined with an Orbitrap LC-MS/MS system. Identification of differentially expressed proteins in STEAP1-knockdown PCa cells may advance knowledge of the mechanistic role of STEAP1 in PCa, and provide novel evidence for targeting STEAP1-relevant signalling pathways for PCa treatment.

Chapter 6 explores the effect of anti-androgens and taxanes-based drugs in regulating STEAP1 expression on PCa cells. Moreover, this chapter addresses whether inhibition of STEAP1 may change the sensitivity of PCa cells to anti-androgens and chemotherapeutics, through cell viability, proliferation and apoptosis analysis.

Finally, **Chapter 7** contains an integrative overview of the main findings of this thesis and discusses their potential impact in the context of prostate pathophysiology and PCa treatment.

Chapter 3

THE USEFULNESS OF STEAP1 PROTEIN AS PROGNOSIS BIOMARKER IN PROSTATE CANCER

This chapter was adapted from the published original paper and book chapter published:

Sandra M Rocha, Sílvia Socorro, Luís A Passarinha and Cláudio J. Maia. *"Comprehensive Landscape of STEAP Family Members Expression in Human Cancers: Unravelling the Potential Usefulness in Clinical Practice Using Integrated Bioinformatics Analysis."* Data. May 2022 7(5), 64.

Sandra M Rocha, Jorge Barroca-Ferreira, Luís A Passarinha, Sílvia Socorro, Cláudio J Maia. *"The Usefulness of STEAP Proteins in Prostate Cancer Clinical Practice."* Book Title: Prostate Cancer. ISBN-13: 978-0-6450017-5-4. Exon Publications. 2021 May 27. Chapter 10.

The usefulness of STEAP1 protein as prognosis biomarker in prostate cancer

Abstract

Prostate cancer is a multifactorial disease and the second most common cancer diagnosed in men worldwide. The six transmembrane epithelial antigen of prostate (STEAP) proteins seem to be involved in prostate tumorigenesis. However, the clinical significance of the expression pattern of STEAP1 in PCa development is barely known, as well as its relationship with the expression of the other members of the STEAP family. Herein, the Oncomine database, CANCERTOOL and cBioPortal platform were selected to predict the differential expression levels of STEAP members and clinical prognosis. In particular, the STEAP1 gene is differentially expressed in prostate cancer cells, from benign lesions to metastases. In addition, survival analysis reveals that prostate cancer patients with high levels of STEAP1 have poor survival outcomes. In contrast, high expression of STEAP4 offers a better prognosis.

Keywords: prostate cancer; STEAP1; biomarker; prognosis; survival; bioinformatics.

3.1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second most common cause of cancer-related death in men in the Western world [1]. There are three different stages involved in the development of this disease. PCa develops from precursor lesions, designated prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA), which evolve to carcinoma, and therefore also called pre-neoplastic lesions [2]. However, noncancerous conditions are also detected in men, such as benign prostatic hyperplasia (BPH) [2]. Around 70 to 80% of the diagnosed prostatic adenocarcinomas emerge in the peripheral zone, while BPH commonly evolves in the transition zone [3]. The risk factors for PCa can be endogenous (age, family history, ethnicity, hormones and oxidative stress) or exogenous (dietary factors, physical inactivity, obesity, environmental factors, occupation and smoking). Of all these factors, older age, black race, and a family history of the disease are the best-established risk factors for PCa [4]. Treatment options for men with PCa might include surgery, radiation therapy, cryotherapy, hormone therapy, chemotherapy and immunotherapy [4].

The main biomarker used in clinical practice for PCa screening is the serum level of prostate-specific antigen (PSA). However, several factors may affect PSA levels resulting in a considerable number of false-positives [5]. The low specificity of PSA in the diagnosis of PCa is a clinical problem. There is an urgent need to identify new biomarkers for early detection of the disease, and to improve patients' stratification and better define targeted therapies and clinical management of PCa.

The human six-transmembrane epithelial antigen of prostate (STEAP)1 was the first member of STEAPs family to be discovered in 1999 as a prostate-specific cell-surface antigen highly expressed

in PCa and in many other cancers [6]. The STEAP1 protein is composed of six-transmembrane domains with cytosolic C- and N-terminals connected by three extra- and two intracellular loops preferentially located at the cell-cell junctions of the secretory epithelium of prostate and PCa cells, indicating that it may act as a channel or transporter protein [6]. These features pointed out the role of STEAP1 in cellular communication and in cell adhesion processes [7, 8]. In addition, several studies exploring the role of STEAP1 in cancer cells showed that its overexpression inhibits apoptosis, enhances cell proliferation and invasion, and induces epithelial to mesenchymal transition, ultimately contributing to tumour progression and aggressiveness [9–14]. Cumulative evidence has pointed out STEAP1 as putative biomarker, as well as therapeutic target, in several types of human cancers, particularly in PCa [15–19]. However, the clinical significance of the expression of STEAP1 protein for PCa development is still scarce, as well as its relationship with the expression of the other members of the STEAP family (STEAP2, STEAP3 and STEAP4). Further analysis is needed to better establish the utility of STEAP1 as prognostic biomarker. This chapter provides a discussion on their role in PCa showing their putative role in tumorigenesis and prognosis of PCa, based on public datasets retrieved from the Oncomine [20], CANCERTOOL [21] and cBioPortal [22].

3.2. Methods

3.2.1. Oncomine analysis

The expression levels of *STEAP1* gene in PCa was obtained from different human datasets available in the Oncomine Cancer Microarray database [20] (https://www.oncomine.org/). STEAPs messenger RNA (mRNA) expression was compared between cancer cases and normal patients' samples. Oncomine uses t-test statistics to compare the mean gene expression and to determine whether a gene is significantly over- or underexpressed in cancer cases compared to normal tissue. The analysis carried out provided the p-value, fold-change variation, and rank for STEAP1 transcript. The data obtained was compiled in table, which indicate the total number of samples in the dataset (cancer/normal samples), and the reference of the original publication of the data. Table also showed all the dataset found indicating statistically significant STEAP1 overexpression ($p < 0.05$). The search date was November 2020.

3.2.2. CANCERTOOL and cBioPortal analysis

CANCERTOOL resource [21] (http://web.bioinformatics.cicbiogune.es/CANCERTOOL/) was used to analyse the STEAP1 expression in several patients datasets with PCa. Genomic alteration analysis of STEAP1 mRNA expression in PCa across multiple cancer genetic datasets was carried out using the cBioPortal web resource [22] (https://www.cbioportal.org/). The mRNA expression z-scores relative to samples (log RNA Seq V2 RSEM) of each STEAPs transcripts were assessed using the cBioPortal website tool, with a z-score threshold ± 1.8. The prognostic value of STEAPs transcripts' expression in all the different human cancers was performed and analysed using the GraphPad Prism 8.0.1. software, using the results extracted from the cBioPortal for Cancer Genomics database. The search date was December 2020.

3.3. Results and discussion

3.3.1. STEAP1 in prostate cancer

STEAP family members have been implicated in several human cancers, including PCa. In non-neoplastic prostate, the expression of STEAP1 is higher when compared to the other three family members (STEAP2, STEAP3 and STEAP4) [16]. With the development of PCa, the overall expression of STEAPs is dysregulated or reversed. STEAP1 expression is highly increased in PCa in comparison with non-neoplastic tissues [6, 23]. Also, STEAP2 and STEAP4 expression increase during PCa development compared with non-neoplastic prostate tissue [24, 25]. In contrast to other STEAPs, Porkka *et al.* described that STEAP3 expression decreases with the onset of prostate malignancy [26]. Nowadays, public databases are widely used to corroborate basic research, and the expression levels of STEAP1 was analysed in non-tumoral and tumoral conditions using Oncomine, CANCERTOOL and cBioPortal resources. Oncomine analysis in seven of the thirteen datasets considered demonstrated that STEAP1 expression is significantly increased in prostate carcinoma, as indicated in Table III.1. In prostate adenocarcinoma was also verified a significantly increased of STEAP1 expression in one of the two datasets found in this platform (Table III.1.). Regarding pre-neoplastic PIN lesions, also it was shown an overexpression of STEAP1, and no significant difference was observed in BPH (Table III.1.). In agreement with this analysis, some studies showed the higher STEAP1 expression in malignant disorders of prostate, and its correlation with tumour aggressiveness [6, 23, 27]. STEAP1 staining intensity correlated with tumour grading, suggesting that it is associated with malignant transformation and tumour aggressiveness [23, 27]. In addition, it has also been shown that silencing STEAP1 expression can inhibit the proliferation of PCa cells promoting their apoptosis [9].

Table III.1. Analysis of STEAP1 expression in human disorders relative to prostate.

Gene	Expression level	Fold-change	Rank (Top %)	Dataset	#Samples	*p*-value	Reference
Prostate Carcinoma vs. Normal							
STEAP1	Overexpressed	2.092	1	Singh Prostate	102 (52 / 50)	1.88E-6	[28]
	Overexpressed	2.995	8	Welsh Prostate	34 (25/9)	2.42E-4	[29]
	Overexpressed	1.829	9	Yu Prostate	112 (65/23)	8.08E-4	[30]
	No difference	1.346	12	Holzbeierlein Prostate	54 (40/4)	0.264	[31]
	Ove-expressed	1.551	9	Liu Prostate	57 (44/13)	0.006	[32]
	Overexpressed	2.292	11	Tomlins Prostate	52 (30/22)	0.002	[33]
	Overexpressed	1.391	7	Taylor Prostate 3	185 (131/29)	4.79E-4	[34]
	Overexpressed	2.073	10	Grasso Prostate	122 (59/28)	4.50E-4	[35]
	No difference	1.419	15	Luo Prostate 2	30 (15/15)	0.061	[36]
	No difference	1.842	32	LaTulippe Prostate	35 (23/3)	0.206	[37]
	No difference	1.057	36	Lapointe Prostate	112 (60/40)	0.069	[38]
	No difference	1.212	49	Arredouani Prostate	21 (13/8)	0.172	[39]
	No difference	1.089	51	Varambally Prostate	19 (7/6)	0.376	[40]
Prostate Adenocarcinoma vs. Normal							
STEAP1	Overexpressed	2.221	9	Vanaja Prostate	40 (27/8)	9.61E-4	[41]

	No difference	-1.128	63	Wallace Prostate	89 (69/20)	0.261	[42]
Prostatic Intraepithelial Neoplasia vs. Normal							
STEAP1	Overexpressed	2.661	12	Tomlins Prostate	34 (13/22)	0.005	[33]
Benign Prostatic Hyperplasia Epithelial vs. Normal							
STEAP1	No difference	2.020	30	Tomlins Prostate	26 (4/22)	0.212	[33]

mRNA STEAP1 expression was compared between tumors and normal tissues using the Oncomine database. Expression level of STEAP1, fold-change variation, rank and datasets used are indicated. Statistically significant overexpression is highlighted by red filling.
#Samples - Total number of samples. Numbers in () mean the number of cancer cases vs. normal tissue.

Bioinformatics analysis using the CANCERTOOL software, which uses transcriptomics databases for the most prevalent types of cancers, corroborates these results. Data analysis indicated that STEAP1 expression is significantly increased in PCa cases (Figure III.1.A). Furthermore, the deregulated expression of STEAP1 observed of primary prostate tumours continues with the progression of disease to metastatic PCa, highlighting an increase in this last condition (Figure III.1.A). Another analysis using The Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010) dataset [34] from cBioPortal for Cancer Genomics with a z-score threshold for STEAP1 mRNA expression of ± 1.8, indicated that 17,3% (26 of 150) and 0,7% (1 of 150) of patients have high and low STEAP1 mRNA expression levels, respectively (Figure III.1.B). The aggressiveness and prognosis of PCa is traditionally determined by Gleason score, and a recent study by Burnell *et al.* (2019) showed that STEAP1 staining intensity in non-malignant tissue was weak, increasing slightly in Gleason 6 PCa samples, and strongly from Gleason 7 onwards [19].

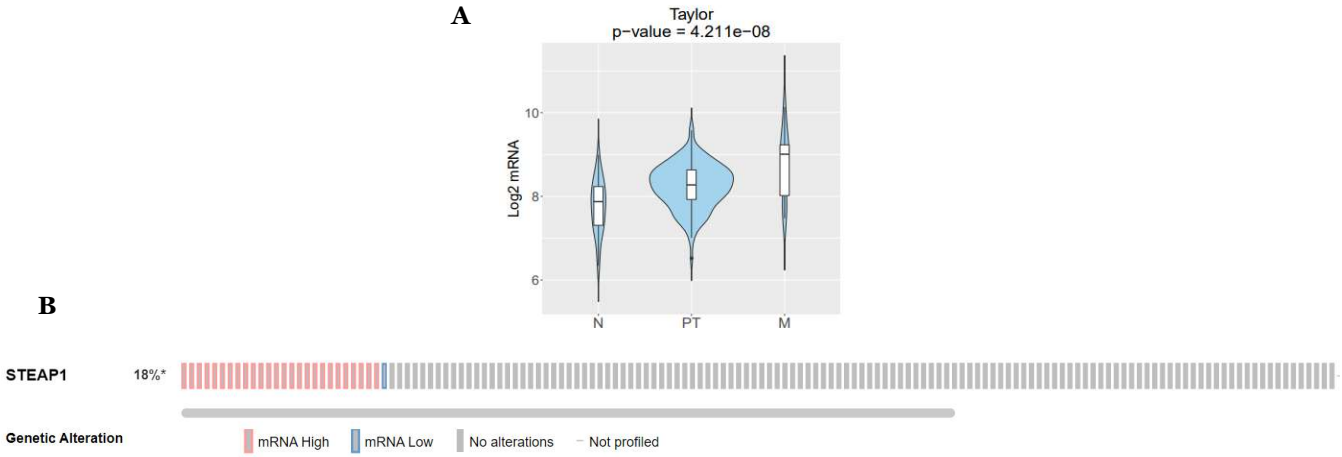

Figure III.1. STEAP1 expression in human PCa. **(A)** Using the CANCERTOOL resource, violin plots show the expression of STEAP1 in non-tumoral tissue (N), primary tumours (PT) and metastatic PCa (M) for Taylor *et al.* [34]. This dataset has a cohort of 179 patients and mean gene expression between the three groups was compared with an ANOVA test. **(B)** OncoPrint visual summary of variation on STEAP1 mRNA expression from Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010) dataset constituted for 240 samples, but only 150 samples have profiled.

3.3.2. Prognostic value of STEAP1 in prostate carcinoma

STEAP family members are relatively new proteins and their potential as prognostic biomarkers have been demonstrated in breast cancer [43], glioblastoma [44, 45], Ewing tumours [46], skin disorders [47] and PCa [19]. A recent study that explored the use of STEAP proteins as possible prognostic indicators in PCa showed that only STEAP4 was overexpressed, and significantly associated with relapse [19]. Strangely in this study, no significant difference was observed in STEAP1 expression with regards to relapse [19]. Thus, the relationship of STEAP1 overexpression with the survival of patients with this pathology was explored. Using Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010) dataset [34] from cBioPortal, indicated that the higher expression of STEAP1 is directly correlated with lower survival of PCa patients (Figure III.2.A, $p = 0.0315$), indicating a poor outcome. Inversely, higher expression of STEAP4 is directly correlated with higher overall survival when compared to the group with unaltered STEAP4 expression (Figure III.2.B, $p = 0.0394$). These findings suggest that STEAP1 and STEAP4 could be indicators of bad and good prognosis, and survival of PCa patients, respectively. However, a study previously referred [19] showed opposite results, indicating that patients with high STEAP4 expression relapsed more quickly than those with medium or low STEAP4 gene expression. Both studies have a relatively small number of samples (Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010) dataset = 43; Burnell study = 36), and this may be the reason for the difference obtained.

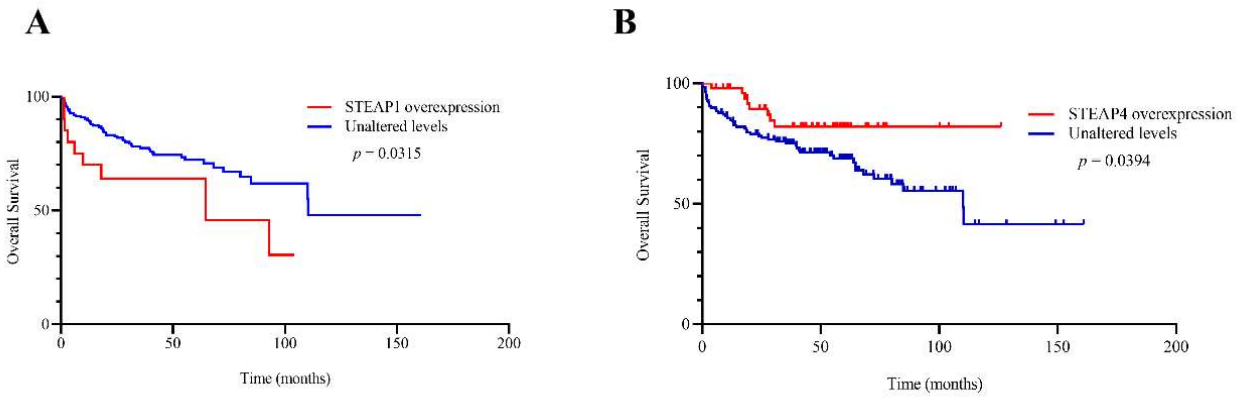

Figure III.2. Correlation between STEAP1 and STEAP4 gene expression and patients' overall inserted in Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010) dataset with 150 samples [34]. **(A)** Patients were stratified in two groups: STEAP1 overexpressed (red line, n=26) and unaltered expression levels (blue line, n=123). Survival analysis showed that high levels of STEAP1 transcript are correlated with lower survival. **(B)** Patients were stratified in two groups: STEAP4 overexpressed (red line, n=56) and unaltered expression levels (blue line, n=87), and high levels of STEAP4 transcript are correlated with higher survival.

To date, there are no studies evaluating the combined association of STEAP1 with different STEAP proteins regarding the prognostic value of PCa. A possible linear association between two variables, in this case, two genes, is analysed by correlation coefficient [48], where -1 and 1 indicate a negative and positive perfect linear relationship, respectively. Using primary prostate tumour tissue dataset from online MERAV database (Metabolic GEne RApid Visualizer, http://merav.wi.mit.edu/

[accessed on December 2020]) a strong positive correlation was found between STEAP1 and STEAP2 expression. This observation is in accordance with data of Grunewald *et al.* (2012), who noted that STEAP1 and STEAP2 seem to be significantly co-overexpressed across 59 cancer cell line entities [17], which suggests that STEAP2 is a likely candidate for heterodimerization with STEAP1. However, it is unknown if the combined expression of these two genes correlates with the overall survival of prostate cancer patients. Using the same dataset retrieved from the cBioPortal [34], it was found that only STEAP1 overexpression is associated with the overall survival of PCa patients when compared with unaltered expression group (Table III.2.). Concerning STEAP2, STEAP3 and STEAP4, no significant relationship was found between higher or low expression levels and the survival of PCa patients with unaltered expression (Table III.2.). These findings contradict the study of Burnell *et al.* (2019) that reported a statistically significant difference between STEAP4 overexpression and overall survival, indicating that patients with higher STEAP4 levels were more likely to relapse earlier than those with medium or low expression levels [19]. The source of the data, the number of patients per group, and the stratification of STEAPs expression may have caused the differences observed.

Table III.2. Expression of STEAP family members correlated with overall survival of prostate cancer patients.

STEAP1 overexpression (n = 20) ***versus***	Unaltered expression (n = 58)	$\boldsymbol{p < 0.024}$
	STEAP2 overexpression (n = 21)	$p < 0.108$
	STEAP3 underexpression (n = 24)	$p < 0.228$
	STEAP4 overexpression (n = 52)	$\boldsymbol{p < 0.017}$
STEAP2 overexpression (n = 21) ***versus***	Unaltered expression (n = 58)	$p < 0.962$
STEAP3 underexpression (n = 24) ***versus***	Unaltered expression (n = 58)	$p < 0.696$
STEAP4 overexpression (n = 52) ***versus***	Unaltered expression (n = 58)	$p < 0.753$

As shown in Table III.2. and considering the possible associations between STEAP1 protein with each other, patients with STEAP1 overexpression displayed significantly lower overall survival compared with patients with overexpression of STEAP4 (Figure III.3.). Furthermore, patients with STEAP1 overexpression presented poor survival outcome when compared with patients overexpressing STEAP4. This association suggest that the overexpression of STEAP4 can be a predictor for patients with PCa since they presented a better survival rate when compared with overexpressing STEAP1. Despite the study limitations, such as the low number of patients in some experimental groups, these findings provide a comprehensive analysis of the STEAP expression in prostate cancer and their application for predicting prognosis of PCa.

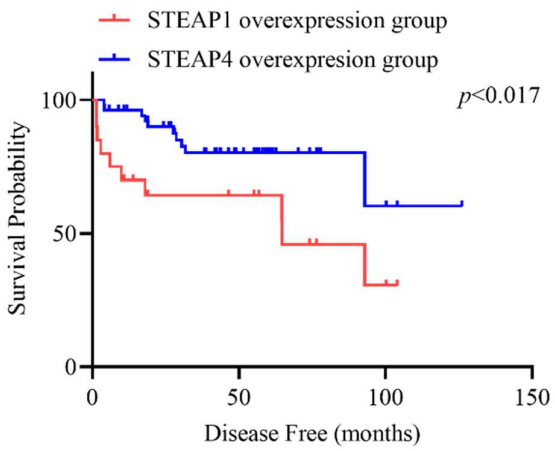

Figure III.3. Correlation between STEAP1 and STEAP4 overexpression of patients from Prostate Adenocarcinoma dataset [34], retrieved from cBioPortal. The samples were stratified into groups: overall survival of PCa patients with STEAP1 overexpression (n =20) *versus* overall survival of PCa patients with STEAP4 overexpression (n = 52) ($p < 0.017$), which was statistically different. Survival curves plotting fractional survival as a function of time was obtained using GraphPad Prisma 8.0.1.

3.4. Conclusion

The development of "omics" and bioinformatics tools allowed us to analyse how the STEAP genes are differentially expressed in human PCa, and their expression levels correlate with patients' overall survival rate. This approach is of paramount relevance considering the use of these proteins as therapeutic targets and/or biomarkers of prognosis. Analysing available scientific literature and multiple public databases show that STEAP1, STEAP2, and STEAP4 are overexpressed in prostate tumours. In contrast, STEAP3 is underexpressed. The differential expressions of these proteins appear to be of prognostic value, especially to STEAP1.

Interestingly, the expression of STEAP1 gene is already changed in benign lesions, overexpressing in prostatic intraepithelial neoplasia, which is the typical pattern in prostate carcinogenesis. This suggest that the deregulation of STEAPs expression levels may be involved in malignant transformation increasing the risk of cancer onset and development.

Overexpression of STEAP1 is associated with poor clinical outcomes, whereas STEAP4 offers better overall survival and progression-free survival. However, further investigations in large scale clinical cohorts are needed to definitively confirm the prognostic value of the STEAPs proteins, and the therapeutic potential of targeting STEAPs for prostate cancer.

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Chapter 4

PROMOTER DEMETHYLATION UPREGULATES STEAP1 GENE EXPRESSION IN HUMAN PROSTATE CANCER: *IN VITRO* AND *IN SILICO* ANALYSIS

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Promoter Demethylation Upregulates STEAP1 Gene Expression in Human Prostate Cancer: *In Vitro* and *In Silico* Analysis

Abstract

The Six Transmembrane Epithelial Antigen of the Prostate (STEAP1) is an oncogene overexpressed in several human tumours, particularly in prostate cancer (PCa). However, the mechanisms involved in its overexpression remain unknown. It is well known that epigenetic modifications may result in abnormal gene expression patterns, contributing to tumour initiation and progression. Therefore, this study aimed to analyse the methylation pattern of the STEAP1 gene in PCa versus non-neoplastic cells. Bisulfite amplicon sequencing of the CpG island at the STEAP1 gene promoter showed a higher methylation level in non-neoplastic PNT1A prostate cells than in human PCa samples. Bioinformatic analysis of the GEO datasets also showed the STEAP1 gene promoter as being demethylated in human PCa, and a negative association with STEAP1 mRNA expression was observed. These results are supported by the treatment of non-neoplastic PNT1A cells with DNMT and HDAC inhibitors, which induced a significant increase in STEAP1 mRNA expression. In addition, the involvement of HDAC in the regulation of STEAP1 mRNA expression was corroborated by a negative association between STEAP1 mRNA expression and HDAC4,5,7 and 9 in human PCa. In conclusion, our work indicates that STEAP1 overexpression in PCa can be driven by the hypomethylation of STEAP1 gene promoter.

Keywords: prostate cancer; STEAP1; DNA methylation; histone deacetylation; bioinformatics.

4.1. Introduction

Prostate cancer (PCa) is the second most common cancer worldwide, and it is the sixth leading cause of cancer death globally [1]. Prostatic carcinogenesis arises from precursor preneoplastic lesions that have the distinct molecular characteristics of normal prostate cells, and may give rise to localized cancer and eventually acquire the potential of invasion and metastasis [2]. The molecular alterations underpinning these cellular events include mutations, gene deletions and amplifications, chromosomal rearrangements and epigenetic modifications in key genes controlling cell fate [3, 4, 5]. The main epigenetic mechanisms are DNA methylation and histone modifications, the main function of which is to ensure the proper regulation of gene expression by changing the chromatin structure [6]. DNA methylation is catalysed by the family of enzymes known as DNA methyltransferases (DNMTs) and by histone modifications, including mainly histone acetylation, which is regulated by two groups of enzymes exerting opposite effects, histone acetyltransferases (HATs) and histone deacetylases (HDACs) [5, 6].

In tumour cells, hypermethylation is observed in promoters of specific genes, particularly tumour suppressor genes, and a global hypomethylation contributes to genomic instability and the

activation of oncogenes [7]. The disruption of epigenetic mechanisms may conduct the deregulation of gene expression, leading to tumour development and progression [6].

The Six Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1) gene was identified as overexpressed in PCa compared to non-malignant tissues [8]. The STEAP1 protein is mainly located in the plasma membrane of epithelial cells, particularly at cell–cell junctions where it may act as an ion channel or transporter protein [8]. In fact, it was reported that STEAP1 may allow the transport of small molecules between adjacent cells, indicating that STEAP1 may be involved in intercellular communication [9, 10]. Several studies have demonstrated the role of STEAP1 in cancer. In Ewing tumours, STEAP1 protein seems to promote cell growth and invasiveness by increasing intracellular reactive oxygen species levels. The oxidative stress that results from STEAP1 overexpression may enhance tumour aggressiveness through the activation of genes involved in cell proliferation and invasion [11]. Additionally, in gastric tumours, it was demonstrated that the upregulation of STEAP1 increased cell proliferation, migration and invasion [12]. In human ovarian and lung cancers, the STEAP1 gene is highly expressed, and it is associated with metastasis and epithelial–mesenchymal transition [12, 13, 14]. It was also demonstrated that the knockdown of STEAP1 expression on prostate tumour cells is associated with antitumor effects, such as enhanced apoptosis, and reduced proliferation, migration and invasion [12, 13, 14, 15]. Previously, it was shown that post-transcriptional and post-translational modifications may contribute to STEAP1 overexpression, as STEAP1 mRNA and protein stability are higher in neoplastic LNCaP cells than in non-neoplastic PNT1A cells [16]. However, these alterations do not justify the overexpression of STEAP1 in tumour cells, suggesting that other mechanisms may be involved. Thus, we hypothesized that epigenetic alterations of the STEAP1 gene result in its overexpression in PCa. The present study aimed to analyse the methylation pattern of the STEAP1 gene in PCa cells. Therefore, we analysed the methylation levels of the STEAP1 gene promoter in prostate cell lines and human samples of PCa. In addition, we analysed the association between methylation levels of STEAP1 and gene expression using publicly available datasets. Additionally, non-neoplastic PNT1A cells were used to demonstrate that demethylation of the STEAP1 gene, as well as a synergistic effect between DNA demethylation and inhibition of class I and II HDACs, induces STEAP1 overexpression.

4.2. Materials and Methods

4.2.1. Prostate Cell Lines and Treatments

The human LNCaP PCa cell line and the immortalized non-neoplastic PNT1A prostate epithelial cell line were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). LNCaP and PNT1A cell lines were cultured at 37 °C in a 5% CO2 atmosphere with RPMI 1640 phenol-red medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, USA). For the treatment with the demethylation and the histone deacetylation drug (epidrugs), approximately 3 × 105 PNT1A cells were seeded in six-well plates until reaching about 60% confluence. After that, PNT1A cells were exposed to one treatment with 5 µM 5-aza-dC (Sigma-Aldrich, USA) for 72 h, and the other with 5

μM 5-aza-dC for 48 h followed by 24 h with 1 μM TSA (Sigma-Aldrich, USA). In the control group, the medium was replaced by RPMI medium with DMSO for 72 h.

4.2.2. Patients and Tissue Sample Collection

Prostate tissue samples from five patients diagnosed with clinically localized PCa and primary treatment with radical prostatectomy, at the Portuguese Oncology Institute of Porto (IPO-Porto), were used in this study. Informed consent was obtained from all participants, according to institutional regulations. This study was approved by the institutional review board (Comissão de Ética para a Saúde-(IRB-CES-IPOFG-EPE 019/08)) of IPO-Porto.

4.2.3. DNA Extraction and Bisulfite Conversion

DNA extraction from PNT1A and LNCaP cells and clinical samples was carried out using the Gentra Puregene Cell Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To evaluate the methylation pattern, 1 μg of genomic DNA was modified using the EZ DNA Methylation-Gold kit (ZYMO RESEARCH, Irvine, CA, USA) according to the manufacturer's instructions. The modified DNA was stored at −80 °C.

4.2.4. Polymerase Chain Reaction (PCR) Amplification and Cloning Products

PCR reactions were performed using 200 ng of bisulfite-modified DNA in 25 μL reaction containing 1 U of TrueStart Hot Start Taq DNA Polymerase (Thermo Scientific, Waltham, MA, USA), 2.5 mM of $MgCl_2$, 10 mM dNTPs and 300 nM of each primer (−338 fw/+74 rv). The primer sequences and characteristics are described in Table IV.1. The PCR products were purified using NucleoSpin Gel and the PCR Clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's instructions, cloned into a pNZY28 vector and transformed in NZYStar Competent Cells. After heat shock, the cells were plated onto LB agar plates containing 100 μg/mL ampicillin, 80 μg/mL X-gal and 0.5 mM IPTG and incubated at 37 °C overnight. All components used in cloning and transformation were purchased from Nzytech, Portugal.

Table IV.1. Primer sequences and respective amplicon size used for amplification of the modified DNA from cell lines or human samples, and for the quantitative real-time PCR.

Primers	Accession Number	Sequence	Amplicon Size (bp)	Target
STEAP1_−338 fw ***STEAP1***_+74 rv	NC_000007.14	5′ AAAGTGTGATTTGGGAATGTTTTT 3′ 5′ TTTTAAGTTAGTTGTAGGTTTT 3′	412	Modified DNA
h***STEAP1***_619 fw h***STEA***P1_747 rv	NM_012449.3	5′ GGCGATCCTACAGATACAAGTTGC 3′ 5′ CCAATCCCACAATTCCCAGAGAC 3′	128	mRNA
h***GAPDH***_74 fw h***GAPDH***_149 rv	NM_002046.7	5′ CGCCAGCCGAGCCACATC 3′ 5′ CGC CCA ATA CGA CCA AAT CCG 3′	75	mRNA
h***β2M***_347 fw h***β2M***_439 rv	NM_004048.4	5′ ATGAGTATGCCTGCCGTGTG 3′ 5′ CAAACCTCCATGATGCTGCTTAC 3′	92	mRNA

4.2.5. DNA Sequencing

Colony screening was performed by PCR, with standard vector primers (T7 and M13), to confirm and amplify the DNA insert. Thus, white colonies were selected and incubated in 10 µL TE buffer at 100 °C for 2 min. Afterwards, 1 µL was used for PCR reaction with Speedy NZYTaq 2x Green Master Mix (Nzytech, Portugal). Sequencing of PCR products was carried out using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Fullerton, USA) according to the manufacturer's instructions. For each sample, the DNA sequencing reaction was performed in both strands with universal sequencing primers (T7 and M13). The sequencing products were separated on an automated capillary DNA sequencer (GenomeLabTM GeXP, Genetic Analysis System; Beckman Coulter, Fullerton, CA, USA). The sequencing data analysis was performed using the Clustal Omega software to align the PCR product sequences with the sequence of *STEAP1* gene modified DNA.

4.2.6. Datasets and Bioinformatic Analysis

Three PCa datasets (GSE52955, GSE76938 and GSE38240) were downloaded from the public repository NCBI Gene Expression Omnibus (GEO) databases (https://www.ncbi.nlm.nih.gov/geo/, assessed on 12 November 2021). All these datasets were based on the GPL13534 platform (Illumina HumanMethylation450 BeadChip). For each dataset, only the samples associated with prostate were selected. Methylation status was determined through the interactive web tool GEO2R, which allows the comparison of the two groups defined, pathologic condition and normal. The methylation status varies between 0 and 1, where 0 means a low degree of methylation and 1 indicates a high degree of methylation. The results were exported, and graphs were constructed with GraphPad Prism 8.0.1. The details of each dataset used in the present study are described in Table IV.2. The correlation of DNA methylation status with *STEAP1* mRNA expression was assessed using another public repository, the Prostate Adenocarcinoma (TCGA, Cell 2015) [17] dataset, available from cBioPortal for Cancer Genomic (https://www.cbioportal.org/, assessed on 12 November 2021). This dataset comprises data from 333 primary prostate carcinomas.

Table IV.2. Dataset used to evaluate the STEAP1 gene methylation profiling.

Dataset	Platform	Sample Type	Disease Condition (n)	Normal Tissue (n)	Reference
GSE52955	GLP13534	Frozen tissue	Cancer (25)	5*	18
GSE76938	GLP13534		Cancer (73)	Adjacent Tissue (63)	19
GSE38240	GLP13534		Cancer Metastasis (8)	4#	20

*Obtained from patients submitted to cystoprostatectomy due to bladder cancer. #Obtained from organ donor with no evidence of prostate cancer.

4.2.7. Total RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR (qPCR)

Total RNA extraction from PNT1A cells treated with epidrugs (5-aza-dC and TSA) was carried out using TRI reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. cDNA synthesis was performed using the NZY First-Strand cDNA Synthesis KIT (Nzytech, Portugal) according to the manufacturer's instructions, and qPCR was carried out to evaluate the expression

of *STEAP1* mRNA (h*STEAP1*) in PNT1A cells treated with 5-aza-dC alone and 5-aza-dC plus TSA. To normalize the expression of the *STEAP1* gene, human GAPDH (h*GAPDH*) and human beta-2-microglobulin (h*β2M*) primers were used as internal controls. qPCR reactions were carried out using 1 µL of cDNA synthesized in a 20 µL reaction containing 10 µL of Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific) and 300 nM of primer for each gene. Fold differences were calculated following the mathematical model proposed by Pfaffl using the formula: 2-(ΔΔCt) [21]. The primer sequence for each gene and respective amplicon sizes used in qPCR are described in Table IV.1.

4.2.8. Statistical Analysis

Data analysis was performed using GraphPad Prism version 8.0.1. for Windows (GraphPad Software, San Diego, CA, USA). The statistical significance of differences in *STEAP1* mRNA expression for the treatment with 5-aza-dC and TSA in non-neoplastic PNT1A cells was assessed by student's *t*-test. Significant differences were considered when $p < 0.05$ compared to control values. All experimental data are shown as mean ± SEM.

4.3. Results

4.3.1. Methylation Analysis of STEAP1 Gene in Neoplastic Tissue/Cells Compared with Non-Neoplastic Cells

To evaluate if DNA methylation plays a role in *STEAP1* gene regulation and if there are alterations in PCa, the methylation pattern of *STEAP1* was determined in PCa tissue samples, LNCaP and PNT1A cells. For this purpose, cytosine-rich regions of *STEAP1* gene promoter and primer design were performed using the Methyl Primer Express Software v1.0 (Applied Biosystems). This analysis indicated that part of the promoter region and the first exon of the *STEAP1* gene contain a large CpG island containing 24 CpG dinucleotides (Figure IV.1.), which could provide a large number of sites for the methylation modification of this gene.

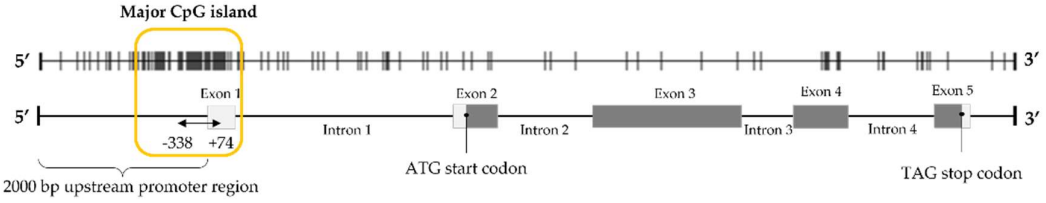

Figure IV.1. Schematic map of the predicted CpG island and indication of the region analysed by bisulfite genomic sequencing within the exon–intron structure of *STEAP1* gene. Vertical bars represent the CG dinucleotides. Parameters used to find CpG islands: minimum length of island: 300 bp; maximum length of island: 2000 bp; C+Gs/total bases > 50%; CpG observed/CpG expected > 0.6.

The methylation pattern of the *STEAP1* was analysed through the bisulfite sequencing PCR method from position −338 (promoter region) to +74 (exon 1). The analysis of the methylation pattern of *STEAP1* revealed some differences between neoplastic and non-neoplastic samples. In non-neoplastic PNT1A cells, the results show that some of the CpG dinucleotides located in the promoter region are methylated, but in PCa tissue or in neoplastic LNCaP cells the CpG dinucleotides are completely demethylated (Figure IV.2.).

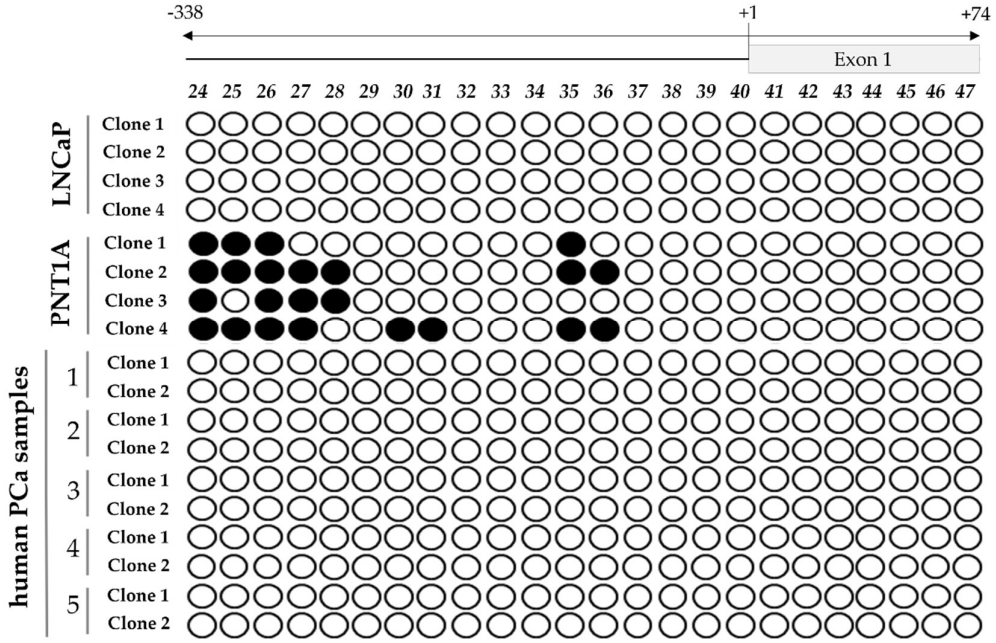

Figure IV.2. Evaluation of the *STEAP1* gene promoter methylation status in LNCaP and PNT1A cells, and human PCa samples. Each circle represents a CpG dinucleotide present in the CpG island identified, and on the top is the position of each CpG site relative to the beginning of the CpG island identified (● methylated CpG and ○ demethylated CpG). Each row represents a different clone.

4.3.2. Analysis of STEAP1 Promoter Methylation Levels in PCa and Normal Prostate Tissues from the GEO database

In order to support and validate the results above, the methylation pattern of *STEAP1* gene promoter methylation was evaluated in datasets from public PCa databases. The GSE52955, GSE76938, and GSE38240 datasets were downloaded and analysed by GEO2R online software. Four CpG probes on the *STEAP1* gene promoter were selected: cg15089950, cg19317433, cg19532731 and cg24286372 located on −314 to −193 bp, −285 to −163 bp, −301 to −180 bp and −250 to −129 bp upstream of the transcription start, respectively. As shown in Figure IV.3., there were significant differences in the levels of CpG methylation between prostate tumour and normal tissue. In three datasets analysed, the results reveal lower methylation levels of the *STEAP1* gene promoter in neoplastic tissue than in normal tissue.

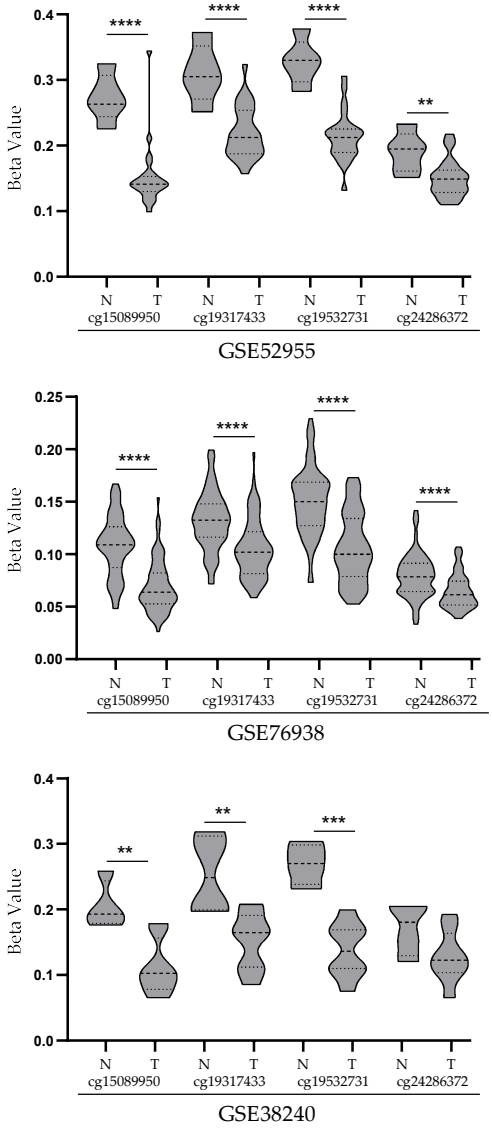

Figure IV.3. *STEAP1* promoter methylation levels (beta value) in GSE52955, GSE76938 and GSE38240 datasets. Each dataset represents methylation levels of *STEAP1* in prostate tumour tissue (T) and normal prostate tissue (N) for the four probes (cg15089950, cg19317433, cg19532731 and cg24286372). Violin plots were obtained with GraphPad Prism 8.0.1, and mean methylation levels between normal and tumour were compared with a student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$ and **** $p< 0.0001$.

4.3.3. Correlation between STEAP1 Gene Promoter Demethylation and Its Expression in PCa Tissue from the TCGA Database

In an attempt to better understand whether DNA methylation status may have an impact on *STEAP1* gene expression, the Prostate Adenocarcinoma dataset was analysed from The Cancer Genome Atlas (TCGA, Cell 2015) [17], accessed through the cBioPortal. In this dataset, differential

methylation levels between the unaltered (n = 286) and altered (n = 43) expression of the *STEAP1* gene were observed (Figure IV.4.A). Additionally, a negative correlation (Spearman coefficient of −0.42, and Pearson coefficient of −0.44) was observed between the methylation levels and the *STEAP1* gene expression (Figure IV.4.B).

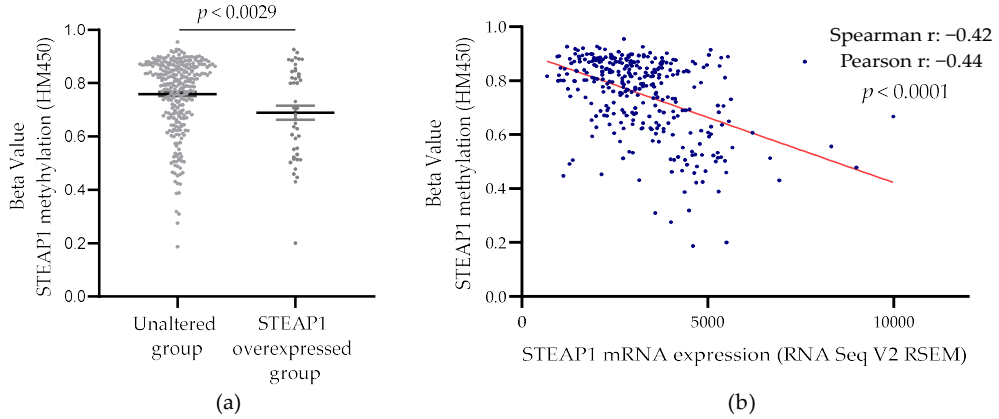

Figure IV.4. *STEAP1* methylation levels in PCa samples with normal and overexpression of *STEAP1* **(A)**, and correlation between methylation levels and *STEAP1* mRNA expression **(B)** in Prostate Adenocarcinoma (TCGA, Cell 2015) [17] dataset (n = 333). Statistical analysis used a student's t-test **(A)** and Spearman and Pearson correlation **(B)**.

4.3.4. Effect of Epigenetic-Modulating Drugs in STEAP1 Gene Expression in Non-Neoplastic PNT1A Cells

In order to support that epigenetic modifications contribute to the regulation of *STEAP1* expression, non-neoplastic PNT1A cells were used to evaluate the effect of DNMT and HDAC inhibitors (5-aza-dC and TSA, respectively) on *STEAP1* mRNA expression by qPCR. As shown in Figure IV.5., treatment with the demethylation agent 5-aza-dC induced a three-fold increase in *STEAP1* mRNA levels when compared to the control group (p < 0.01). Moreover, treatment with both epidrugs (5-aza-dC + TSA), which contributes to demethylation and histone hyperacetylation, induced a strong increase (15-fold variation relative to control, p < 0.001) in *STEAP1* mRNA levels.

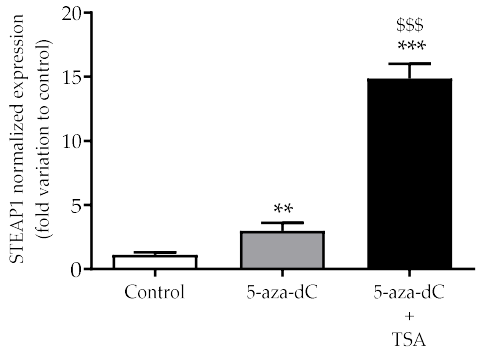

Figure IV.5. Effect of treatment with 5-aza-dC and TSA (DNMT and HDAC inhibitors, respectively) on *STEAP1* mRNA expression in PNT1A cells. Relative *STEAP1* mRNA expression was determined by qPCR analysis after normalization with the GAPDH and β2M housekeeping genes. Results are expressed as fold-variation relative to the control group. Error bars indicate mean ± SEM (n = 6). $^{**}p < 0.01$ and $^{***}p < 0.001$ relative to control. $^{\$\$\$}p < 0.001$ relative to 5-aza-dC.

4.3.5. Analysis of Co-Expression between STEAP1 mRNA Expression and HDACs

The TSA drug is a potent and specific inhibitor of HDAC classes I and II, which include ten isoforms (HDAC1–HDAC10) [22]. Considering that the results above suggest a negative association between histone deacetylation and *STEAP1* mRNA expression, we intended to analyse the association between *STEAP1* mRNA expression and *HDAC* mRNA expression, using the Prostate Adenocarcinoma (TCGA, Cell 2015) [17] dataset. Of the ten isoforms analyzed, *STEAP1* mRNA expression showed a positive association with *HDAC8*, as highlighted in Table IV.3. On the other hand, *STEAP1* mRNA expression was negatively associated with *HDAC4,5,7* and *9* (Table IV.3.).

Table IV.3. Association of *STEAP1* mRNA expression with class I and II HDACs, showing spearman's rank for each comparison and the respective *p*-value. The associations with significant values are highlighted in bold.

***STEAP1* Correlated**	**spearman's Correlation**	***p*-Value**
HDAC1	−0.011	0.834
HDAC2	−0.005	0.929
HDAC3	0.089	0.105
HDAC4	**−0.242**	**8.07e-6**
HDAC5	**−0.305**	**1.35e-8**
HDAC6	0.049	0.366
HDAC7	**−0.336**	**3.31e-10**
HDAC8	**0.255**	**2.34e-6**
HDAC9	**−0.294**	**4.53e-8**
HDAC10	−0.064	0.245

4.4. Discussion

The methylation pattern observed in normal tissues undergoes relevant modifications in cancer, leading to changes in the regulation of the transcription of numerous genes [23]. Recent studies have shown that *STEAP1* acts as an oncogene, showing that its overexpression in several

human cancers contributes to tumour progression and aggressiveness through the inhibition of apoptosis and stimulation of cell proliferation, invasion and epithelial–mesenchymal transition [11, 12, 13, 14, 15]. Although the stability of the *STEAP1* gene and protein is higher in LNCaP PCa cells than in PNT1A cells, contributing to STEAP1 overexpression, other mechanisms underlying its overexpression in cancer must be involved. As epigenetics has been pointed out as a major hallmark in cancer, affecting genes involved in all cellular pathways [24, 25], our main goal was to assess whether epigenetic mechanisms are involved in the regulation of the STEAP1 gene expression in PCa, and if there are changes between normal and PCa cells.

As a first approach, two different cell lines were chosen, neoplastic LNCaP and non-neoplastic PNT1A, which express high and low levels of STEAP1, respectively [16], to analyse the methylation status of the *STEAP1* gene. Furthermore, five human PCa samples were also analysed. The major CpG island located from position −338 of the promoter region to position +74 of the first exon of *STEAP1* revealed differences in the methylation status. While in non-neoplastic PNT1A cells, the CpG dinucleotides near the transcription start site are methylated, in neoplastic LNCaP cells and PCa samples these are demethylated. This result suggests that demethylation of the *STEAP1* gene promoter may lead to its overexpression in PCa. Hypomethylation/demethylation-dependent overexpression of several oncogenes has already been described in several cancer types. In PCa cells, Wingless-related MMTV integration site 5A, S100 calcium-binding protein P and cysteine-rich protein 1 were found to be hypomethylated [26]. hsa-miR-191 was also hypomethylated in 63% of hepatocellular carcinoma tissue samples, associated with its increased expression [27], and demethylation of the miR-128a promoter region drives the upregulation of miR-128a expression in the human T lymphocyte Jurkat cell line [28].

The possibility that demethylation of the *STEAP1* gene promoter may be involved in its overexpression in PCa was also corroborated by a bioinformatic analysis, using public datasets from the GEO database and cBioPortal platform. This analysis showed that CpG dinucleotides in the *STEAP1* gene of PCa samples have low levels of methylation, negatively correlated with *STEAP1* mRNA expression. These results suggest, once more, that demethylation of the *STEAP1* gene promoter may contribute to its overexpression in PCa. Our results are in line with other studies that also analysed genes of the STEAP family, which showed that epigenetic alterations may be responsible for changes in gene expression. Using combined analysis of GEO and TCGA datasets, it was shown that *STEAP3* is hypomethylated and consequently upregulated in glioblastoma, and may be used as a potential methylation-based prognostic biomarker. In addition, the authors suggested that *STEAP3* is a potential target for glioblastoma treatment [29]. Another gene that was also reported to be deregulated due to epigenetic changes is the *STEAP4* gene. Tamura et al. showed no CpG methylation in the *STEAP4* promoter region in LNCaP cells, suggesting that demethylation may activate the expression of the *STEAP4* gene in PCa cells [30]. On the other hand, a more recent study showed that *STEAP4* was hypermethylated and downregulated in the hepatocellular carcinoma when compared to the non-tumour liver tissues [31]. This study also demonstrated that expression of *STEAP4* was restored with a DNA methyltransferase (DNMTs) inhibitor and a histone deacetylase (HDACs) inhibitor, suggesting that aberrant DNA methylation suppressed the expression of the *STEAP4* gene [31].

It is well established that there is a crosstalk between DNA methylation and histone modifications in the control of gene expression [32, 33]. Aberrant CpG-island methylation by recruiting DNMTs and HDACs might be directly targeted by consequence of gene expression alterations [34]. Therefore, it was hypothesized that treatment with both DNMT and HDAC inhibitors might enhance *STEAP1* expression in cells with reduced levels of STEAP1. Thus, the non-neoplastic PNT1A cell line was used to evaluate the effects of 5-aza-dC (DNMT inhibitor) plus TSA (HDAC inhibitor) on the expression of STEAP1. The results indicate an increase in *STEAP1* gene expression in response to treatment with DNMT and HDAC inhibitors, suggesting a synergistic effect of combined hypomethylation and histone hyperacetylation.

HDACs are enzymes that remove acetyl groups from the tails of histones, resulting in a more closed chromatin structure and repression of gene expression [22, 35, 36]. Class I of the HDAC family includes HDAC1, 2, 3 and 8; class II includes HDAC4, 5, 6, 7, 9 and 10 [36]. To the best of our knowledge, the role played by both HDAC class I and II enzymes in the regulation of *STEAP1* gene expression is still unknown. So, we aimed to explore the association between HDAC and *STEAP1* gene expression. Our results reveal a positive association of *HDAC8* and a negative association of *HDAC4,5,7* and *9* with *STEAP1* mRNA expression. In fact, the HDAC family modulates several genes involved in cancer development/progression via angiogenesis, cell adhesion, migration and invasion [22]. Some studies support our results, showing that increased expression of autotaxin in PCa cell lines is mediated by the downregulation of *HDAC7* and *HDAC3* [37]; additionally, *HDAC5* is downregulated in human cancer, namely PCa [38], and decreased expression of *HDAC4* increases the growth of PCa cells [39]. On the other hand, there are also studies showing an overexpression of HDAC in several types of cancer, suggesting the use of HDAC inhibitors as a promising class of compounds for cancer treatment [40, 41, 42, 43]. Thus, the effects that can be triggered by these inhibitors on oncogenes should not be ignored and more studies are required to clarify their effectiveness in cancer treatment.

To summarize, this study showed that the *STEAP1* gene is hypomethylated in PCa cells when compared to non-neoplastic cells, contributing to the overexpression of *STEAP1* in PCa. Furthermore, our results suggest a putative involvement of *HDCA4,5,7* and *9* in the regulation of *STEAP1* in PCa. Considering the complexity of the mechanisms associated with HDAC, more studies are required to clarify their role in *STEAP1* regulation, as well as to elucidate this association with PCa development and progression.

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Informed Consent Statement: Informed consent was obtained from all participants involved in the study, according to institutional regulations.

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Chapter 5

QUANTITATIVE PROTEOME ANALYSIS REVEAL A NEW ROLE FOR STEAP1 IN PROSTATE CANCER LNCAP CELLS

This chapter was submitted as:

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Proteomic analysis of STEAP1 Knockdown in human LNCaP prostate cancer cells

Abstract

Prostate cancer (PCa) continues to be one of the most common cancers in men worldwide. The six transmembrane epithelial antigen of the prostate 1 (STEAP1) protein is overexpressed in several types of human tumours, particularly in PCa. Our research group has demonstrated that STEAP1 overexpression is associated with PCa progression and aggressiveness. Therefore, understanding the cellular and molecular mechanisms triggered by STEAP1 overexpression will provide important insights to delineate new strategies for PCa treatment. In the present work, a proteomic strategy was used to characterize the intracellular signalling pathways and the molecular targets downstream of STEAP1 in PCa cells. A label-free approach was applied using an Orbitrap LC-MS/MS system to characterize the proteome of STEAP1-knockdown PCa cells. More than 6700 proteins were identified, of which a total of 526 proteins were found differentially expressed in scramble siRNA versus STEAP1 siRNA (234 proteins up-regulated and 292 proteins down-regulated). Bioinformatics analysis allowed us to explore the mechanism through which STEAP1 exerts influence on PCa, revealing that endocytosis, RNA transport, apoptosis, aminoacyl-tRNA biosynthesis, and metabolic pathways are the main biological processes where STEAP1 is involved. By immunoblotting, it was confirmed that STEAP1 silencing induced the up-regulation of cathepsin B, intersectin-1, and syntaxin 4, and the down-regulation of HRas, PIK3C2A, and DIS3. These findings suggested that blocking STEAP1 might be a suitable strategy to activate apoptosis and endocytosis, and diminish cellular metabolism and intercellular communication, leading to inhibition of PCa progression.

Keywords: STEAP1; endocytic pathway; intercellular communication; proteomics; label-free quantification; prostate cancer.

5.1. Introduction

Prostate cancer (PCa) is one of the most common cancers among men worldwide and represents the sixth leading cause of cancer deaths [1]. The progression of PCa is characterized by the transition from the androgen-responsiveness stage to an androgen-insensitive stage, which represents a poor prognosis [2]. Despite the overall advances in PCa management, clinical PCa therapy requires better biomarkers and treatment. Taking into account that some oncoproteins are the drivers of most cellular responses and therapeutic targets for drug delivery, an analysis of the proteome of PCa cells in response to knockdown of oncoproteins might lead to clarifying the molecular mechanisms underlying PCa progression, as well as to identify putative biomarkers.

The Six-transmembrane epithelial antigen of the prostate 1 (STEAP1) is a cell surface protein that is overexpressed in many human cancers, namely in PCa [3–5]. In normal tissue, STEAP1 expression is almost exclusively restricted to the prostate [4], making it a very interesting immunotherapeutic target in PCa and a promising biomarker of prognosis [6, 7]. Due to its location at the plasma membrane of epithelial cells, there are several studies suggesting that STEAP1 may

play an important role as an ion channel or transporter protein at the cell–cell junctions where it may be involved in cell-cell communication [4, 8]. In addition, several studies also unrevealed the role of STEAP1 in cancer cells showing that its overexpression inhibits apoptosis, enhances cell proliferation and invasion, and induces epithelial to mesenchymal transition, ultimately contributing to tumour progression and aggressiveness [9–15]. Briefly, STEAP1 participates in molecular transport, cell growth, immune responses, and several biological processes. However, it is necessary to better understand the mechanisms by which STEAP1 performs its functions on processes underlying tumorigenesis, cancer cell migration, and metastasis.

Proteomic techniques are used to estimate the complete set of proteins in cells, tissues, or biofluids [16, 17]. Early studies were mostly based on two-dimensional gel electrophoresis followed by MALDI-TOF mass spectrometric analysis, but they were capable of assessing a limited number of proteins [18–20]. More reproducible and sensitive mass-spectrometry-based methods, along with improved computational analysis, are currently used for high-throughput and comprehensive proteomics analysis. Furthermore, quantitative proteomics offers an additional information layer to understand molecular events in cancer by identifying and comparing qualitatively several proteins [21]. Therefore, and considering that STEAP1 is an important oncoprotein involved in PCa progression, this study aimed to find expression changes in the proteome of PCa LNCaP cells induced by STEAP1 knockdown using a label-free quantitative (LFQ) method. Our outcomes provide an opportunity to advance our knowledge of the mechanistic role of STEAP1 in PCa and provide novel evidence for the development of new treatments based on targeting STEAP1-relevant signalling pathways.

5.2. Experimental Procedures

5.2.1. Cell Culture and STEAP1 gene silencing

The human prostate adenocarcinoma cell line (LNCaP) was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). LNCaP cells were maintained in RPMI-1640 phenol red medium, supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and 1% penicillin/ streptomycin (Gibco, CA, USA) at 37 °C in an atmosphere equilibrated with 5% CO2. To experiments, LNCaP cells were seeded in T75 flasks with an antibiotic-free medium. At 40% confluence, LNCaP cells were transfected with 20 nM of a small interfering RNA (siRNA) targeting STEAP1 (s226093, Ambion, USA) or scramble siRNA (4390846, Ambion, USA) for 72 h, using Lipofectamine 3000 (Thermo Fisher Scientific, USA) following the manufacturer's instructions. At the end of experiment, which was performed in four independent assays, cells were trypsinized and harvested until further use.

5.2.2. RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR was carried out to confirm the STEAP1 gene knockdown in the LNCaP cells. Total RNA was obtained using TRIzol Reagent (GRiSP, Oporto, Portugal) according to the manufacturer’s

instruction, and the RNA pellet was resuspended in 50 µL of DEPC treated-water. The expression levels of STEAP1 and the percentage of STEAP1 gene knockdown were determined using Power SYBR Green RNA-to-CT, 1-Step Kit (Applied Biosystems, USA) on the CFX connect Real-time system (Bio-Rad, Hercules, USA). qPCRs were performed with 0,2 µL of RNA in 10 µL of total reaction with specific primers for STEAP1 (sense: 5' GGC GAT CCT ACA GAT ACA AGT TGC 3' and anti-sense: 5' CCA ATC CCA CAA TTC CCA GAG AC 3') and beta-2-microglobulin (β2M, sense: 5′ ATG AGT ATG CCT GCC GTG TG 3′ and anti-sense: 5′ CAA ACC TCC ATG ATG CTG CTTAC 3′). The amplified PCR fragments were analysed by melting curves. β2M housekeeping was used as internal control to normalize gene expression. Fold differences were calculated following the mathematical model proposed by Pfaffl [22]. Data processing and statistical analyses (t-test, $p < 0.05$) were performed using GraphPad Prism software (version 8, San Diego, USA).

5.2.3. Protein extraction and immunoblotting

LNCaP cells were homogenized in the appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris) supplemented with 10% phenylmethylsulfonyl fluoride (PMSF) and 1% protease cocktail. The total protein extract was recovered after a 14,000-rpm centrifugation for 20 min at 4°C. Protein quantification was measured using the Pierce 660 nm protein assay reagent (Thermo Scientific, USA). Approximately twenty micrograms of total protein were resolved on 10% TGX Stain-Free polyacrylamide gels (BioRad, USA) and then transferred into a PolyVinylidene DiFluoride (PVDF) membrane (BioRad, USA) using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA). After blockage with 5% milk solution, membranes were incubated overnight at 4 °C with primary antibodies. After that, membranes were incubated with secondary antibodies. Once membranes were rinsed thoroughly within TBS-T, protein bands were visualized using the ChemiDoc MP Imaging System (Bio-Rad) after the incubation with Clarity Western ECL Substrate (BioRad, USA). The detection and normalization volume of the bands was done using the Image Lab 5.1 software (Bio-Rad, USA) following the user guide. Briefly, a multichannel image was opened and configured with two channels: channel 1 for the target protein blot and channel 2 for the stain-free gel image. The Analysis Tool box was used to detect bands and lanes, which were adjusted as necessary. The normalization channel was set as the stain-free image, and the normalized volumes are displayed in the Analysis Table on the Tool bar. The intensity value of the target protein band was adjusted to account for variations in the total protein load on the gel. Data processing and statistical analyses (t-test, $p < 0.05$) were performed using GraphPad Prism software (version 8, San Diego, USA). Primary antibodies used were as follows: rabbit anti-STEAP1 antibody (1:1000, D8B2V, Cell Signaling Technology, USA), mouse anti-HRas antibody (1:500, F235: sc-29 Santa Cruz, USA), mouse anti-cathepsin B (1:1000, H-5: sc-365558, Santa Cruz, USA), mouse anti-PI 3-kinase C2α (1:500, G-5: sc-365290, Santa Cruz, USA), mouse anti-intersectin 1 (1:1000, 29: sc-136242, Santa Cruz, USA), mouse anti-DIS3 (1:1000, H3: sc-398663, Santa Cruz, USA) and mouse anti-syntaxin 4 (1:1000, QQ-17: sc-101301, Santa Cruz, USA). Secondary antibodies were anti-rabbit IgG HRP (1:10000, #7074, Cell Signaling Technology, USA) or anti-mouse-IgGκ BP-HRP (1:10000, sc-516102; Santa Cruz Biotechnology, USA).

5.2.4. Proteomic Acquisition and analysis

To identify proteins differentially expressed between two experimental groups (STEAP1 siRNA condition and scramble siRNA condition), one-hundred micrograms of total protein of each group (four biological replicates) were extracted as previously described (section 5.2.3). For proteomic analysis, each sample was processed following the solid-phase-enhanced sample-preparation protocol described in [23]. Enzymatic digestion was performed with trypsin/LysC (2 micrograms) overnight at 37 °C at 1000 rpm. The resulting peptide concentration was measured by fluorescence. Each sample was analyzed by nanoLC-MS/MS using an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) at i3s Proteomics Scientific Platform (Porto, Portugal), as previously described by Osório and co-workers [24]. For monitoring mass spectrometry instrument performance, regular quality control was performed by a parallel injection of 100 ng of mass spec-compatible human peptide standard (V6951, Promega), as described in [25]. Five hundred nanograms of peptides were loaded on a 50 cm × 75 µm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific, Bremen, Germany) with 0.1% formic acid (FA) (mobile phase A) at 10 µL/min. After 3 min loading, peptides were separated at a flow rate of 250 nL/min using a 150 min gradient ranging from 2.5 % to 99% mobile phase B (80 % acetonitrile (ACN) in 0.1% FA) and this condition was maintained for 10 min. From 170 to 180 min, the column was equilibrated to return to initial conditions (0.1% FA). Data acquisition was performed using a data-dependent method, in full scan positive mode, scanning 380 to 1580 m/z. Survey scans were acquired at a resolution of 70,000 at m/z 200, with an automatic gain control (AGC) target of 3 × 106 and a maximum injection time of 120 ms. The top 10 most intense ions from each MS1 scan were selected and fragmented (normalized collision energy of 27%). Resolution for HCD spectra was set to 35,000 at m/z 200, with an AGC target of 2 × 105 and a maximum ion injection time in 110 ms. Isolation of precursors was performed with a window of 2.0 m/z and with an exclusion duration of 45 s. Precursor ions with unassigned, single, or eight and higher charge states were excluded.

5.2.5. Data and statistical analysis and functional enrichment

The raw data file was searched against Homo Sapiens UniProtKB reviewed database (downloaded in July 2020, 75,069 entries) using two search engines (MSPepSearch and Sequest HT) within Proteome Discoverer v2.5 software (Thermo Scientific, Bremen, Germany) [24]. Search parameters were set as follows: cysteine carbamidomethyl was defined as fixed modification; methionine oxidation (M), N-term acetylation, asparagine (N) and glutamine (Q) deamidation, Gln→pyro-Glut and the loss of methionine and methionine+acetyl as variable modifications. Mass tolerances were set at 10 ppm for precursor and 0.02 Da for-fragment ions, whereas the maximum of missed cleavage was set to 2. The false discovery rate (FDR) was calculated using the processing node Percolator (maximum delta Cn 0.05; decoy database search target) and the validation of proteins, peptides, and peptide spectral matches (PSMs) peptides with an FDR≤1%. Precursor ion quantitation was also performed in Proteome Discoverer using the “Minora” feature in the processing method and the "Feature Mapper” and “Precursor Ions Quantifier” nodes in the consensus step. The

following parameters were set in the "Precursor Ions Quantifier" node: unique+razor peptides were used for quantitation, precursor abundance was based on intensity, normalization mode was based on the total peptide amount, and Min. # replicate features in the sample group was set to 50%. The Feature Mapper node was used to create features from unique peptide-specific peaks by applying a chromatographic retention time alignment with a maximum shift of 10 min and 10 ppm of maximum mass tolerance to allow the mapping of features from different sample files. The table of non-normalized abundances was exported from Proteome Discoverer only considering the abundance values of the master protein (i.e., one quantitative value per protein group).

Perseus software platform (version 1.6.10.0) [26] was used to analyze and quantify differentially expressed proteins between LNCaP scramble siRNA and LNCaP STEAP1 siRNA groups. For this purpose, the raw abundances were normalized by dividing the intensity of each protein by the total protein intensity in that specific sample and multiplying it by the median of the total intensity of all samples. Contaminant proteins and proteins quantified with a single peptide were removed from the statistical analysis. Data were logarithmized (Log2), filtered by valid values (min 70% of valid values), and missing values were imputed with random numbers from a normal distribution (width=0.3, shift=1.8). Two-sample t-tests were performed for differentiating the two groups in terms of protein expression. A permutation-based method was used to correct for multiple hypothesis testing with the number of randomizations set to 250 and a false discovery rate (FDR) < 1% (q-value).

Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed proteins were performed using Cytoscape software with the ClueGo plugins (version 2.5.7) [27] and the Database for Annotation, Visualization and Integrated Discovery website (DAVID, https://david.ncifcrf.gov/) [28]. The network specificity was adjusted to medium, and the GO levels were set from 3 (Min) to 8 (Max level) to perform the functional enrichment considering the more representative pathways. Enrichment/depletion (two-side hypergeometric test) and multiple hypothesis testing correction Benjamini-Hochberg were applied for statistical analysis. To discover the biological protein-protein associations, the proteins were analysed in Search Toll for the Retrieval of Interacting Genes (STRING, https://string-db.org/, version 11) [29] to obtain the protein-protein interaction networks.

5.3. Results

5.3.1. Validation of STEAP1 knockdown efficiency in LNCaP cells

STEAP1 gene expression in LNCaP cells was silenced using a siRNA technology. Gene silencing was evaluated for 72 h after transfection through qPCR and western blot (Figure V.1.). STEAP1 mRNA and protein expression levels were significantly lower in the STEAP1 siRNA group, 67.2% ± 0.04 and 76.4% ± 0.05, respectively.

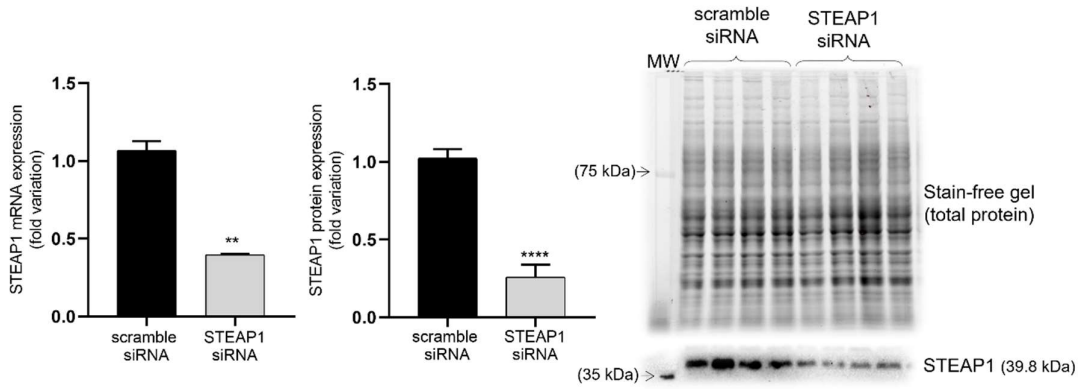

Figure V.1. STEAP1 mRNA and protein expression levels in neoplastic human LNCaP prostate cancer cells after transfection with siRNA targeting STEAP1 or scramble siRNA for 72 h of transfection. Relative STEAP1 mRNA expression was determined by qPCR after normalization with the β2M housekeeping gene. Normalization of the STEAP1 protein input was performed using the stain-free gel. Results are expressed as fold variation expression to the scramble siRNA group and representative blots are shown in the right panel. Error bars indicate mean ± SEM (n=4). $^{**}p< 0.01$; $^{****}p< 0.0001$.

5.3.2. Identification of differentially expressed proteins in LNCaP cells knocked-down for STEAP1

Using an LFQ approach with Orbitrap LC-MS/MS, a total of 6703 proteins were identified with 65102 peptides, of which 59989 correspond to unique peptides (Supplementary Table 1). After the removal of common contaminants, the use of at least three unique peptides and the FDR set to ≤ 0.01, the number of identified proteins decreased to 4701 (Supplementary Table 2). Comparing STEAP1 siRNA versus scramble siRNA, 526 proteins were found to be differentially expressed (q-value<1 %), of which 292 proteins were upregulated and 234 downregulated (Supplementary Table 3). Among the global list of 526 differentially expressed proteins, the top 30 most up and downregulated proteins are presented in Table V.1.

Table V. Top 30 up and downregulated proteins in STEAP1 siRNA comparatively to scramble siRNA samples. A positive fold change logarithmized value indicates an increase in protein abundance (highlighted in light red), while a negative fold change logarithmized value represent protein with decreased expression (highlighted in light green). Further details can be found in Supplementary Table 3.

Accession No.	Protein Name	Gene Symbol	Log2 Fold-Change	# Unique Peptides	Student 't T-test q-value
O95816	BAG family molecular chaperone regulator 2	BAG2	6,331	3	0,006
Q9Y4C2	TRPM8 channel-associated factor 1	TCAF1	6,316	9	0,006
Q9BTT6	Leucine-rich repeat-containing protein 1	LRRC1	5,712	4	0,004
Q9NVA1	Ubiquinol-cytochrome-c reductase complex assembly factor 1	UQCC1	5,406	3	0,004
P51689	Arylsulfatase D	ARSD	5,252	3	0,006
Q08499	cAMP-specific 3'.5'-cyclic phosphodiesterase 4D	PDE4D	5,023	3	0,004
P11166	Solute carrier family 2. facilitated glucose transporter member 1	SLC2A1	4,982	5	0,003
Q7Z422	SUZ domain-containing protein 1	SZRD1	4,869	4	0
Q13496	Myotubularin	MTM1	4,803	3	0
Q96MW1	Coiled-coil domain-containing protein 43	CCDC43	4,561	3	0,007
Q9NRD5	PRKCA-binding protein	PICK1	4,457	5	0,004
Q9NRL2	Bromodomain adjacent to zinc finger domain protein 1A	BAZ1A	4,276	3	0,008
P38935	DNA-binding protein SMUBP-2	IGHMBP2	4,010	3	0,001
O95425	Supervillin	SVIL	3,921	5	0
Q96PU8	Protein quaking	QKI	3,571	8	0,008
Q9BXW6	Oxysterol-binding protein-related protein 1	OSBPL1A	3,531	3	0,005
Q9NRY5	Protein FAM114A2	FAM114A2	3,507	15	0
Q15811	Intersectin-1	ITSN1	3,333	18	0,005
Q96RU3	Formin-binding protein 1	FNBP1	3,310	6	0,006
Q9UMY1	Nucleolar protein 7	NOL7	3,206	3	0,001
Q9BQE5	Apolipoprotein L2	APOL2	3,200	5	0,006
Q08722	Leukocyte surface antigen CD47	CD47	3,145	4	0,010
Q15642	Cdc42-interacting protein 4	TRIP10	3,117	3	0,006
Q8N5M1	ATP synthase mitochondrial F1 complex assembly factor 2	ATPAF2	3,098	9	0,000
O76041	Nebulette	NEBL	3,060	3	0,005
Q9H974	Queuine tRNA-ribosyltransferase accessory subunit 2	QTRT2	3,043	4	0,007
Q86TB9	Protein PAT1 homolog 1	PATL1	2,984	6	0
Q96D71	RalBP1-associated Eps domain-containing protein 1	REPS1	2,937	9	0,007
P55212	Caspase-6	CASP6	2,803	3	0,005
Q5GLZ8	Probable E3 ubiquitin-protein ligase HERC4	HERC4	2,802	11	0
Q8NDA8	Maestro heat-like repeat-containing protein family member 1	MROH1	-2,523	4	0
O60282	Kinesin heavy chain isoform 5C	KIF5C	-2,575	5	0,004
Q4J6C6	Prolyl endopeptidase-like	PREPL	-2,592	4	0,001
P28702	Retinoic acid receptor RXR-beta	RXRB	-2,637	3	0,006
Q9UPP1	Histone lysine demethylase PHF8	PHF8	-2,663	3	0
Q69YU5	Uncharacterized protein C12orf73	C12orf73	-2,680	3	0
A3KMH1	von Willebrand factor A domain-containing protein 8	VWA8	-2,796	16	0,003
Q9BY49	Peroxisomal trans-2-enoyl-CoA reductase	PECR	-2,805	7	0,006
Q9BZC7	ATP-binding cassette sub-family A member 2	ABCA2	-3,006	3	0
O95758	Polypyrimidine tract-binding protein 3	PTBP3	-3,070	7	0
Q9BYV8	Centrosomal protein of 41 kDa	CEP41	-3,092	6	0,010
Q6NUK4	Receptor expression-enhancing protein 3	REEP3	-3,114	3	0,006
Q9HBH1	Peptide deformylase. Mitochondrial	PDF	-3,167	7	0,001
Q07817	Bcl-2-like protein 1	BCL2L1	-3,174	3	0,006
Q9H4K7	Mitochondrial ribosome-associated GTPase 2	MTG2	-3,179	3	0,001
O94875	Sorbin and SH3 domain-containing protein 2	SORBS2	-3,372	4	0
Q96E29	Transcription termination factor 3. mitochondrial	MTERF3	-3,398	3	0,005

The differences between the two experimental groups also can be observed in the Volcano plot as depicted in Figure V.2. Of the 89 proteins that showed the highest levels of expression in LNCaP cells knocked-down for STEAP1 (coloured in red, FDR < 0.001), caspase-6 (CASP6), intersectin-1 (ITSN1), histidine-tRNA ligase (HARS), nucleolar protein 7 (NOL7), focal adhesion kinase 1 (PTK2), solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1), leucine-rich repeat-containing protein 1 (LRRC1), protein FAM114A2 (FAM114A2), ubiquinol-cytochrome-c reductase complex assembly factor 1 (UQCC1), BAG family molecular chaperone regulator 2 (BAG2), huntingtin (HTT), leukocyte surface antigen CD47 (CD47), apolipoprotein L2 (APOL2), SUZ domain-containing protein 1 (SZRD1), DNA-binding protein SMUBP-2 (IGHMBP2) and arylsulfatase D are highlighted in Fig. 2. On the other hand, of the 65 proteins that showed the lowest levels of expression in LNCaP cells knocked-down for STEAP1 (coloured in green, FDR < 0.001), beta-actin-like protein 2, (ACTBL2), CDK-activating kinase assembly MAT1 (NMAT1), TBC1 domain family member4 (TBC1D4), von Willebrand factor A domain-containing protein 8 (VWA8), molybdopterin synthase catalytic subunit (MOCS2), testis-specific Y-encoded-like protein 1 (TSPYL1), 1-acyl-sn-glycerol-3-phosphate acyltransferase alpha (AGPAT1), serine/threonine-protein kinase SMG1 (SMG1), sorbin and SH3 domain-containing protein 2 (SORBS2), a-kinase anchor protein 17A (AKAP17A) and Ras-related protein Rab-3B (RAB3B) are highlighted in Figure V.2.

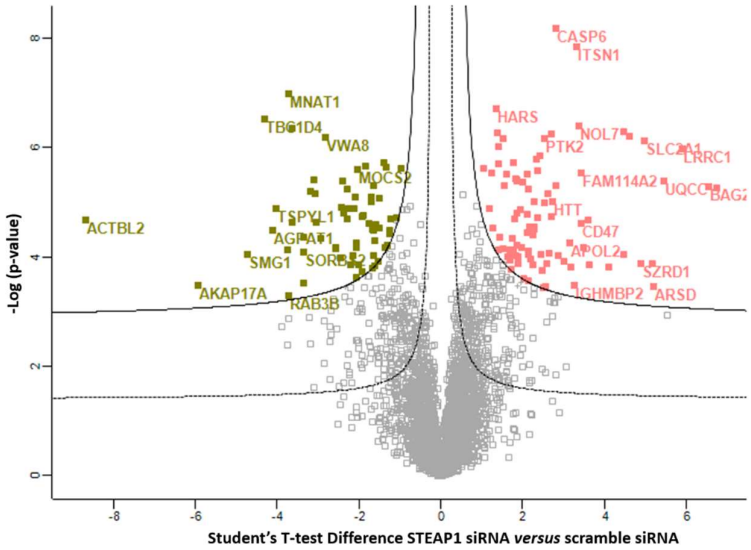

Figure V.2. Volcano plot displays the proteins found differentially expressed between the experimental groups, highlighting some proteins upregulated (red) and downregulated (green) in the STEAP1 siRNA group compared with the scramble siRNA group.

5.3.3. Functional enrichment analysis of differentially expressed proteins

To gain into the biological roles and pathways of the differentially expressed proteins, a combination of several bioinformatics tools was used. Biomolecular interaction network enrichment of differentially expressed proteins was constructed by Cytoscape software v3.8.0 (ClueGo, app v2.5.7, *p*-Value ≤ 0.01), consisting of 60 nodes and 235 edges (Figure V.3.). This analysis indicated that the differential proteins are highly correlated and share common biological terms, such as intracellular transport, RNA processing, organelle organization, cellular protein-containing complex assembly, and metabolic process.

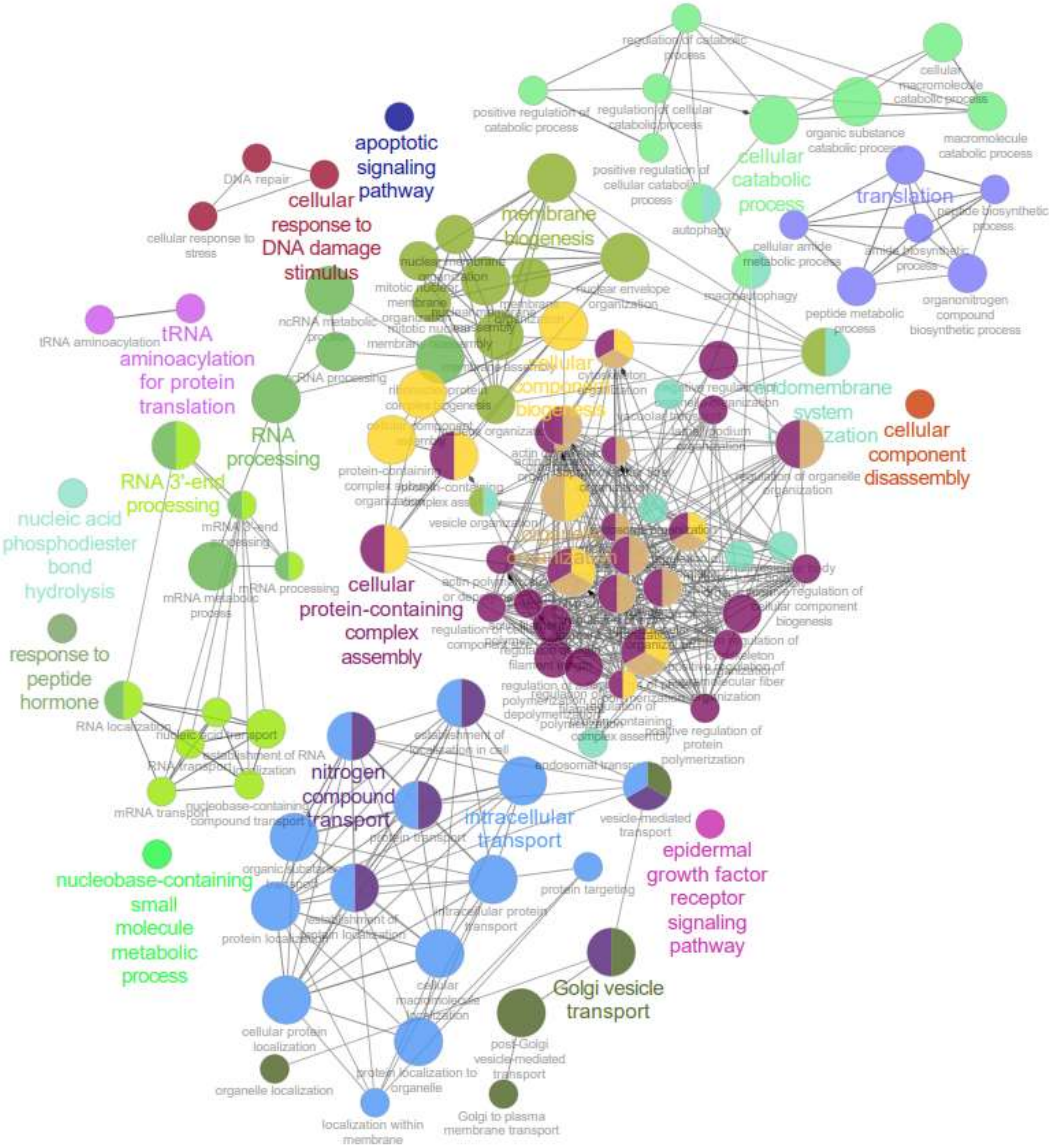

Figure V.3. Functionally grouped network of enriched categories generated in ClueGo (Cytoscape) for the proteins found differentially expressed in STEAP1 siRNA group compared with scramble siRNA group (kappa score threshold = 0.4 and pV ≤ 0.01). The node size represents the term enrichment most significant.

The 526 proteins differentially expressed in the STEAP1 siRNA group compared with scramble siRNA group were classified according to the related GO terms for biological process, molecular function, and cellular component, using DAVID Bioinformatic Database 6.8. As can be seen in Figure V.4., which shows proteins grouped according to their biological process (green bars), the differentially expressed proteins were mainly related to vesicle-mediated transport, protein transport, apoptotic process, and positive regulation of GTPase activity. Furthermore, functional enrichment for biological process indicated that STEAP1 silencing results in the overexpression of proteins involved in intracellular protein transport, membrane organization, oxidation-reduction process, actin filament organization, and apoptotic pathways. On the other hand, RNA binding proteins and enzymes required for protein biosynthesis were found underexpressed with STEAP1 silencing. A significant part of differentially expressed proteins also participates in cell-cell adhesion and endocytosis. Regarding the analysis of molecular function (Figure V.4., orange bars), both over and underexpressed proteins were mainly involved in protein, ATP, and poly(A) RNA binding. However, enzymes with trans-2-enoyl-CoA reductase activity were found underexpressed after STEAP1 knockdown. The categorization according to cellular component (Figure V.4., blue bars) showed that most of the differential proteins were largely localized intracellularly, namely in the cytoplasm and nuclear involvement. Interestingly, many of them were associated with extracellular exosomes (110 proteins).

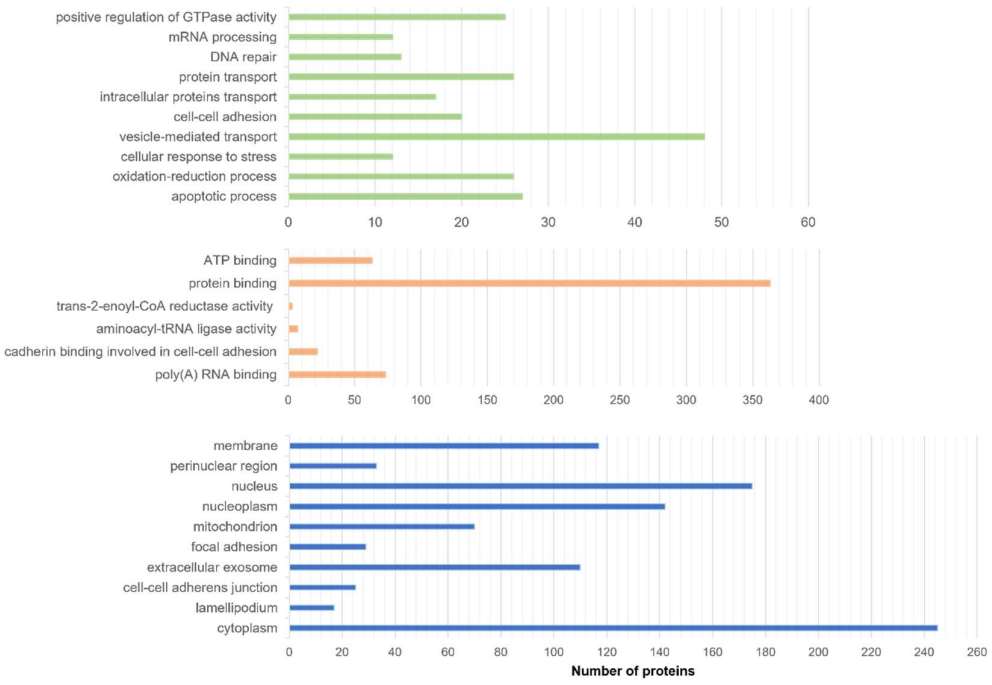

Figure V.4. Classification of the proteins found differentially expressed in STEAP1 siRNA group compared with scramble siRNA group, according to Gene Ontology (GO) terms using David online software. GO annotation for biological process, molecular function, and cellular component are represented at green, orange and blue, respectively.

In addition, protein-protein interaction (PPI) networks functional enrichment analysis was performed by STRING with the highest confidence (0.9). Figure V.5. showed the network enriched in 684 interactions between the 526 proteins found differentially expressed in the STEAP1 siRNA group compared with scramble siRNA, with a PPI enrichment *p*-Value < 7.77e-16. Many of the proteins share interactions among them, indicating that these molecules may play key roles in diverse pathways. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) classification, the differential proteins are involved in pathways such as endocytosis, RNA transport, apoptosis, aminoacyl-tRNA biosynthesis, and metabolic pathways. Of the highlighted pathways, the metabolic pathway is the one in which the most proteins are involved and is associated with oxidative phosphorylation, nucleotide biosynthesis process, oxidoreductase activity, galactose catabolic process, inositol phosphate metabolism and amino sugar metabolism. Several proteins involved in oxidative phosphorylation pathway were found overexpressed when STEAP1 is knocked-down, including ATP synthase subunit (ATPAF2 and ATP5L), V-type proton ATPase (ATP6V1E1 and ATP6V0A1), cytochrome b-c1 complex subunit Rieske (UQCRFS1) and ubiquinol-cytochrome-c reductase complex (UQCC1). However, enzymes with oxidoreductase activity were found underexpressed with silencing STEAP1. Among them, it was found glutathione S-transferase (LANCL1), NADPH:adrenodoxin oxidoreductase (FDXR), short-chain specific acyl-CoA dehydrogenase (ACADS), glutaryl-CoA dehydrogenase (GCDH) and enoyl-[acyl-carrier-protein] reductase (MECR). Proteins involved in purine and pyrimidine biosynthesis process (DTYMK, POLR3A, POLD1, AGPAT1, PTBP3, and MTAP) and amino sugar metabolism, namely galactose catabolic process (FPGT, UAP1L1 and GLB1) were also found as underexpressed, suggesting that silencing of STEAP1 decreased cell growth. Moreover, it was observed that the proteins involved in cysteine and methionine metabolism were decreased (CTH, TKT, AHCYL1, CHDH and MTAP), suggesting that STEAP1 knockdown interferes with the biosynthesis of amino acids. Also, proteins related to inositol phosphate metabolism were found underexpressed with the silencing of STEAP1 (ISYNA1 and PIK3C2A). On the other hand, some proteins associated with carbon metabolism (glycolysis/gluconeogenesis and pentose phosphate pathway), including GOT2, ENO2 and PGM2 were found overexpressed.

The proteins colored in green represent proteins specifically involved in apoptosis (Figure V.5.). It is important to highlight that some proteins involved in this biological process were found with high levels of expression in STEAP1-knockdown condition (LMNB1, CASP6, ATM, CTSB, CASP7, XIAP, DFFA, BAD, CAPN1). Curiously, the levels of oncogene GTPase HRas were found to decrease, suggesting that STEAP1 can act through the HRas signaling pathway.

Aminoacyl-tRNA biosynthesis and RNA transport are other two pathways where the differential proteins are involved. Threonine--tRNA ligase (TARS2) and phenylalanine--tRNA ligase (FARS2) are two enzymes found underexpressed in LNCaP proteome silenced to STEAP1, and nucleoporin (NUP188) was also found with ratio -3.620-fold change between scramble siRNA and STEAP1 siRNA. Other proteins related to RNA transport were also decreased in LNCaP cells

knocked-down for STEAP1, such as SUMO1, CYFIP1, THOC5 and EIF4A3, indicating a decrease in overall protein synthesis, and consequently, reduction of cell growth.

According to KEGG pathways, from the list of differentially expressed proteins, endocytosis is the one of terms with the greatest significant enrichment. Several components of the endocytic machinery were found overexpressed in LNCaP proteome without STEAP1, including epidermal growth factor receptor substrate 15-like 1 (EPS15L1), dynamin-2 (DNM2), endophilin-B2 (SH3GLB2), AP-2 and AP-3 complex (AP2M1 and AP3S1), cdc42-interacting protein 4 (TRIP10) and intersectin-1 (ITSN1). Components of the ESCRT-I complex, a regulator of the vesicular trafficking process (MV12A, VPS37B, VPS37A, VPS33B, CHMP4A, and VTA1), and of the SNARE family (SNAP29, STX16, STX4 and VTI1B) were also found overexpressed. Curiously, multivesicular body subunit MVB12A and exosome complex exonuclease RRP44 (DIS3) were found underexpressed in STEAP1 silencing-LNCaP cells. Furthermore, the levels of myc box-dependent-interacting protein 1 (BIN1), which may be involved in the regulation of synaptic vesicle endocytosis and may act as a tumor suppressor, was found to increase with the silencing of STEAP1. As previously indicated, the levels of HRas, involved in the activation of Ras protein signal transduction, were found to diminish with the silencing of STEAP1. These results suggest that LNCaP cells with higher levels of STEAP1 alter endocytic trafficking and recycling to enhance their survival, proliferative, and migratory properties.

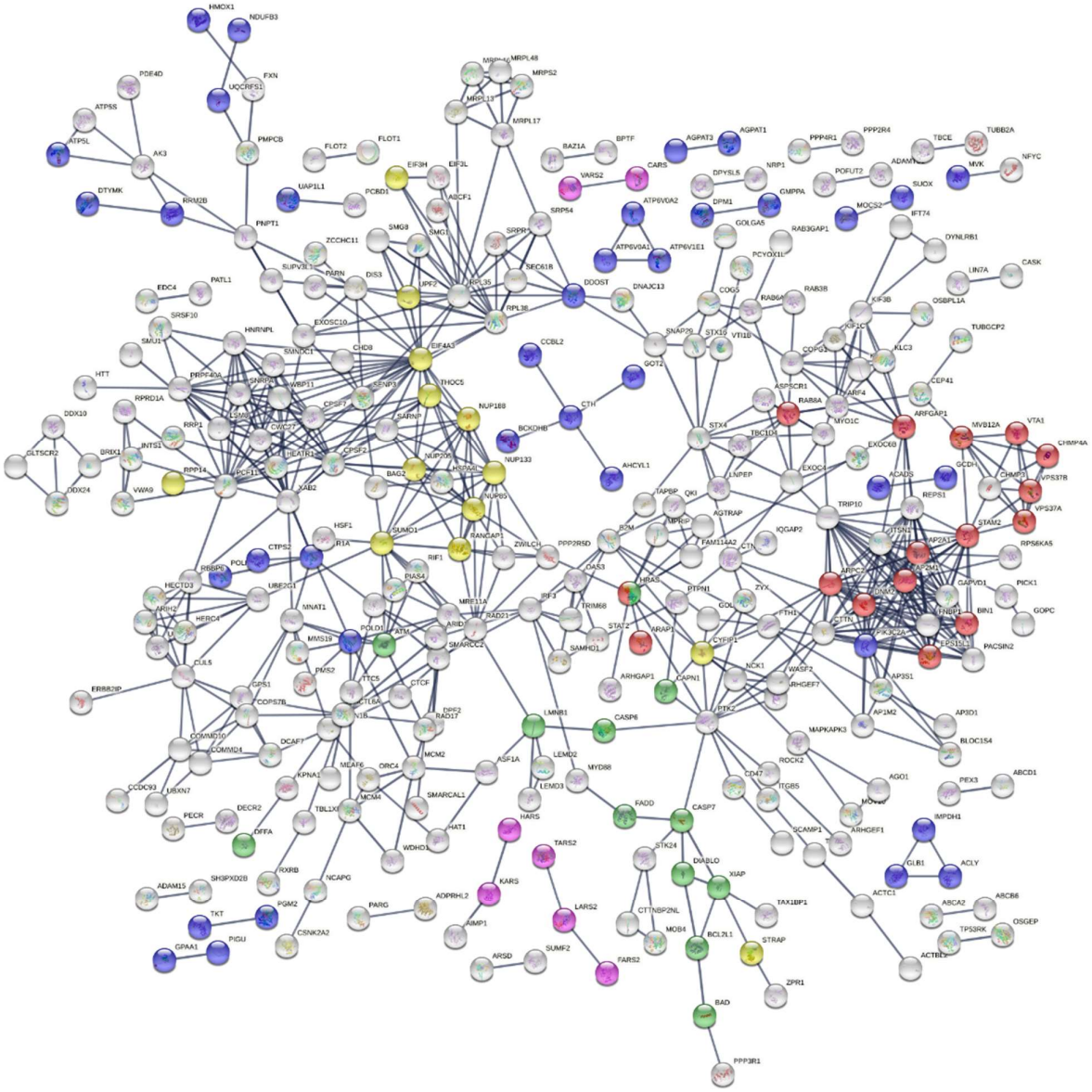

Figure V.5. Protein-protein interaction network of the proteins found differentially expressed in STEAP1 siRNA group compared with scramble siRNA group, based on highest confidence, predicted using STRING 11.0. Proteins highlighted with red, yellow, green, pink and blue are involved in endocytosis (FDR < 0.0004), RNA transport (FDR < 0.0017), apoptosis (FDR < 0.017), aminoacyl-tRNA biosynthesis (FDR < 0.02) and metabolic pathways (FDR < 0.042), respectively.

5.3.4. Validation of target protein by immunoblotting

Six proteins identified were subjected to verification to confirm the levels measured in proteome analysis. Western blot assay was used to validate changes of HRas, cathepsin B, PIK3C2A, intersectin-1, DIS3 and syntaxin 4 induced by loss of STEAP1 in LNCaP cells. These proteins were selected considering their roles in metabolism, apoptosis and vesicle-mediated transport, sharing

interactions in different molecular/cellular pathways. As shown in Figure V.6., the changes in protein intensity confirmed the reliability of our proteomic results. Unpaired *t*-test showed a significant increase in the levels of an activated form of cathepsin B, intersectin-1, and syntaxin 4 in the STEAP1 siRNA group (2.3-, 3.6- and 1.9-fold change, respectively), which corroborates the results obtained in proteomic analysis (0.46-, 3.33- and 1.85-fold change, respectively). Analysis of HRas, PIK3C2A and DIS3 were also consistent with the results obtained in the LC-MS/MS analysis (-1.63-, -0.95- and -2.46-fold change, respectively), showing that these proteins were downregulated in STEAP1 siRNA samples (-0.6-, -0.6- and -0.5-fold change, respectively).

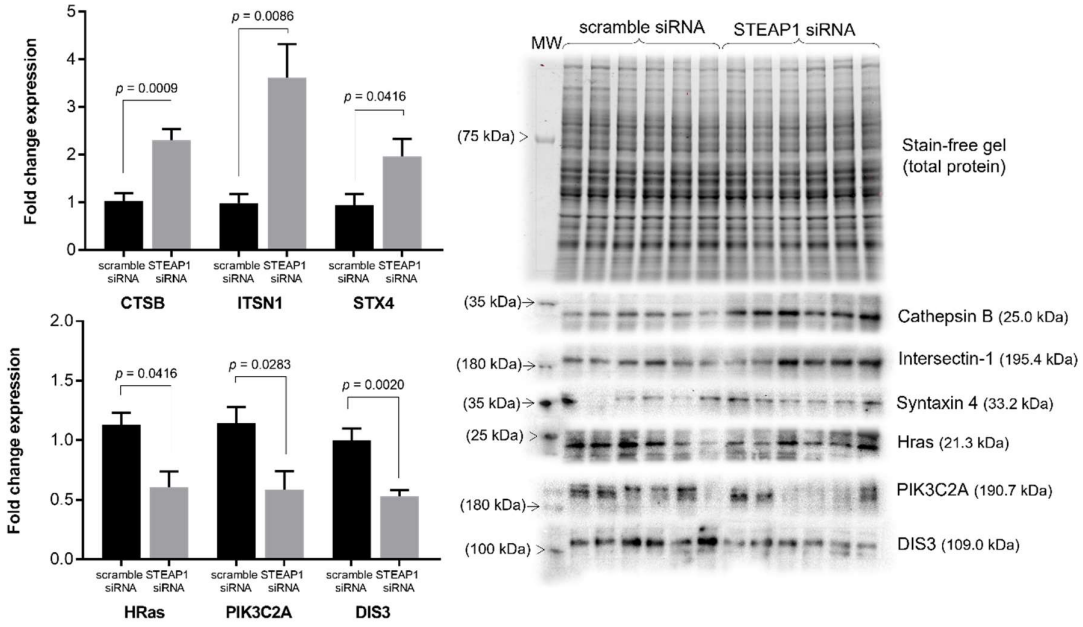

Figure V.6. Western Blot analysis of cathepsin B (CTSB), intersectin-1 (ITSN1), syntaxin 4 (STX4), HRas, PIK3C2A and DIS3 in samples from two groups used in this study (STEAP1 siRNA versus scramble siRNA). Normalization of the protein input were performed using the stain-free gel. Results are expressed as fold variation expression to the scramble siRNA group and representative blots are shown in the right panel. Error bars indicate mean ± SEM (n=3 in duplicate).

5.4. Discussion and Conclusion

Over recent years, the discovery and validation of new protein biomarkers are gaining growing interest in both research and clinical practice, having a huge impact on early cancer detection, diagnosis improvement, recurrence prevention, therapeutic response monitoring and increased survival outcome [30, 31]. Many researchers have contributed to the characterization of the human prostatic proteome in cancer [32–35]. In addition, to understand the basic biology of PCa, proteomics techniques have been used to identify how the signaling pathways in tumor cells are altered, improving our knowledge of potential new target pathways for PCa therapy [32].

The STEAP1 protein is overexpressed in cancer microenvironments, particularly in PCa, being overexpressed in more than 80% of total PCa cases in comparison with non-neoplastic tissues

[4, 5, 15]. Our previously work showed that knockdown of STEAP1 facilitates apoptosis of PCa cells and attenuates their cell growth [9]. These results allowed us to establish the hypothesis that STEAP1 is a key protein in PCa pathogenesis and emphasize the need for investigating the underlying mechanisms in its oncogenic role. Thus, the aim of this study was to unravel the molecular and cellular mechanisms associated with STEAP1 actions in PCa. For this purpose, the proteome-wide alteration of STEAP1 silencing-LNCaP cell lysates was measured using a LFQ-based Orbitrap LC-MS/MS proteomic approach, followed by a systematic bioinformatic analysis to quantify the differentially expressed proteins.

The LFQ analysis indicated that the reduced levels of STEAP1 induced a wide change of protein expression in LNCaP cells, allowing to quantify 526 proteins (292 up-regulated and 234 down-regulated) as differentially expressed in the STEAP1 siRNA group compared to the scramble siRNA group. Combination of the PPI from STRING with GO/KEGG analysis contributed to a better insight into biological processes/pathways changes induced by STEAP1. Endocytosis, RNA transport, apoptosis, aminoacyl-tRNA biosynthesis and metabolic pathways are the biological processes strongly associated with the 526 proteins identified as differentially expressed. Considering these results, six proteins involved in these biological processes were chosen, namely HRas, cathepsin B, PIK3C2A, syntaxin 4, intersectin-1, and DIS3. HRas, a GTPase proto-oncoprotein, is overexpressed in many types of cancers promoting tumor growth, differentiation and survival [36-39]. STEAP1 silencing induced a decrease in HRas protein expression, indicating that the oncogenic effect of STEAP1 in PCa can be mediated by HRas signaling pathway. The phosphatidylinositol 3-kinase (PI3K) pathway regulates various cellular processes such as cell growth, proliferation, apoptosis and intracellular trafficking, allowing cancer progression [40]. Nguyen *et al.* identified that one of the components of the PI3K, PIK3C2A, was found increased in mice with PTEN gene inactive, one of the most frequent alterations in human PCa [41]. In our results, PIK3C2A protein levels diminished with knockdown of STEAP1, showing that STEAP1 inhibition may attenuate the growth and proliferation of PCa cells through PI3K pathway. These results are consistent with the findings in the other types of cancer, such as Ewing tumor [10], ovarian cancer [11] and liver cancer [13]. Beyond the blocking of STEAP1 can induce a reduction in cellular metabolism, this study identified for the first time that some proteins involved in oxidative phosphorylation in mitochondria are increased with STEAP1 silencing, namely ubiquinol-cytochrome-c reductase complex assembly factor 1 (UQCC1) and cytochrome b-c1 complex subunit Rieske, mitochondrial (UQCRFS1). Warburg effect is well described in PCa and other cancer types as a hallmark of cancer characterized by increased glycolysis to obtain energy instead of oxidative phosphorylation in mitochondria [42]. Some studies have explored mechanisms to reverse the Warburg effect by inducing oxidative phosphorylation in mitochondria [43, 44]. Therefore, it is liable to speculate that the STEAP1 knockdown may reverse the Warburg effect through the same mechanism. This premise is also supported by decreased expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) protein in LNCaP cells knocked-down for STEAP1, which is associated with a high rate of glycolysis and it is generally overexpressed in PCa [45].

Autophagy and apoptosis are catabolic pathways essential for cellular homeostasis. Cathepsins are engaged in these biochemical pathways being able to either favor or limit tumor

growth [46]. The material destined for degradation can enter into the lysosomes primarily via endocytosis, autophagy, or phagocytosis, and is degraded through the action of several hydrolases, including cathepsin B [47]. This lysosomal cysteine protease was also recently implicated in apoptosis [48]. Thus, our study observed that the knockdown of STEAP1 up-regulated cathepsin B, supporting its role in attenuating PCa progression. Intersectin-1 interacts with a multitude of partners to regulate endocytosis as well as a variety of signal transduction pathways [49]. Furthermore, this endocytosis-related gene has been reported as downregulated in PCa metastasis [50], suggesting that the process of endocytosis is altered during the development of PCa and metastization. One of the essential regulatory proteins in endocytosis is epidermal growth factor substrate 15 (EPS15), which binds to adaptor protein 2 (AP2) and interacts with other endocytic proteins such as intersectin-1 [51]. The present study indicated that EPS15L1, AP2 complex subunits and intersectin-1 were overexpressed by knockdown of STEAP1, indicating a negative regulation by STEAP1 expression. These findings suggested that knockdown of STEAP1 can promote endocytosis of the epidermal growth factor (EGF) receptor (EGFR) and thus decrease the EGF/EGFR signaling cascade, one of the most important pathways of proliferation, migration, differentiation, apoptosis in mammalian cells [52].

Syntaxin 4 is one of the components of SNARE family that play a fundamental role in the development, invasion and metastasis of cancer cells [53]. The role of syntaxin 4 in the regulation of several exocytic pathways have been previously described [54]. Perrotta *et al.* demonstrated that the blockade of syntaxin 4-dependent exocytosis inhibits CD95 receptor clustering and internalization, caspase activation and loss of mitochondrial membrane potential, leading to inhibition of apoptosis and maintenance of the cells in a proliferative state [55]. In our work, it was verified that STEAP1 silencing induced up-regulation of syntaxin 4 expression levels in PCa cells, suggesting that STEAP1 may exert an influence on apoptosis of PCa cells via regulating the exocytic process.

There are several biological mechanisms that extracellular vesicles use to bind and/or be internalized by recipient cells, which are coordinated by multiples proteins responsible for extracellular vesicles biogenesis [56]. Despite many proteins involved in vesicular traffic, endocytosis and exocytosis system are increased in response to STEAP1 silencing, the fusion of multivesicular bodies with the plasma membrane and exosome release appears to be compromised. DIS3, also known as Rrp44 or EXOSC11, is a highly conserved RNA exoribonuclease and a catalytic subunit of the exosome, whose aberrant expression has been implicated in a range of different cancers [57]. In our study, STEAP1 silencing in LNCaP cells induced down-regulation of DIS3 expression levels, suggesting it can inhibit intercellular communication through exosomes. It is well established that STEAP1 is involved in intercellular communication [8, 58, 59]. Nevertheless, our results showed for the first time that STEAP1 may regulate the formation of exosomes, which are important players in intercellular communication that can promote cell growth and survival, modulating the tumor microenvironment to increase the invasive and metastatic activity [56]. In fact, recent data demonstrated that STEAP1 is also present on PCa cell culture-derived extracellular vesicles [60, 61].

In conclusion, significant changes in proteome of human LNCaP occur in response to STEAP1 knockdown, revealing that this protein may be associated with PCa progression through the deregulation of proteins involved in metabolism, apoptosis, endocytosis, and intercellular

communication by vesicles. However, the use of only one PCa cell line and one STEAP1-targeting siRNA are the main limitations of this study. Therefore, further studies should be conducted to investigate the mechanisms by which STEAP1 regulate HRas, cathepsin B, PIK3C2A, intersectin-1, syntaxin 4 and DIS3, as well as their association in human PCa samples. Although more studies are required, this study opened new perspectives regarding that the role of STEAP1 in PCa progression may be mediated by changes in vesicle-mediated transport Altogether, the data herein obtained unravels potential molecular mechanisms triggered by STEAP1 in PCa progression, supporting the role of STEAP1 as a molecular target for PCa treatment.

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Data availability statement: All data supporting the findings of this study are provided within the paper and its supplementary information. The fasta file of the human proteomes (UniProt Homo sapiens UP000005640) was downloaded from UniProt. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the Proteomics Identification Database (PRIDE) [62], partner repository with the dataset identifier PXD037834, with project DOI: 10.6019/PXD037834.

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5.5. References

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Chapter 6

EFFECT OF STEAP1-KNOCKDOWN IN THE RESPONSE OF PROSTATE CANCER CELLS TO ANTI-ANDROGENIC AND CHEMOTHERAPEUTIC DRUGS

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STEAP1 regulation and its influence modulating LNCaP prostate cancer cells response to bicalutamide, enzalutamide and apalutamide

Abstract

Anti-androgen drugs are the standard pharmacological therapies for treatment of non-metastatic prostate cancer (PCa). However, the response of PCa cells may depend on the anti-androgen used and often patients become resistant to treatment. Thus, studying how the anti-androgen drugs affect oncogenes expression and action, and the identification of the best strategy for combined therapies are essential to improve the efficacy of treatments. The Six Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1) is an oncogene associated with PCa progression and aggressiveness, though its relationship with the androgen receptor signaling is not clear. This study aimed to evaluate the effect of anti-androgens in regulating STEAP1 expression and investigate whether silencing STEAP1 can make PCa cells more sensitive to anti-androgen drugs. For this purpose, wild-type and STEAP1 knockdown LNCaP cells were exposed to bicalutamide, enzalutamide and apalutamide. Bicalutamide decreased the expression of STEAP1, but enzalutamide and apalutamide induced its expression. However, decreased cell proliferation and increased apoptosis were observed in response to all drugs. Overall, the cellular and molecular effects were similar between LNCaP wild-type and LNCaP-STEAP1 knockdown cells, except for c-myc expression levels, where a cumulative effect between anti-androgen treatment and STEAP1 knockdown was observed. The effect of STEAP1 knockdown alone or combined with anti-androgens in c-myc levels must be addressed in future studies.

Keywords: Bicalutamide; Enzalutamide; Apalutamide; STEAP1; Prostate cancer.

6.1. Introduction

Prostate cancer (PCa) is the most frequently diagnosed neoplasm in men in the Western world [1]. PCa incidence has been rising in the last decades due to the increase in population aging, obesity caused by dietary habits, and lifestyle, among others [2, 3]. The development and progression of PCa is initially dependent on the stimulatory action of androgens, which validate the use of therapies reducing the biosynthesis of androgens and/or antagonizing the action of androgens through the androgen receptor (AR) [4]. Bicalutamide (second generation), and enzalutamide and apalutamide (third generation) are anti-androgens that antagonize the AR activity, inhibiting gene expression associated with PCa progression, and consequently having therapeutic benefits in PCa [5]. However, several patients become resistant to androgen-deprivation therapy (ADT), giving rise to the so-called castrate-resistant PCa [6]. Clinical trials have investigated the efficacy of ADT in combination with other drugs, as strategies for better management of PCa and slowing the progression to castrate-resistant stage [7]. The identification of new therapeutic targets, in

combination with the ADT, remains a fundamental aspect to improve PCa treatment and to identify novel predictive biomarkers for response to ADT.

The human Six-Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1) is highly expressed in several types of cancer, with special emphasis on PCa where the STEAP1 protein expression levels are 5- to 10- fold higher compared to other cancer types [8]. The mechanisms underlying the overexpression of STEAP1 in cancer remain poorly explored, but epigenetic changes associated with increased expression levels in PCa were identified in the STEAP1 promoter region [9]. Among non-tumoral tissues, STEAP1 is almost restricted to the epithelial cells of prostate gland, which makes it a promising biomarker and/or therapeutic target for PCa [8, 10]. The potential role of STEAP1 in cancer progression has been studied extensively and its oncogenic role highlighted [11–13]. However, the physiological role of STEAP1 and the specific actions driven the carcinogenic process remain to be clarified. Nevertheless, the STEAP1 protein seems to act as a channel for small molecules, being involved in intercellular communication [14]. In addition, there is evidence of the formation of STEAP1-STEAP2 heterotrimers, which seem to be associated with the activity of metal reductase and superoxide synthase enzymes [15]. This is in accordance with a previous study demonstrating that STEAP1 actions, favoring cancer progression, are associated with oxidative stress [16]. Several research groups have demonstrated the contribution of STEAP1 to tumor progression, namely, by its involvement in promoting cell proliferation, migration and invasion [17–21]. Regarding PCa, it was shown that silencing the STEAP1 gene reduces androgen-dependent PCa cell viability and proliferation, and increase the apoptosis rate. In addition, the anti-proliferative and pro-apoptotic effects triggered by STEAP1 knockdown are not abrogated by the treatment with dihydrotestosterone (DHT), suggesting that STEAP1 inhibition might be a good option of treatment to prevent the effects of DHT in PCa [17]. The effect of androgens in regulating STEAP1 expression has also been studied, with contradictory results. Some studies found STEAP1 as androgen-stimulated, androgen-inhibited, or androgen-independent in cell lines of PCa [8, 22–24]. Concerning LNCaP cells, DHT down-regulates STEAP1 expression, but this effect should not involve directly the AR because no androgen response elements are found in promoter region of the STEAP1 gene [23, 25]. This indirect action of AR in down-regulating STEAP1 means that *de novo* protein synthesis should be required [23]. In human PCa biopsies, it was found an association between STEAP1 overexpression and metastasis, with the presence of more aggressive tumors, and the majority of patients becoming resistant to treatment with anti-androgens [26]. In addition, it was detected a marginally positive significant association between STEAP1 overexpression and presurgical prostate specific antigen (PSA) levels, indicating a potential crosstalk between STEAP and AR [26]. Taking into account the use of anti-androgens in PCa treatment, and the potential of STEAP1 as therapeutic target, associated with the putative relationship between STEAP1 and AR, it was hypothesized that STEAP1 expression may be regulated by anti-androgens.as Also, it was envisaged that blocking the action of STEAP1 may sensitizes PCa cells to treatment with anti-androgens drugs. Therefore, the main goal of the present study was to investigate the effect of several types of anti-androgens, bicalutamide, enzalutamide and apalutamide, in LNCaP wild-type (LNCaP-WT) and LNCaP-STEAP1 knockdown cells.

6.2. Material and Methods

6.2.1. Cell culture

Human prostate adenocarcinoma cell line (LNCaP) was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and maintained in Roswell Park Memorial Institute medium (RPMI)-1640 (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Biochrom AG, Leonorenstraße, Berlin, Germany) and 1% penicillin/streptomycin (Gibco, Life technologies, Carlsbad, CA, USA), in a humidified chamber at 37 °C and a 5% CO2 atmosphere.

6.2.2. STEAP1 knockdown and experimental design

LNCaP cells at 50% confluence in T-flasks or multiwell plates were transfected with 20 nM of a small interfering RNA (siRNA) targeting the STEAP1 (s226093, Ambion, Carlsbad, CA, USA) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 24 h in Opti-Minimum Essential Medium (MEM) medium (Invitrogen) as recommended by the manufacturer. These cells are referred as LNCaP-STEAP1 knockdown. As a control for STEAP1-knockdown, a scramble siRNA sequence (4390846, Ambion) was used, and these control cells are designated as LNCaP-WT cells. The sequences of STEAP1 and scramble siRNA were not provided by the manufacturer. 24 h after transfection, the cells were stimulated with anti-androgens, 100 µM bicalutamide (Sigma Aldrich), 10 µM enzalutamide (Sigma Aldrich) and 10 µM apalutamide (Alfa Aesar, Massachusetts, EUA). All drugs stock solutions were prepared in dimethyl sulfoxide (DMSO). Cells were harvested at 24 h after drugs treatment, and the efficiency of STEAP1 knockdown expression was analyzed by quantitative real-time PCR (qPCR) and Western blot.

6.2.3. MTT assay

In order to determine the viability of LNCaP cells silenced for STEAP1 and exposed to the three anti-androgenic drugs, 3-(4,5-dimethylthiazol-2- thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich) was used according to the manufacturer's instructions. Briefly, 100 µL of MTT solution at 0.5 mg/mL concentration was added to cells. After 1 h of incubation at 37ºC, the MTT solution was removed and 100 µL DMSO was added for solubilization of the formazan crystals. Next, the optical density was measured at 570 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA).

6.2.4. Ki-67 fluorescence immunocytochemistry

Fluorescent immunocytochemistry of the proliferation marker Ki-67 was used to estimate the proliferation index between LNCaP cells knocked down for STEAP1 and LNCaP-WT, both treated with three drugs. LNCaP cells were fixed with 4% paraformaldehyde (PFA) for 10 min and permeabilized with 0.1% Triton X-100 during 5 min. A blocking step was performed by incubating cells with 20% FBS in phosphate buffer saline (PBS) containing 0.1% tween®-20 (PBS-T) for 1 h at room temperature, and then, cells were incubated with rabbit anti-Ki-67 (1:50, nº16667, Abcam,

Cambridge, UK). The Alexa Fluor 546 goat anti-rabbit IgG (1:1000, Invitrogen) were used as secondary antibodies. The specificity of the staining was assessed by omission of the primary antibody. Cell nuclei were stained with Hoechst 33342 (5 μg/mL, Invitrogen) for 5 min. Coverslips were washed and mounted onto microscope slides with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using a AxioImager Z2 Optical Microscopy (Carl Zeiss, Göttingen, Germany). Proliferation was determined the percentage of Ki-67-positive cells out of the total number of Hoechst-stained nuclei in eight randomly selected fields per microscope cover glass.

6.2.5. Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay

Cells were fixed with 4% PFA for 10 min and then, permeabilized in 1% Triton X-100 with PBS-T for 5 min. Forty microliters of TUNEL reaction mixture (Roche, Mannheim, Germany) was added to each sample for 1 h at room temperature in the dark. Cells were washed in PBS and incubated for 5 min in Hoechst-33342 (5 μg/mL, Invitrogen). Coverslips were then mounted using Dako fluorescent mounting medium (Dako) and analyzed by fluorescence microscopy using the AxioImager Z2 Optical Microscopy (Carl Zeiss, Ger-many). The percentage of apoptotic cells was estimated by counting the number of TUNEL-positive cells and Hoechst-stained nuclei in eight randomly selected 40× magnification fields in each coverslip. The ratio between the number of TUNEL-positive cells and total number was calculated.

6.2.6. RNA extraction and Reverse Transcription real time quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA from LNCaP cells was obtained using TRI reagent (Grisp, Porto, Portugal) according to the manufacturer's instructions. The RNA pellet was dried, resuspended in 20 μL of DEPC treated water and storage at -80°C. In order to assess the quantity of total RNA, its optical density was determined by measuring absorbance at 260 and 280 nm on a nanospectrometer (Pharmacia Biotech, Ultrospec 3000, Denmark). Total RNA integrity was verified by agarose gel electrophoresis. RT-qPCR was used to determine the expression levels of STEAP1 and p21 genes, using Power SYBR Green RNA-to-CT, 1-Step Kit (Applied Biosystems, Waltham, Massachusetts, USA) on the CFX connect real-time system (Bio-Rad). RT-qPCRs were performed with 0,2 μg of RNA in 10 μL of total reaction with specific primers for STEAP1 (sense: 5' GGC GAT CCT ACA GAT ACA AGT TGC 3' and anti-sense: 5' CCA ATC CCA CAA TTC CCA GAG AC 3'), p21 (sense: 5' TCC AGC GAC CTT CCT CAT C 3' and anti-sense: 5' AGC CTC TAC TGC CAC CAT C 3'), KLK3 (sense: 5' ACC AGA GGA GTT CTT GAC CCC 3' and anti-sense 5' CCC CAG AAT CAC CCG AGC AG 3') and beta-2-microglobulin housekeeping (β2M, sense: 5′ ATG AGT ATG CCT GCC GTG TG 3′ and anti-sense: 5′ CAA ACC TCC ATG ATG CTG CTT AC 3′). All primers were synthesized by the services of STAB VIDA (Caparica, Lisbon, Portugal) and were previously characterized with qPCRs optimized by our research group in previous papers [17, 23]. After cDNA synthesis at 48°C for 30 min, qPCR was performed with the following steps: an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing temperature at 60°C for 30 sec and extension at 72°C for

20 sec. The amplified PCR fragments were analyzed by melting curves. β2M housekeeping was used as internal control to normalize gene expression. Fold differences were calculated following the mathematical model proposed by Pfaffl [27].

6.2.7. Protein extraction and Western blot

LNCaP cells were lysed on an appropriate volume of Radioimmunoprecipitation assay (RIPA) (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris) supplemented with 10% phenylmethylsulfonyl fluoride (PMSF) and 1% protease cocktail. The total protein extract was obtained after centrifugation of the cell lysate for 20min at 14,000 rpm at 4°C. Quantification of the total protein was measured using the Pierce 660 nm Protein assay reagent (Thermo Scientific, Waltham, MA, USA). Approximately 20 μg of total protein were resolved on 10% TGX Stain-Free polyacrylamide gels (Bio-Rad), scanned in the ChemiDoc™ MP Imaging System (Bio-Rad), and then, transferred into a PolyVinylidene DiFluoride (PVDF) membrane (Bio-Rad). After blockage with 5% milk solution, membranes were incubated overnight at 4 °C with following antibodies: rabbit anti-STEAP1 (1:1000, D8B2V, Lot 1, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-p-AKT (1:500, ref. 9271S, Lot 14, Cell Signaling Technology), rabbit anti-AKT (1:500, ref 9272S, Lot 27, Cell Signaling Technology), rabbit anti-p-ERK (1:500, ref. 9101S, Lot 12, Cell Signaling Technology), rabbit anti-ERK (1:500, Ref 9102S, Lot 27, Cell Signaling Technology), rabbit anti-p-c-myc (1:500, ref. 13748S, Lot 4, Cell Signaling Technology), rabbit anti-Bcl-2 (1:1000, ref. #2876S, Lot 6, Cell Signaling Technology), rabbit anti-c-myc (1:500, A-14:sc-789, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Bax (1:1000, ref. 2772S, Lot 11, Cell Signaling Technology), and rabbit anti-p53 (1:1000, FL-393:sc-6243, Lot #L2713, Santa Cruz Biotechnology, Santa Cruz). Membranes were incubated with secondary antibodies anti-rabbit IgG- Horseradish Peroxidase (HRP, 1:15000, Sigma-Aldrich). After this, immunoreactivity was visualized using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.) after the incubation with enhanced chemiluminescence (ECL) substrate (Bio-Rad Laboratories, Inc.). Total protein normalization was carried out using the Image Lab 5.1 software (Bio-Rad Laboratories, Inc.), by opening a multichannel image, configure two channels: channel 1, target protein blot and channel 2, stain-free gel image. Normalization icon from the Analysis Tool box was used to detect bands and lanes and after were adjusted if needed. Stain-free image was selected as normalization channel and the normalized volumes are indicated in the Analysis Table on the tool bar. The target protein band intensity value is adjusted for variation in the protein total load on the gel, following other studies [28, 29].

6.2.8. Caspase-3-like activity assay

The caspase-3-like activity was determined after the cleavage of the labeled substrate by the detection of the chromophore p-nitroaniline (pNA), measured spectrophotometrically at 405 nm. 25 μg of total protein extract was incubated with a reaction buffer (25 mM 4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid (HEPES), 0.1% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), 10% sucrose, supplemented with 10 mM dithiothreitol (DTT), pH 7.5) and 2 mM of caspase-3 substrate (N-Acetyl-Asp-Glu-Val-Asp pNA, Ac-DEVD-pNA, Sigma-Aldrich) for 2h at 37 °C. The amount of generated pNA was calculated by extrapolation with a standard curve of free pNA.

6.2.9. Statistical analysis

All experimental data are shown as mean ± standard error of the mean (SEM). Statistical significance of differences among experimental groups were evaluated by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, using GraphPad Prism v7.01 (GraphPad Software, Inc., USA). $p < 0.05$ was considered statistically significant.

6.3. Results

6.3.1. Effect of bicalutamide, enzalutamide and apalutamide on STEAP1 expression in LNCaP-WT and LNCaP-STEAP1 knockdown cells

The effect of anti-androgens in regulating STEAP1 gene expression was evaluated in LNCaP-WT and LNCaP-STEAP1 knockdown cells. After stimulation with bicalutamide (100 μM), enzalutamide (10 μM) or apalutamide (10 μM) for 24 h, the STEAP1 mRNA and protein expression was evaluated by qPCR and Western blot, respectively. As can be seen in Figure VI.1., using a siRNA targeting STEAP1 silenced STEAP1 gene confirmed both at mRNA (87% ± 0.008 vs. scramble siRNA, Figure VI.1.A) and protein (80% ± 0.053 vs. scramble siRNA, Figure VI.1.B) levels. The efficiency and validation of the anti-androgen treatment was shown by analyzing expression levels of AR the target gene KLK3 that encodes the PSA protein. Figure VI.1.C showed that suppressing androgen actions with bicalutamide, enzalutamide or apalutamide significantly reduced KLK3 expression (0.15 ± 0.01, 0.16 ± 0.03- and 0.16 ± 0.01-fold variation, respectively). Also, it was verified that STEAP1 knockdown decreased PSA levels (0.573 ± 0.04- vs. 0.964 ± 0.06-fold variation, Figure VI.1.C).

When LNCaP-WT cells were treated with bicalutamide, there was a significant decrease in STEAP1 mRNA expression (0.59 ± 0.23-fold variation, Fig. 1A), but no significant effect was observed at STEAP1 protein level (0.73 ± 0.020-fold variation, p=0.108, Fig. 1B). On the other hand, enzalutamide and apalutamide increased the expression of STEAP1 mRNA (1.79 ± 0.018 and 1.77 ± 0.275-fold variation, respectively, Fig. 1A). Concerning the STEAP1 protein levels, the increase of STEAP1 in LNCaP-WT cells was observed with the treatment of apalutamide (1.89 ± 0.177-fold variation, Fig. 1B), but not with enzalutamide (1.47 ± 0.167-fold variation, p=0.108, Fig. 1B).

Regarding the effect of anti-androgens in LNCaP-STEAP1 knockdown, no significant differences in STEAP1 mRNA and protein expression were verified in comparison with LNCaP STEAP1-knockdown without treatment with anti-androgens.

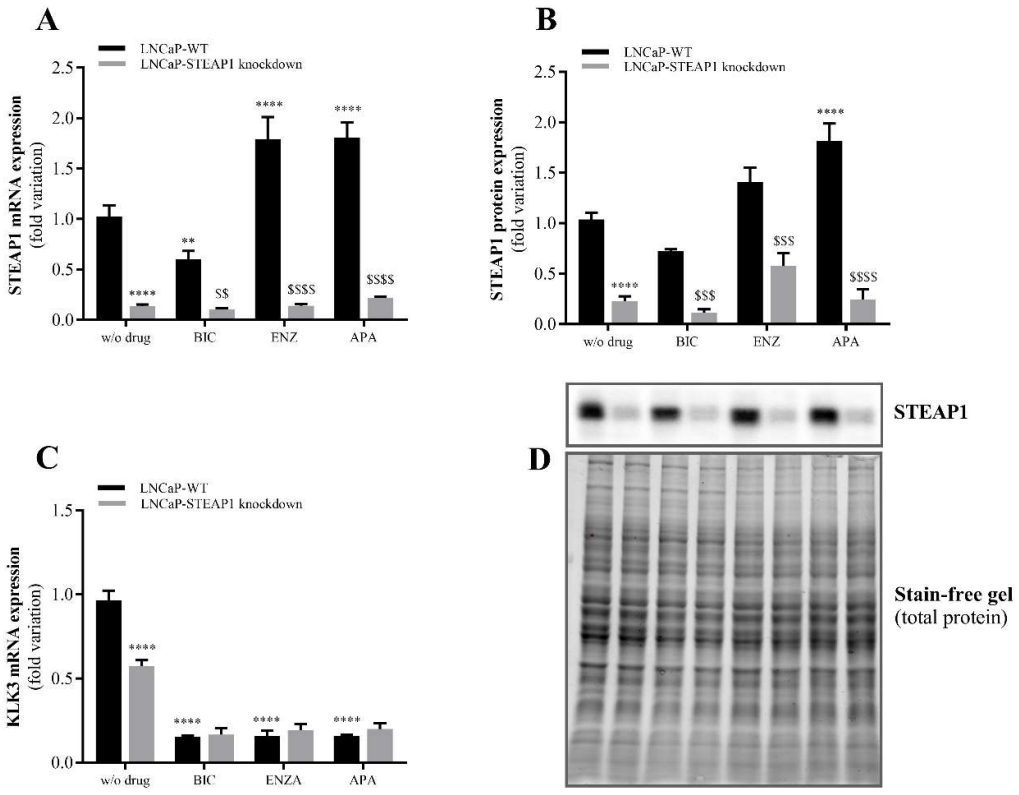

Figure VI.1. Effect of bicalutamide, enzalutamide and apalutamide on the expression of STEAP1 and KLK3 in LNCaP-WT and LNCaP-STEAP1 knockdown prostate cancer cells. STEAP1 and KLK3 expression levels in LNCaP cells following transfection with siRNA for 24 h following treatment with 100 µM of bicalutamide, or 10 µM of enzalutamide or 10 µM of apalutamide for 24 h. Relative **(A)** STEAP1 mRNA, **(B)** STEAP1 protein and **(C)** KLK3 mRNA expression were determined by reverse transcription-quantitative PCR following normalization with the β2M housekeeping gene and western blot after normalization with total protein as represented in (D). Representative immunoblots are also showed in **(D)**. Results are expressed as fold-variation relative to LNCaP-WT (control group). Error bars indicate mean ± standard error of the mean (n≥3). $^{**}p<0.01$ and $^{****}p<0.0001$ *vs*. the LNCaP-WT condition; $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ and $^{\$\$\$\$}p<0.0001$ *vs*. LNCaP-WT plus respective drug. STEAP1, six transmembrane epithelial antigen of the prostate 1; KLK3, kallikrein related peptidase 3; WT, wild type; siRNA, small interfering RNA; BIC, bicalutamide; ENZA, enzalutamide; APA, apalutamide; β2M, β-2-microglobulin.

6.3.2. Cell viability and proliferation of LNCaP-STEAP1 knockdown cells in response to anti-androgenic drugs

The effect of bicalutamide, enzalutamide and apalutamide anti-androgenic drugs on viability and proliferation of LNCaP-STEAP1 knockdown cells were determined by the MTT assay and immunofluorescent labelling of Ki-67, respectively. Twenty-four hours after knockdown of STEAP1, LNCaP cells were exposed to bicalutamide, enzalutamide and apalutamide drugs. Viability of LNCaP cells markedly decreased upon STEAP1 silencing (47,2% ± 11.8 reduction *vs*. scramble siRNA, Figure VI.2.A). 100 µM of bicalutamide, 10 µM of enzalutamide and 10 µM of apalutamide significantly decreased the viability of LNCaP-WT cells (59.55% ± 5.5, 50.68% ± 3.7, and 46.1% ± 6.7 of reduction, respectively, Figure VI.2.A). The silencing of STEAP1 in LNCaP cells did not significantly change cell

viability when treated with anti-androgenic drugs (Figure VI.2.A). The results of Ki-67 fluorescent immunocytochemistry were similar to the results observed for the MTT assay, also showing that the number of Ki-67-positive cells relative to total cells (Hoechst-positive) was significantly decreased in the LNCaP-STEAP1 knockdown cells when compared to LNCaP-WT cells (0.553 ± 0.03- *vs.* 1.01 ± 0.003-fold variation, Figure VI.2.B). Administration of bicalutamide, enzalutamide and apalutamide drugs in LNCaP-WT cells significantly decreased the number of Ki-67 positive cells compared to control (0.630 ± 0.06-, 0.511 ± 0.01- and 0.500 ± 0.01-fold variation, respectively, Figure VI.2.B). However, the STEAP1 gene silencing in LNCaP cells did not significantly change the Ki-67-positive cells number obtained when treated with anti-androgenic drugs (Figure VI.2.B). The representative fluorescent immunocytochemistry images of Ki-67-labelled LNCaP cells in all experimental conditions were represented in Figure VI.2.C.

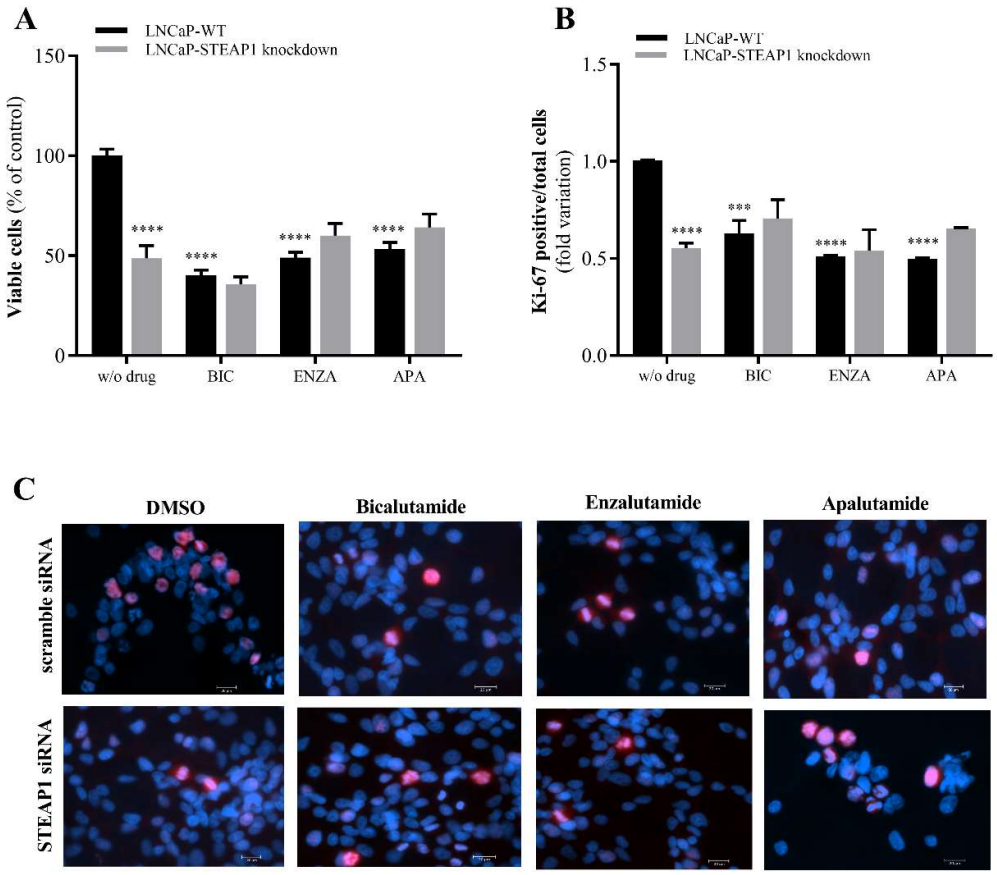

Figure VI.2. Effect of bicalutamide, enzalutamide and apalutamide in cell viability and proliferation of LNCaP-WT and LNCaP-STEAP1 knockdown cells. Cell viability and proliferation of LNCaP cells following transfection with siRNA for 24 h following treatment with bicalutamide (100 µM), enzalutamide (10 µM) and apalutamide (10 µM) for 24 h. **(A)** Percentage of viable cells was determined by the MTT assay. **(B)** Ki-67 positive cells relative to the total cell number after different conditions were obtained by the immunofluorescence analysis of Ki-67 assay; eight randomly selected fields per microscope cover glass were assessed. **(C)** Representative fluorescent immunocytochemistry images of Ki-67 labelled cells (red staining) and Hoechst 33342 stained nuclei (blue) were obtained with the AxioImager Z2 fluorescence microscope (magnification, x400). Results are expressed as percentage of control and fold-variation relative to the LNCaP-WT condition. Error bars indicate mean ± standard error of the mean (n≥2). ***p<0.001 and ****p<0.0001 *vs.*

the LNCaP-WT condition. STEAP1, six transmembrane epithelial antigen of the prostate 1; siRNA, small interfering RNA; WT, wild type; BIC, bicalutamide; ENZA, enzalutamide; APA, apalutamide.

6.3.3. Analysis of survival pathways in LNCaP-STEAP1 knockdown cells in response to anti-androgenic drugs

To understand the decreased viability and proliferative activity of LNCaP cells in response STEAP1 knockdown associated with anti-androgenic action, the expression of proteins related with cell survival pathways was evaluated. Figure VI.3 shows the Western blot analysis for the expression of the active phosphorylated AKT, ERK and c-myc isoforms, respectively to the expression of total proteins. The results showed that p-AKT/AKT and p-ERK/ERK ratio decreased in LNCaP-STEAP1 knockdown cells when compared to control (0.487 ± 0.04- vs. 0.989 ± 0.01-fold variation and 0.701 ± 0.02- vs. 1.02 ± 0.024-fold variation, respectively, Figure VI.3.A and B). Treatment of LNCaP-WT cells with 100 μM of bicalutamide, 10 μM of enzalutamide and 10 μM of apalutamide also decreased the p-AKT/AKT ratio relatively to control group (0.748 ± 0.003- vs. 0.989 ± 0.01-, 0.402 ± 0.058- vs. 0.989 ± 0.01-, and 0.676 ± 0.05- vs. 0.989 ± 0.01-fold variation, respectively, Figure VI.3.A). The silencing of STEAP1 did not alter the p-AKT/AKT ratio of LNCaP cells treated with anti-androgens (Figure VI.3.A). No statistically significant differences were found in the p-ERK/ERK ratio in LNCaP-WT or LNCaP-STEAP1 knockdown cells, both treated with bicalutamide, enzalutamide or apalutamide (Figure VI.3.B).

Regarding the levels of phosphorylated c-myc (p-c-myc) and c-myc, an increase of p-c-myc/c-myc ratio was observed in LNCaP-STEAP1 knockdown when compared to the LNCaP-WT cells (1.978 ± 0.16- vs. 1.002 ± 0.01-fold variation, Figure VI.3.C). Bicalutamide-, enzalutamide- and apalutamide-treated group in LNCaP-WT cells, high levels of p-c-myc/c-myc ratio was found in comparison with scramble siRNA group (1.659 ± 0.02- vs. 1.002 ± 0.01-, 1.883 ± 0.003- vs. 1.002 ± 0.01-, and 1.668 ± 0.03- vs. 1.002 ± 0.01-fold variation, respectively, Figure VI.3.C). The p-c-myc/c-myc ratio in response to bicalutamide, enzalutamide and apalutamide drugs was higher in LNCaP-STEAP1 knockdown cells than in LNCaP-WT cells (2.315 ± 0.04- vs. 1.659 ± 0.02-fold variation to bicalutamide, 2.320 ± 0.01- vs. 1.883 ± 0.003-fold variation to enzalutamide, 2.451 ± 0.12- vs. 1.668 ± 0.03-fold variation to apalutamide, Figure VI.3.C).

Summarizing, anti-androgen treatment significantly decreased p-AKT/AKT and increased p-c-myc/c-myc ratio expression levels in LNCaP-WT cells. Moreover, a slight additive effect was observed in p-c-myc/c-myc ratio in LNCaP cells knocked-down for STEAP1 treated with bicalutamide and apalutamide.

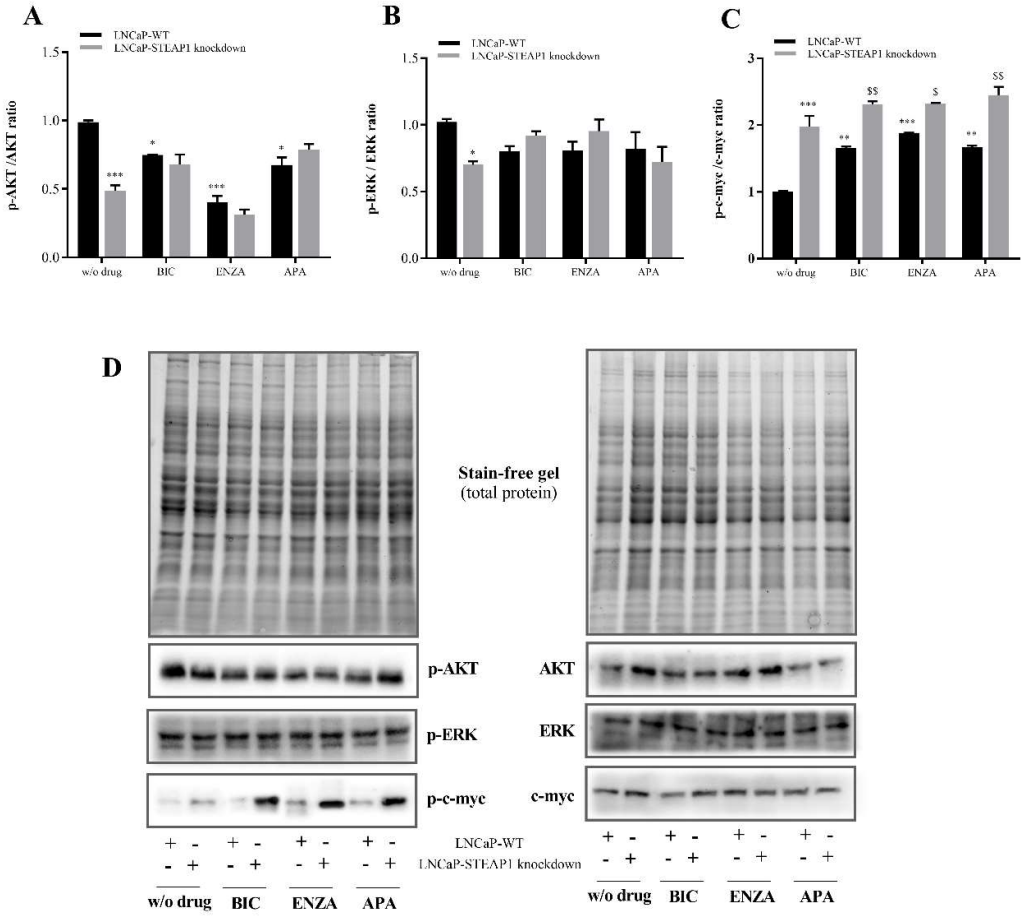

Figure VI.3. Effect of bicalutamide, enzalutamide and apalutamide on the expression of p-AKT, AKT, p-ERK, ERK, p-c-myc and c-myc in LNCaP-WT and LNCaP-STEAP1 knockdown cells. LNCaP cells transfected with siRNA targeting STEAP1 or scramble siRNA. 24 h after transfection, LNCaP cells were treated with 100 µM of bicalutamide, or 10 µM of enzalutamide or 10 µM of apalutamide for 24 h. Ratio of phosphorylated forms and total protein of **(A)** AKT, **(B)** ERK and **(C)** c-myc were determined by western blotting after independent normalization with total protein load on gel as represented in **(D)** together with representative immunoblots. Results are expressed as fold-variation relative to LNCaP-WT (control group). Error bars indicate mean ± standard error of the mean (n≥2). *p<0.05, **p<0.01 and ***p<0.001 *vs.* the LNCaP-WT condition; $^{\$}p$<0.05 and $^{\$\$}p$<0.01 *vs.* LNCaP-WT plus respective drug. STEAP1, six transmembrane epithelial antigen of the prostate 1; siRNA, small interfering RNA; WT, wild type; p-, phosphorylated; BIC, bicalutamide; ENZA, enzalutamide; APA, apalutamide.

6.3.4. Analysis of apoptotic pathways in LNCaP-WT and LNCaP-STEAP1 knockdown cells treated with anti-androgenic drugs

To determine whether the diminished viability/proliferation of LNCaP-STEAP1 knockdown cells in response to anti-androgens treatment is a consequence of increased apoptosis, the expression levels and activity of several apoptotic markers were evaluated. The knockdown of STEAP1 significantly increased the expression of several regulators involved in the apoptosis pathway, the Bax/Bcl-2 was 1.7-fold variation increased (Figure VI.4.A), and the expression of p53 protein and p21 mRNA were also increased (2.004 ± 0.08- and 2.161 ± 0.16- fold variation, respectively, Figure VI.4.B

and VI.4.C). A remarkable end-point of apoptosis is the activation of caspase-3, and our results showed an increased activity of caspase-3 in LNCaP-STEAP1 knockdown (1.944 ± 0.27-fold variation, Figure VI.4.D) when compared to LNCaP-WT cells. Also, the number of TUNEL-positive cells relative to total cells was significantly increased in the LNCaP-STEAP1 knockdown cells when compared to the scramble siRNA group (1.951 ± 0.12- *vs*. 1.002 ± 0.004-fold variation, Figure VI.4.E). Treatment with 100 μM of bicalutamide, 10 μM of enzalutamide and 10 μM of apalutamide triggered an increased expression of apoptotic regulators (Figure VI.4.). The expression levels of pro- and anti-apoptotic members (Bax and Bcl-2 respectively) led to enhanced Bax/Bcl-2 ratio in response to anti-androgen drugs in LNCaP-WT cells (1.6 ± 0.08- to bicalutamide, 1.3 ± 0.07- to enzalutamide, and 1.6 ± 0.08-fold variation to apalutamide, Figure VI.4.A). However, in LNCaP-STEAP1 knockdown cells, there appears to be a potentiating effect of Bax/Bcl-2 ratio with apalutamide treatment (1.9 ± 0.04- *vs*. 1.6 ± 0.08-fold variation, Figure VI.4.A), but not with bicalutamide and enzalutamide.

The treatment of LNCaP-WT cells with anti-androgenic drugs significantly increased the expression of p53 protein (1.615 ± 0.05- to bicalutamide, 1.689 ± 0.16- to enzalutamide, and 1.720 ± 0.12-fold variation) and p21 mRNA (2.119 ± 0.12- to bicalutamide, 2.396 ± 0.001- to enzalutamide, and 1.810 ± 0.09-fold variation), as observed in Figure VI.4.B and VI.4.C, respectively. The knockdown of STEAP1 did not alter the effect of bicalutamide and enzalutamide in expression of p53 protein (1.559 ± 0.14- *vs*. 1.615 ± 0.05- and 1.813 ± 0.16 *vs*. 1.720 ± 0.12-fold variation, respectively) and p21 mRNA (1.738 ± 0.23- *vs*. 2.119 ± 0.12- and 1.902 ± 0.05 *vs*. 1.810 ± 0.09-fold variation, respectively). However, the enzalutamide treatment seems to have less effect in LNCaP-STEAP1 knockdown than in LNCaP-WT cells (1.366 ± 0.02- *vs*. 1.689 ± 0.16-fold variation to p53 levels and 1.605 ± 0.23 *vs*. 2.396 ± 0.001-fold variation to p21 levels, Figure VI.4.B and VI.4.C).

The activity of caspase-3 significantly increased in LNCaP-STEAP1 knockdown cells treated with bicalutamide, enzalutamide and apalutamide (2.11 ± 0.27-, 1.849 ± 0.24- and 1.836 ± 0.07-fold variation, respectively, *vs*. 0.999 ± 0.002-fold variation, Figure VI.4.D). And this effect did not significantly alter with silencing of STEAP1 (Figure VI.4.D).

The results of TUNEL fluorescent immunocytochemistry assay showed that the number of TUNEL-positive LNCaP-STEAP1 knockdown cells were significantly increased with the treatment of bicalutamide, enzalutamide and apalutamide when compared to LNCaP-WT cells (2.154 ± 0.13-, 1.801 ± 0.32- and 1.809 ± 0.22-fold variation, respectively, *vs*. 1.002 ± 0.004-fold variation, Figure VI.4.E). No significant effect was observed in response to anti-androgen drugs in LNCaP-STEAP1 knockdown cells in comparison with LNCaP-WT cells (Figure VI.4.E).

In sum, anti-androgen treatment in LNCaP-WT cells increased the expression and activity of several apoptosis regulators. Also, silencing of STEAP1 did not significantly change the effect observed by bicalutamide, enzalutamide and apalutamide treatment.

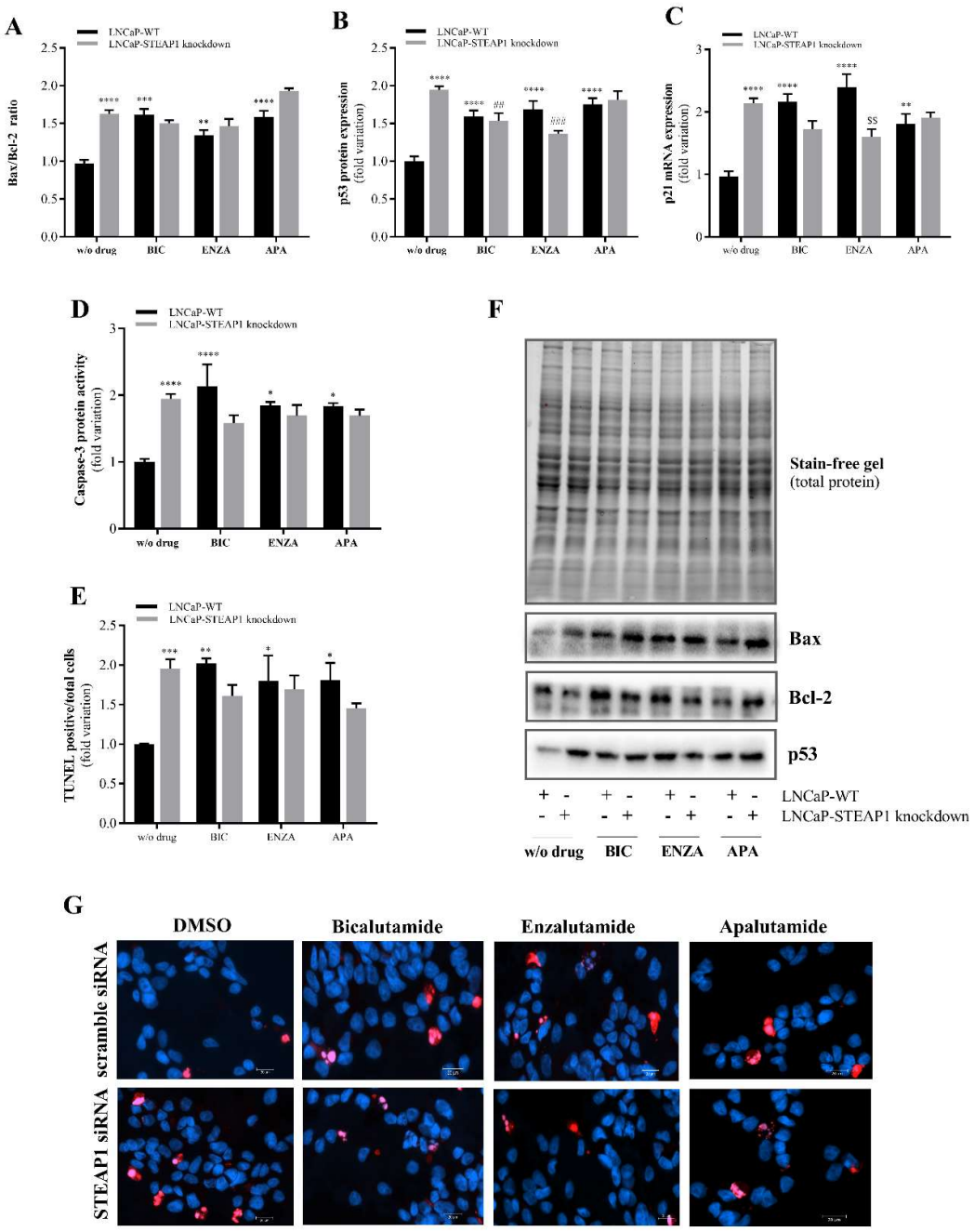

Figure VI.4. Effect of bicalutamide, enzalutamide and apalutamide on the expression of several apoptotic regulators in LNCaP-WT and LNCaP-STEAP1 knockdown cells. LNCaP cells were transfected with siRNA targeting STEAP1 or scramble siRNA. 24 h following transfection, LNCaP cells were treated with 100 µM of bicalutamide, or 10 µM of enzalutamide or 10 µM of apalutamide for 24 h. **(A)** Bax/Bcl-2 protein ratio, **(B)** p53 protein expression and **(C)** p21 mRNA expression were determined by western blotting and reverse transcription-quantitative PCR, respectively. **(D)** Caspase-3 activity was measured spectrophotometrically by the release of the product pNA and **(E)** immunofluorescence analysis of TUNEL-positive cells was determined by the TUNEL assay being the results expressed as the mean of TUNEL-positive cells (red staining) relatively to the total cell number [Hoechst 33342 (blue) staining]. **(F)** Relative protein expression was normalized with total protein load on gel as represented in and relative mRNA expression was normalized with the β2M housekeeping gene. Representative immunoblots are also shown. **(G)** Representative microscopy images showing TUNEL and Hoechst staining in the different groups were obtained in the AxioImager Z2 fluorescence microscope (magnification, x400). Results are expressed as fold-variation relative

to LNCaP-WT (control group). Error bars indicate mean ± standard error of the mean (n≥2). *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$ *vs*. the LNCaP-WT condition; ##$p<0.01$ and ###$p<0.001$ *vs*. the LNCaP-STEAP1 knockdown condition; $$$p<0.01$ *vs*. LNCaP-WT plus respective drug. STEAP1, six transmembrane epithelial antigen of the prostate 1; siRNA, small interfering RNA; WT, wild type, β2M, β-2-microglobulin; BIC, bicalutamide; ENZA, enzalutamide; APA, apalutamide.

6.4. Discussion

In the recent decades, the use of ADT to treat PCa patients has dramatically increased [30, 31]. However, ADT treatment alone becomes insufficient for the management of PCa, since most patients with this pathology progress to the castration-resistant disease within a few years [32, 33]. A way of improving PCa treatment is to evaluate combined action with other putative therapeutic targets. There are several proteins that are dysregulated in PCa, including STEAP1 [8]. This transmembrane protein has been implicated in several forms of cancer due to its overexpression in malignant tissue compared with their non-malignant counterparts [11, 13, 34]. Considering the oncogenic role of STEAP1 in PCa, associated with a lack of studies focusing on impact of ADT treatment in PCa cells overexpressing STEAP1, the main goals of the present study were to evaluate the effect of anti-androgens on expression of STEAP1 and to investigate if the sensitivity of PCa cells to anti-androgen drugs can be improved in response to STEAP1 knockdown. Thus, the effect of bicalutamide, enzalutamide and apalutamide was evaluated in LNCaP-WT and LNCaP-STEAP1 knockdown cells.

Deregulated cell proliferation and apoptosis are a well-established cancer hallmarks and of the first deregulated mechanisms underlying cancer progression [35]. The silencing of STEAP1 was confirmed 24 hours following transfection (Figure VI.1), decreasing the viability and proliferation of LNCaP cells (Figure VI.2 and 3) and accompanied by an increasing of apoptosis (Figure VI.4). These results are in accordance with those previously described by our research group [17]. Treatment of PCa cells with an antibody against the STEAP1 protein was associated with inhibition of cell growth [36], supporting the results herein described our results and the role of STEAP1 as an oncoprotein. The high activity of caspase-3, an effector caspase activated by intrinsic and extrinsic pathway [37] and the high number of TUNEL-positive cells, an established marker of apoptosis by detection of free 3′-OH termini in single-stranded breaks in high-molecular-weight nuclear DNA fragments [38], highlighted the enhanced apoptosis of LNCaP cells in response to STEAP1 knockdown. The intrinsic apoptotic pathway should be involved considering the up- and downregulation of pro- and anti-apoptotic Bax and Bcl-2 proteins, respectively (Figure VI.4). Furthermore, the inhibition of cell proliferation and the apoptotic effect triggered by the knockdown of STEAP1 was also supported by the upregulation of p53 and p21 levels (Figure VI.4), which are involved in cell cycle arrest at G1 and S phase [39]; p53 is also an important inducer of the apoptosis intrinsic pathway [40]. The diminished viability and proliferation of LNCaP cells in response to STEAP1 knockdown was corroborated by the downregulation of p-AKT/total AKT and p-ERK/total ERK ratios, two oncogenic survival pathways associated with cancer progression [41, 42]. These results also supported the hypothesis that reduced activity of AKT may induce the expression of p53. In fact, AKT interacts with the ubiquitin E3 ligase Mdm2, which controls the expression levels and activity of p53 [43, 44]. AKT

enhances Mdm2-mediated p53 ubiquitination and degradation, leading to cell survival [45]. Therefore, it is likely to assume that silencing of STEAP1 in LNCaP cells decreased AKT activity, with increased levels of p53, which is associated with the suppression of cell growth and activation of apoptosis. The precise mechanism underlying the role of STEAP1 in the activation of AKT and ERK pathways remains to be elucidated, though it is possible that AKT and ERK activation may be mediated by increased levels of oxidative stress induced by STEAP1 overexpression [16]. This hypothesis is supported by reports demonstrating AKT and ERK activation in response to the increased levels of reactive oxygen species in LNCaP cells [46]. However, and in order to overcome the limitation of this study, additional studies should be carried out to clarify the relationship between STEAP1 and AKT/ERK using specific inhibitors, as well as its association with oxidative stress.

The transcription factor c-myc is a master regulator of the transcriptional program that controls cell survival and proliferation [47]. Increasing evidence demonstrates that c-myc signaling has a tumor-promoting role and is able to significantly increase proliferation and metastasis of tumors [47-49]. Iijima *et al.* [21] showed that the knockdown of STEAP1 leads to cell-growth inhibition in liver cancer by targeting the suppression of c-myc. Unexpectedly, it was observed that silencing of STEAP1 increased c-myc expression in LNCaP PCa cells. Although c-myc is associated with PCa progression, there are several studies showing that it is among the most robust inducers of apoptosis in hematologic diseases, as well as in solid tumors such as breast and lung cancer [50-54]. Murphy *et al.* [55] showed that activation of the p53-mediated apoptotic intrinsic pathway requires high levels of c-myc. Thus, the knockdown of STEAP1 in LNCaP cells increased the p-c-myc/total c-myc ratio, which may activate mechanisms of surveillance, such as p53 induction. These changes ultimately culminate in apoptosis, as a way to eliminate cancer cells. On the other hand, the increased levels of c-myc may also be a strategy of cancer cells to overcome the inhibitory effect triggered by STEAP1 knockdown. This possibility cannot be ignored, and more studies should be addressed in the future to clarify the relationship between STEAP1 and c-myc.

It is well documented that the treatment of PCa with anti-androgens, such as bicalutamide, enzalutamide and apalutamide, result in blockage of PCa cell growth due to antagonistic effects on AR transactivation [30, 56]. At present, it is being evaluated the use of anti-androgens in combination with other therapeutic targets. To date, no studies have focused on effect of anti-androgens on expression of STEAP1 or the effect of combined action between anti-androgens and STEAP1 inhibition in PCa treatment. Therefore, the present study intended to determine the effect of anti-androgens in STEAP1 expression, as well as to evaluate the hypothesis that silencing STEAP1 may improve the effectiveness of anti-androgen therapy.

The present study observed that AR inhibition in LNCaP-WT cells affected STEAP1 expression (Figure VI.1). Overall, bicalutamide decreased STEAP1 expression, but enzalutamide and apalutamide increased the levels of STEAP1. Using microarray analysis, Carter *et al.* [57] also showed that STEAP1 is downregulated in LNCaP cells treated with bicalutamide. In contrast to the findings of the present study, Doran *et al.* [25] showed that treatment of CWR22 PCa cells with enzalutamide and apalutamide represses STEAP1 mRNA and protein expression. Beyond the slightly different concentrations used in that Doran study and the present study, the effect may differ between cell

lines. Although the precise explanation for different effects between bicalutamide and enzalutamide/apalutamide in STEAP1 expression is unclear, and the differences observed might be due to different affinities of these drugs to AR. In fact, it the conformational dynamics of AR with bicalutamide, enzalutamide and apalutamide was evaluated and it was shown that enzalutamide and apalutamide induce different conformational changes in AR compared with bicalutamide [58]. In addition, point mutations in AR, namely F877L and T878A, are associated with resistance to enzalutamide and apalutamide, but not to bicalutamide [59-61]. Considering that T878A mutation in AR is found in LNCaP cells, one could hypothesize that upregulation of STEAP1 in response to enzalutamide and apalutamide may occur as a mechanism of resistance [62]. However, further studies should be performed to clarify the role of STEAP1 in resistance to these drugs.

As expected, anti-androgen drugs efficiently reduced the expression of the KLK3 gene, which is an AR target gene. It suggested that the effect of anti-androgen on STEAP1 expression was dependent on other factors besides the expression of AR. Nevertheless, it should be highlighted that in LNCaP cells, the knockdown of STEAP1 inhibited the expression of the KLK3 gene. This result is in accordance with the results previously described by our research group, demonstrating that the proliferative effect of DHT is abrogated in LNCaP cells knocked down for STEAP1 [17]. Moreover, these findings are also supported by Ihlaseh-Catalano *et al.* [26], who describe a positive strong trend between STEAP1 and PSA levels. The present study explored the cellular pathways of proliferation and apoptosis of anti-androgen treatment in LNCaP cells. Data obtained from LNCaP-WT cells treated with bicalutamide, enzalutamide and apalutamide showed a reduction on cell viability, determined by MTT assay, and cell proliferation, as indicated by the estimated cell proliferation index assessed by the Ki-67 fluorescent immunocytochemistry (Figure VI.2). Anti-androgen effects modulating PCa cells behavior has been underpinned by alterations on key protein targets associated with cell proliferation, survival and oncogenic pathways, namely the AKT and ERK signaling pathway [41, 42, 63]. The results of the present study showed a significant decreased of p-AKT/AKT ratio in response to bicalutamide, enzalutamide and apalutamide treatment in LNCaP-WT cells (Figure VI.3), but no significant differences in p-ERK/ERK ratio were observed. Altogether, these results suggested that treatment of LNCaP cells with anti-androgen drugs inhibited the signaling pathways associated with cell proliferation and survival in an independent manner of their effect in expression of STEAP1, at least in early treatment phase.

Considering the coordinated action of c-myc and AR in PCa development [64, 65], the effect of anti-androgens was evaluated. The results showed an increased expression of p-c-myc with bicalutamide, enzalutamide and apalutamide exposure of LNCaP-WT cells (Figure VI.3). These findings are in line with a recent study showing that androgen deprivation *in vitro* and castration *in vivo* leads to rapid and persistent increases in c-myc expression [66]. This observation suggests that decreased AR activity can be compensated by increased levels of c-myc, contributing to progression to castrate-resistant PCa following ADT.

Anti-androgen treatment in LNCaP-WT cells was also characterized by the increased expression and activity of several apoptosis regulators (Figure VI.4). These results are the according with other studies describing the apoptotic effect of anti-androgens in PCa cells [67-69]. Furthermore, a few reports have shown that administration of anti-androgens in combination with

other anti-cancer drugs trigger cytotoxic effects in PCa [70-72]. The co-administration of enzalutamide and abiraterone (an inhibitor of the steroidal enzyme CYP17A1) inhibits the proliferation and promotes the apoptosis of LNCaP cells [70] and enzalutamide combined with AS602801 (an inhibitor of c-Jun N-terminal kinase) synergistically kills PCa cells, decreasing their migration and invasion capacity [71]. In addition, apalutamide, in combination with autophagy inhibitors, provides a significantly elevated anti-tumor effect in LNCaP cells [72]. However, the present study is the first, to the best of the authors' knowledge, to evaluate the combined effect of STEAP1 knockdown with anti-androgen therapy on PCa cells. LNCaP-STEAP1 knockdown cells treated with bicalutamide, enzalutamide and apalutamide exhibited a decrease on cell viability and proliferation, but no significant differences were observed in comparison with the effect of these anti-androgens in LNCaP-WT cells (Figure VI.2). Similar data was observed regarding the effect of bicalutamide and enzalutamide in p-AKT/AKT and p-ERK/ERK ratios (Figure VI.3). These observations are in accordance with increased expression and activity of regulators/effectors of apoptosis in response to anti-androgen treatment and no significant differences between LNCaP-WT and LNCaP-STEAP1 knockdown cells were detected (Figure VI.4). Thus, the results suggested that there is no synergistic effect between the silencing of STEAP1 and anti-androgens treatment, at least in LNCaP cells treated for 24 hours with anti-androgen drugs.

Unexpectedly, an additive effect of c-myc expression levels in LNCaP cells knocked-down for STEAP1 and treated with bicalutamide, enzalutamide and apalutamide was observed (Figure VI.3). To the best of the authors' knowledge, there are no studies corroborating these discoveries and further studies are needed to improve understanding of the role of c-myc in response to combined action between anti-androgen and STEAP1 knockdown in PCa cells.

In conclusion, the present findings showed that anti-androgen drugs affected the regulation of STEAP1 expression, but inhibition of STEAP1 did not alter the response of LNCaP cells to anti-androgen treatment. Although the levels of STEAP1 in LNCaP cells did not seem to change the effect of anti-androgens in cell proliferation and apoptosis, the synergic effect in p-c-myc levels deserves attention in future studies. Despite the limitations concerning the use of only one PCa cell line and the unique concentration and time of exposure tested for the anti-androgen drugs, the present study strengthened the potential use of STEAP1 knockdown in PCa therapy, as well as opening new avenues of research aimed at exploring the mechanisms underlying the role of STEAP1 in human PCa. Further studies deepening the role of STEAP1 in response to anti-androgen drugs and investigating its actions in the development of PCa resistance to treatments would be fundamental for improved management of the disease.

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STEAP1 knockdown decreases the sensitivity of prostate cancer cells to paclitaxel, docetaxel and cabazitaxel

Abstract

The Six Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1) protein has been indicated as an overexpressed oncoprotein in prostate cancer (PCa), associated with tumor progression and aggressiveness. Taxane-based antineoplastic drugs such as paclitaxel, docetaxel, or cabazitaxel, have been investigated in PCa treatment, namely for the development of combined therapies with the improvement of therapeutic effectiveness. This study aimed to evaluate the expression of STEAP1 in response to taxane-based drugs and assess whether the sensitivity of PCa cells to treatment with paclitaxel, docetaxel, or cabazitaxel may change when the STEAP1 gene is silenced. Thus, wild-type and STEAP1 knockdown LNCaP and C4-2B cells were exposed to paclitaxel, docetaxel or cabazitaxel, and STEAP1 expression, cell viability, and survival pathways were evaluated. The results obtained showed that STEAP1 knockdown or taxane-based drugs treatment significantly reduced the viability and survival of PCa cells. Relatively to the expression of proliferation markers and apoptosis regulators, LNCaP cells showed a reduced proliferation, whereas apoptosis was increased. However, the effect of paclitaxel, docetaxel, or cabazitaxel treatment was reversed when combined with STEAP1 knockdown. Besides, these chemotherapeutic drugs may stimulate the cell growth of PCa cells knocked down for STEAP1. In conclusion, this study demonstrated that STEAP1 expression levels might influence the response of PCa cells to chemotherapeutics drugs, indicating that the use of paclitaxel, docetaxel, or cabazitaxel may lead to harmful effects in PCa cells with decreased expression of STEAP1.

Keywords: Prostate cancer; Paclitaxel; Docetaxel; Cabazitaxel; STEAP1.

6.6. Introduction

The Six-Transmembrane Epithelial Antigen of the Prostate (STEAP1) protein has been identified as up-regulated in several human cancers, with particle emphasis on prostate cancer (PCa) [1–6]. STEAP1 act as a metalloreductase contributing to the generation of reactive oxygen species, which induces intracellular oxidative stress and inflammation [7–10]. Several researchers exploring the role of STEAP1 in cancer have been describing that its overexpression inhibits apoptosis, enhances cell proliferation, migration and invasion, and induces epithelial to mesenchymal transition, ultimately contributing to tumour progression and aggressiveness [11–15]. Moreover, STEAP1 expression levels in human PCa were reported to be 5- to 10-fold higher compared to other cancer types [1]. Due to its high tumour specificity and membrane-bound localization, STEAP1 is currently considered an oncogene and a promising therapeutic target for PCa [6, 17, 18].

Chemotherapy is a type of treatment often used to treat cancer cells, which utilizes powerful chemicals to kill fast-growing cells [19, 20]. Chemotherapeutic drugs can be used alone or in

combination with other types of treatments to treat a wide variety of cancers. Different chemotherapeutic agents with distinct mechanisms of action are available [19], which includes taxanes, an important class of anti-microtubule agents [21]. Taxanes exert anti-cancer effects by binding tubulin and affecting microtubule polymerization, which results in mitotic arrest and induction of apoptosis in the highly proliferating cancer cells [22]. Paclitaxel was the first taxane to receive regulatory approval for use as an anti-cancer therapy in the United States [23]. Later, docetaxel was produced as a second-generation semisynthetic taxane analogue with better tolerability and cytotoxicity [24]. Cabazitaxel is a novel third-generation semisynthetic analogue of docetaxel, which was investigated as a promising agent for the treatment of castration-resistant PCa (CRPC) [25]. In the last years, several studies have shown that cabazitaxel is effective in improving the life-quality of CRPC patients [25–28]. However, the clinical benefit of these taxanes-based chemotherapeutics administration is limited in CRPC treatment, showing a modest effect in patient survival, triggering toxicity in normal tissues, and some studies have even reported death of patients [29, 30]. Therefore, it is crucial to evaluate the effect of chemotherapeutic drugs modulating the mechanisms of cell cycle control, namely the expression of oncogenes, to define better the best treatment protocols and the effectiveness of combined therapies for PCa treatment. Also, it is critical to understand if manipulating oncogenes expression can impact the response to chemotherapeutics. The present study aimed to evaluate the expression of STEAP1 in response to taxane-based drugs, and to determine if the sensitivity of PCa cells to treatment with chemotherapeutic drugs changes when STEAP1 gene is knocked-down. For this purpose, human neoplastic LNCaP cells with different levels of STEAP1 expression were exposed to paclitaxel, docetaxel or cabazitaxel. Alterations in cell viability, proliferation, and apoptosis, as well as on the expression of STEAP1 and target regulators of cell proliferation and apoptosis, were assessed.

6.7. Material and Methods

6.7.1. Cell line and culture conditions

Human prostate cancer adenocarcinoma LNCaP cell line known to overexpress STEAP1 [1] was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained in RPMI-1640 phenol-red medium (Sigma Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Gibco, Grand Island, USA), at 37 ºC in an atmosphere equilibrated with 5% CO2.

6.7.2. Small-interfering RNA transfection and treatments

At 50% confluence, LNCaP cells were transfected with 20 nM of a small interfering RNA (siRNA) targeting STEAP1 (STEAP1 siRNA, s226093, Ambion, Carlsbad, CA, USA), or scramble siRNA (s4390846, Ambion) for 24 hours. For this purpose, the appropriate quantity of scramble- and STEAP1 siRNA was diluted in Opti-MEM® (mix A). Simultaneously, lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was diluted in Opti-MEM® (mix B), according to the manufacturer's

instructions. After incubation for 5 minutes at room temperature (RT), mix A and B were combined, and the formation of siRNA:lipofectamine complexes occurred for additional 20 minutes at RT. Then, the complexes were added to cells. After 24 hours transfection, LNCaP cells were treated with chemotherapeutics drugs, 5nM of paclitaxel (Alfa Aesar, Haverhill, Massachusetts, EUA), 20 nM of docetaxel (Sigma Aldrich, St. Louis, USA) and 1nM of cabazitaxel (Sigma Aldrich, St. Louis, USA), for additional 24 hours. All drugs were prepared with DMSO and concentrations were selected according to the literature [31, 32, 33]. The efficiency of STEAP1 expression knockdown was evaluated by quantitative real-time PCR (qPCR) and Western blot.

6.7.3. Reverse Transcription Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from LNCaP cells using TRI reagent (Grisp, Lisboa, Portugal) in accordance with the manufacturer's protocol. Total RNA was quantified by spectrophotometry at 260 and 280 nm (Pharmacia Biotech, Ultrospec 3000, Denmark), and its integrity using an agarose gel electrophoresis. 200 ng of total RNA was used for cDNA synthesis and the expression of STEAP1 and p21 genes was determined using Power SYBR Green RNA-to-CT, 1-Step Kit (Applied Biosystems, USA) and the CFX connect real-time system (Bio-Rad, Hercules, USA). RT-qPCRs were performed in a final volume of 10 µL with STEAP1 (sense: 5' GGC GAT CCT ACA GAT ACA AGT TGC 3' and anti-sense: 5' CCA ATC CCA CAA TTC CCA GAG AC 3'), p21 (sense: 5' TCC AGC GAC CTT CCT CAT C 3' and anti-sense: 5' AGC CTC TAC TGC CAC CAT C 3'), and β-2-microglobulin (β2M) (sense: 5′ ATG AGT ATG CCT GCC GTG TG 3′ and anti-sense: 5′ CAA ACC TCC ATG ATG CTG CTTAC 3′) specific primers. The annealing temperature was 60 ºC for all primer sets and samples were run in triplicate for three independent experiments. β2M housekeeping was used as internal control to normalize gene expression. Normalized expression values were calculated following the model proposed by Pfaffl [34] using the formula: 2-ΔΔCt.

6.7.4. Protein extraction and Western blot

LNCaP cells were homogenized in radioimmunoprecipitation assay buffer (RIPA, 150 mM NaCl, 1% Nonidet-P40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris) supplemented with 10% PMSF and 1% protease inhibitors cocktail, kept on ice for 30 minutes with occasional vortex. Next, the homogenized was centrifuged at 14,000 rpm for 20 minutes at 4 °C. Total protein was quantified using the Pierce 660 nm Protein assay reagent (Thermo Scientific, USA). 20 µg of total protein were resolved on 10% TGX Stain-free polyacrylamide gels (Bio-Rad), scanned in the ChemiDoc™ MP Imaging System (Bio-Rad) and then eletrotransferred to a PVDF membrane (BioRad). After blocking with 5 % milk solution, membranes were incubated with rabbit anti-STEAP1 (1:1000, D8B2V, Cell Signaling Technology, Danvers, Massachusetts, EUA), rabbit anti-pAKT (1:500, ref. #9271, Cell Signaling Technology), rabbit anti-pERK (1:500, ref. #9101, Cell Signaling Technology), rabbit anti-p-c-myc (1:500, ref. #13748, Cell Signaling Technology), rabbit anti-Bcl-2 (1:1000, ref. #2876, Cell Signaling Technology), rabbit anti-Bax (1:1000, ref. #2772, Cell Signaling Technology), and rabbit

anti-p53 (1:1000, FL-393:sc-6243, Santa Cruz Biotechnology) overnight at 4ºC. Anti-rabbit IgG-HRP (1:15000, Sigma-Aldrich) was used as secondary antibody. Membranes were incubated with the ECL substrate (Bio-Rad) and scanned using the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were obtained by densitometry analysis using the Image Lab 5.1 software (Bio-Rad) and normalized to the total protein on the gel [35].

6.7.5. Cell viability assay

STEAP1 siRNA- and scramble siRNA-transfected LNCaP cells (25.000 cells/well) for 24 hours and treated with paclitaxel, docetaxel and cabazitaxel drugs (24 hours) were grown in 96-well plates and cell viability determined by the colorimetric MTT assay. In brief, MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), at 0.5 mg/mL final concentration, was added to the cell culture medium and the reaction occurred in the dark at 37 ºC for 1 hour. Next, the MTT solution was carefully removed, and the formazan crystals were solubilized with 100 µL of DMSO. The absorbance of the solution was determined at 570 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The absorbance value is proportional to the number of viable cells in each experimental group.

6.7.6. Ki-67 fluorescent immunocytochemistry

LNCaP cells were fixed with 4% paraformaldehyde and permeabilized with 1% triton X-100 for 5 minutes at RT. In order to block non-specific binding sites, the cells were incubated with PBS containing 0.1% (w/v) Tween-20 and 20% FBS for 1 hour. After washing, cells were incubated for 1 hour at RT with rabbit anti-Ki-67 (1:50, nº16667, Abcam) and incubated with the Alexa Fluor 546 goat anti-rabbit IgG (1:1000, Invitrogen) secondary antibody for 1 hour at RT. Cells were washed with PBS and stained with Hoechst-33342 (5 µg/mL, Invitrogen) for 5 minutes. Then, the coverslips were rinsed with PBS, mounted using Dako (Invitrogen) and visualized by fluorescence microscopy (Zeiss AxioImager A1). The index of proliferation was determined by counting the number of Ki-67-staining cells and Hoechst-stained nuclei in ten randomly selected 40× magnification fields for each coverslip. The ratio between the number of Ki-67-stained cells and total number of nuclei was determined.

6.7.7. Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay

TUNEL analysis was performed using the In Situ Cell Death Detection Kit, TMR red kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Briefly, LNCaP cells were fixed with 4% paraformaldehyde for 30 minutes at RT and then permeabilized with 1% Triton X-100 in phosphate buffer saline (PBS-T). Cells were stained with TUNEL reaction mixture for 1 hour at RT in the dark. After washing in PBS-T, cell nuclei were stained with Hoechst-33342 (5 µg/mL, Invitrogen) for 5 minutes. Coverslips were mounted in Dako mounting medium (Invitrogen) and fluorescence microscopy images acquired using a Zeiss AxioImager A1 (Carl Zeiss) microscope. The

apoptotic index was determined by counting the number of TUNEL-positive cells and Hoechst-stained nuclei in ten randomly selected 40× magnification fields for each coverslip.

6.7.8. Caspase-3-like activity assay

The caspase-3-like activity was determined spectrophotometrically by detecting the presence of the yellow product p-nitroaniline (p-NA), upon cleavage of caspase-3-substrate (Ac-DEVD-p-NA). 25 µg of total protein extract was incubated with a reaction buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose, and 10 mM DTT) and 2 mM Ac-DEVD-p-NA. The reaction was left to occur two hours at 37 °C and the absorbance measured at 405 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The amount of generated p-NA was calculated by extrapolation with a standard curve with known concentrations of p-NA.

6.7.9. Statistical analysis

The statistical significance of all experimental groups was assessed by Student's t-test or by ANOVA followed by the Sidak's multiple comparisons test. Significant differences were considered when $p < 0.05$ (*, #), $p < 0.01$ (**, ##), $p < 0.001$ (***, ###) and $p < 0.0001$ (***, ####). All experimental data are shown as mean ± S.E.M. The Graphpad Prism 7.01 program (GraphPad Software, USA) was used for this analysis.

6.8. Results

6.8.1. Effect of Paclitaxel, Docetaxel and Cabazitaxel on STEAP1 Expression in PCa Cells

To investigate the biological role of STEAP1 related to the chemotherapy of PCa, the protein levels of STEAP1 in different PCa cell lines (LNCaP, PC3, DU145, 22RV1, C4-2B and VCaP) were quantified by Western blot. The results showed that LNCaP and C4-2B cells express the STEAP1 protein (Figure VI.5), being even higher in LNCaP cells.

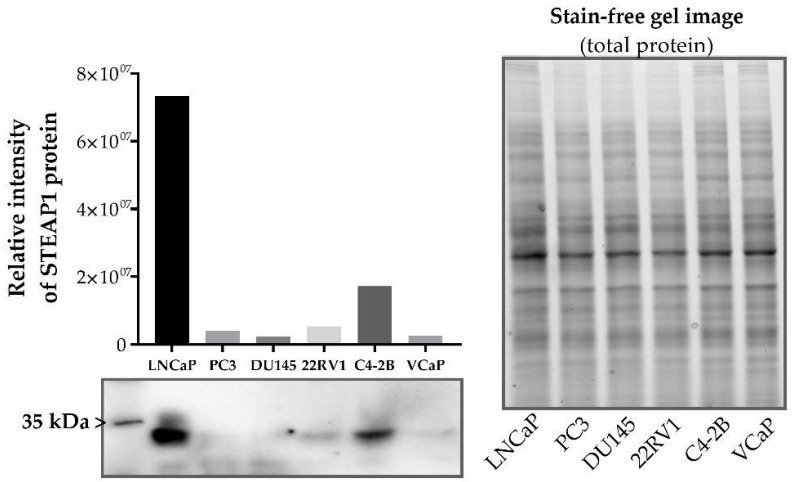

Figure VI.5. Comparison of STEAP1 expression levels in different PCa cell lines. Relative Immunoreactivity of STEAP1 in PCa cell lines (LNCaP, PC3, DU145, 22RV1, C4-2B and VCaP) by Western blot.

The silencing of the STEAP1 gene in LNCaP and C4-2B cells was performed, and the effect of chemotherapeutics paclitaxel, docetaxel, or cabazitaxel in restoring STEAP1 expression was evaluated. The levels of STEAP1 mRNA and protein were determined by RT-qPCR and Western blot, respectively. As indicated in Figure VI.6, the STEAP1 mRNA and protein levels were significantly diminished in the STEAP1 siRNA group of both cell lines (87 ± 0.01% and 80 ± 0.05% reduction for mRNA and protein, respectively, to LNCaP cells; 68 ± 0.003% and 45 ± 0.01% reduction for mRNA and protein, respectively, to C4-2B cells) compared to scramble siRNA.

LNCaP and C4-2B cells transfected with scramble siRNA or STEAP1 siRNA were treated with 5 nM paclitaxel, 20 nM docetaxel or 1 nM cabazitaxel for 24 h. No significant differences were observed in STEAP1 mRNA expression of LNCaP cells with normal STEAP1 expression levels (scramble siRNA group) upon treatment with paclitaxel or cabazitaxel treatment (Figure VI.6a). However, paclitaxel and cabazitaxel induced a significant increase in STEAP1 protein expression compared with the scramble siRNA condition (1.845 ± 0.19- vs. 1.016 ± 0.06- and 1.536 ± 0.27- vs. 1.016 ± 0.06-fold variation, respectively, Figure VI.6c). In opposition, docetaxel treatment significantly decreased STEAP1 mRNA levels in LNCaP cells transfected with scramble siRNA (0.629 ± 0.15- vs. 1.085 ± 0.04-fold variation, Figure VI.6a). Relatively to C4-2B cells, no significant differences were observed in STEAP1 mRNA and protein expression upon treatment with paclitaxel, docetaxel and cabazitaxel in scramble siRNA or STEAP1 siRNA conditions (Figure VI.6b and 6d). None of the tested chemotherapeutics drugs altered the effect of silencing STEAP1 in LNCaP and C4-2B cells, concerning the expression of STEAP1 mRNA and protein (Figure VI.6).

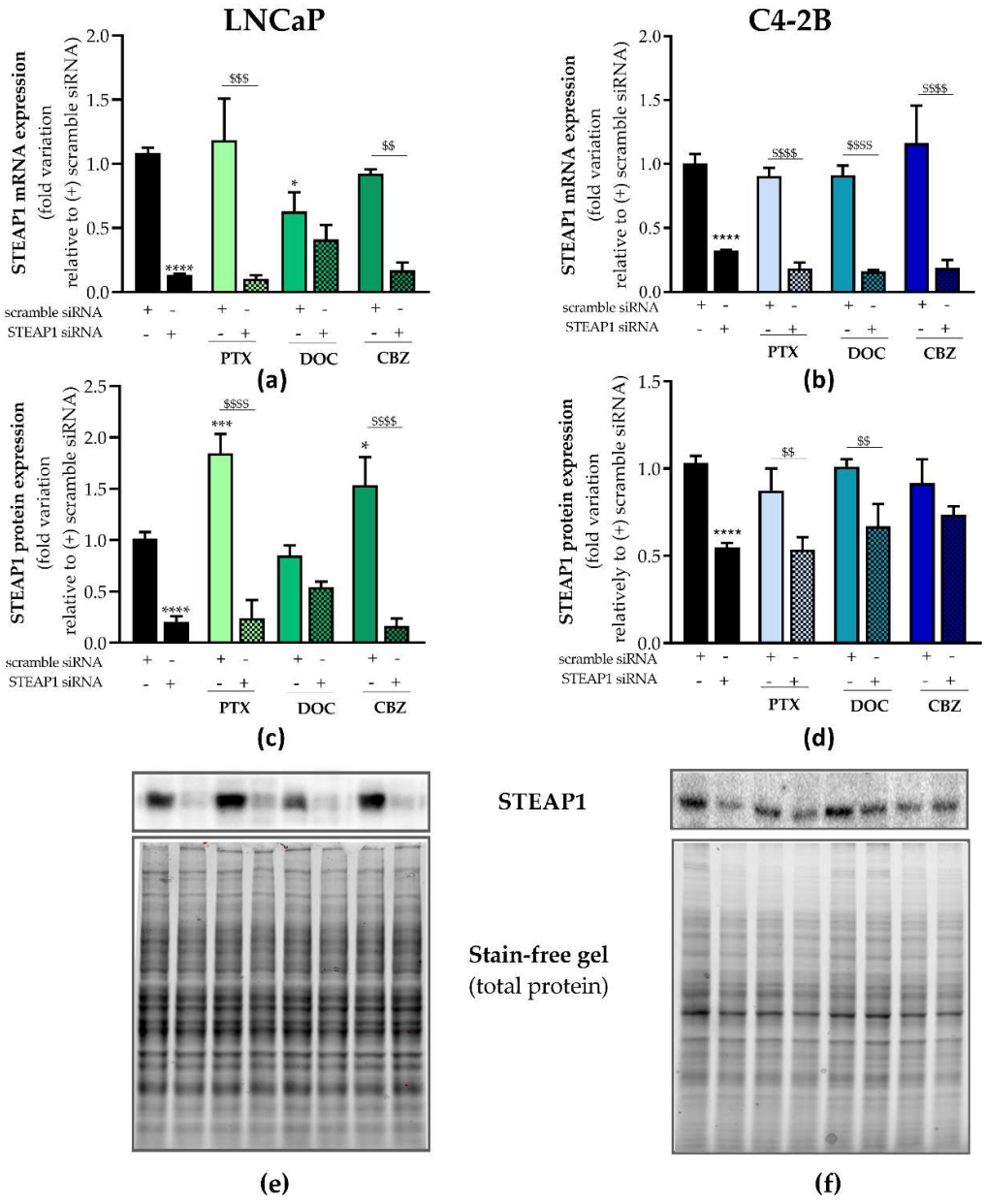

Figure VI.6. STEAP1 silencing in LNCaP and C4-2B cells and effect of taxane-based drugs on STEAP1 expression. Human neoplastic LNCaP and C4-2B prostate cells were transfected with scramble or STEAP1 small interfering RNA (siRNA) for 24 h and treated with 5 nM paclitaxel (PTX), 20 nM docetaxel (DOC) or 1 nM cabazitaxel (CBZ) for an additional 24 h. (a,b) Relative STEAP1 mRNA expression determined by RT-qPCR after normalization with the β2M housekeeping gene. (c,d) Relative STEAP1 protein expression determined by Western blot analysis after normalization with total protein. (e,f) SDS-PAGE gels' representative image. The symbol "+" or "-"means presence or absence of the types of siRNA used in each experimental group. Results are represented as fold-variation in comparison to scramble siRNA (control group). Error bars show mean ± S.E.M (n ≥ 3). * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ in comparison with the scramble siRNA group; and $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ and $^{\$\$\$\$}$ $p < 0.0001$.

6.8.2. Effect of STEAP1 Gene Knockdown Associated with Taxane-Based Drugs on PCa Cells Viability

The viability of scramble or STEAP1 siRNA-transfected PCa cells after treatment with paclitaxel docetaxel or cabazitaxel was determined by the MTT assay. STEAP1-knockdown diminished the viability of LNCaP and C4-2B cells by 47.2 ± 11.8% and 48.7 ± 12.9%, respectively (Figure VI.7). Also, paclitaxel (5 nM), docetaxel (20 nM) and cabazitaxel (1 nM) significantly decreased the viability of mock-transfected (scramble siRNA) LNCaP cells (49.33 ± 12.8%, 32.97 ± 5.1% and 47.06 ± 9.5%, respectively) and C4-2B cells (46.2 ± 11.9%, 74.6 ± 0.6% and 61.4 ± 4.9%, respectively), compared to scramble siRNA control (Figure VI.7). Paclitaxel-, docetaxel-, and cabazitaxel-treated LNCaP cells knocked down for STEAP1 exhibited approximately two-fold higher viability than mock-transfected LNCaP cells treated with chemotherapeutic drugs (represented with $, Figure VI.7). In addition, these drugs stimulated the cell viability in LNCaP cells knocked down for STEAP1 when compared to the respective control group (represented with #, Figure VI.7). Concerning C4-2B cells, a similar effect was only observed with the cabazitaxel treatment.

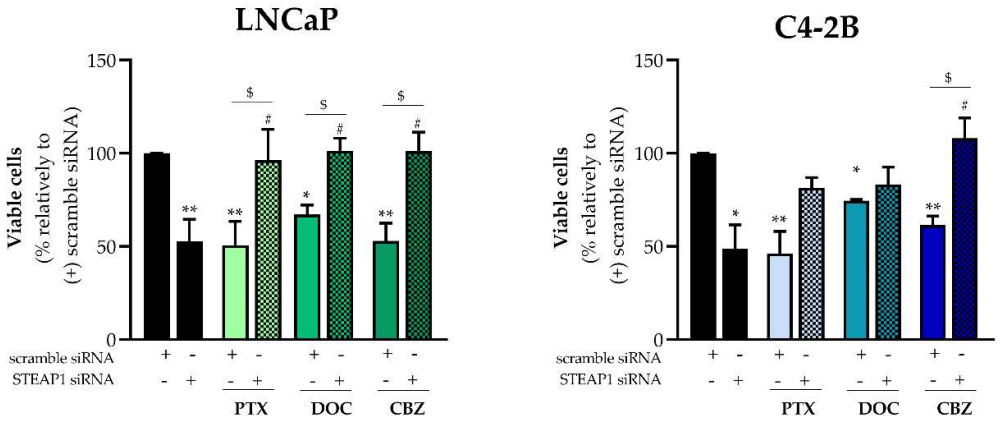

Figure VI.7. Effect of STEAP1 knockdown and paclitaxel, docetaxel, or cabazitaxel treatment on PCa cell viability. Human neoplastic LNCaP and C4-2B prostate cells were transfected with scramble or STEAP1 small interfering RNA (siRNA) for 24 h, and treated with 5 nM paclitaxel (PTX), 20 nM docetaxel (DOC) or 1 nM cabazitaxel (CBZ) for an additional 24 h. The symbol "+"or "-"means presence or absence of the type of siRNA used in each experimental group. The percentage of LNCaP and C4-2B viable cells was determined by MTT assay. Results are expressed as fold variation relative to the scramble siRNA group (control condition). Error bars show mean ± S.E.M (n ≥ 2). * $p < 0.05$ and ** $p < 0.01$ in comparison to the scramble siRNA group; # $p < 0.05$ when compared with the STEAP1 siRNA group; and $ $p < 0.05$.

6.8.3. Effect of STEAP1 Knockdown and Chemotherapeutic Drugs in Survival Pathways

To better understand how STEAP1 knockdown reduces PCa cell viability and suppresses the effect of taxane-based drugs, the expression of target proteins associated with cell survival pathways was evaluated. The results of Western blot analysis demonstrated that the expression of phosphorylated-AKT (pAKT) and -ERK (pERK) isoforms decreased in LNCaP and C4-2B cells knocked down for STEAP1 relative to the scramble siRNA transfected cells (0.709 ± 0.02- vs. 1.001

± 0.002-fold variation and 0.834 ± 0.01- vs. 1.02 ± 0.024-fold variation, respectively to LNCaP cells, Figure VI.8a and 8b; 0.678 ± 0.05-vs. 1.009 ± 0.003-fold variation and 0.710 ± 0.006- vs. 1.034 ± 0.03-fold variation, respectively to C4-2B cells, Figure VI.8d and e). Treatment of scramble siRNA transfected-cells with 5 nM paclitaxel, 20 nM docetaxel and 1 nM cabazitaxel significantly decreased pAKT in LNCaP cells (0.738 ± 0.04- vs. 1.001 ± 0.002-, 0.490 ± 0.01- vs. 1.001 ± 0.002-, and 0.546 ± 0.10- vs. 1.001 ± 0.002-fold variation, respectively, Figure VI.8a) and in C4-2B cells (0.695 ± 0.03- vs. 1.009 ± 0.003-, 0.670 ± 0.03- vs. 1.009 ± 0.003-, and 0.7 ± 0.002-vs. 1.009 ± 0.003-fold variation, respectively, Figure VI.8d). The same treatment also significantly decreased pERK in LNCaP cells (0.804 ± 0.01- vs. 1.020 ± 0.02-, 0.865 ± 0.04- vs. 1.020 ± 0.02-, and 0.677 ± 0.04- vs. 1.020 ± 0.02-fold variation, respectively, Figure VI.8b) and in C4-2B cells (0.771 ± 0.005- vs. 1.034 ± 0.03-, 0.720 ± 0.02- vs. 1.034 ± 0.03-, and 0.772 ± 0.005- vs. 1.034 ± 0.03-fold variation, respectively, Figure VI.8e) relative to the scramble siRNA control group. However, the silencing of STEAP1 in LNCaP cells significantly abolished the effect of paclitaxel, docetaxel, and cabazitaxel in suppressing pAKT. Furthermore, it should be highlighted that chemotherapeutic drugs increased the pAKT expression two-fold when the STEAP1 gene was knocked down in LNCaP cells (Figure VI.8a). Regarding pERK expression levels in LNCaP cells, the silencing of STEAP1 did not reverse the effect of paclitaxel or docetaxel, whereas the down-regulation of pERK in cabazitaxel-treated LNCaP cells knocked down for STEAP1, was reversed (0.677 ± 0.05- vs. 0.867 ± 0.06-fold variation, Figure VI.8b). Relatively to C4-2B cells, the silencing of STEAP1 also significantly reversed the effect of docetaxel and cabazitaxel in pAKT expression (0.67 ± 0.03- vs. 0.941 ± 0.03-, and 0.7 ± 0.002- vs. 0.896 ± 0.003-fold variation, respectively, Figure VI.8d). Also, docetaxel or cabazitaxel induced a significantly increased expression of pAKT in C4-2B cells knocked down for STEAP1.

Regarding the c-myc transcription factor, the levels of phosphorylated c-myc (p-cmyc) were significantly increased in LNCaP and C4-2B cells upon silencing the STEAP1 gene. p-c-myc expression in the STEAP1 siRNA group when compared to the scramble siRNA control was 1.846 ± 0.13- vs. 1.000 ± 0.03-fold variation and 2.401 ± 0.323- vs. 0.993 ± 0.02-fold variation (Figure VI.8c and 8f, respectively). Treatment of scramble siRNA mock-transfected LNCaP cells with paclitaxel, docetaxel, and cabazitaxel strongly induced the expression of p-c-myc (30.848 ± 1.07- vs. 1.000 ± 0.03-, 7.401 ± 4.67- vs. 1.000 ± 0.03-, and 21.077 ± 0.06- vs. 1.000 ± 0.03-fold variation, respectively, Figure VI.8c). The silencing of STEAP1 in LNCaP cells significantly reduced the cabazitaxel-induced p-c-myc expression (9.508 ± 1.440- vs. 21.077 ± 0.06-fold variation, Figure VI.8c), but no differences were found in the response to paclitaxel or docetaxel (Figure VI.8c). However, the expression of p-c-myc was significantly induced by paclitaxel, docetaxel, or cabazitaxel in LNCaP cells silenced for STEAP1 when compared to LNCaP cells silenced for STEAP1 (Figure VI.8c). Concerning C4-2B cells, the effect was similar to that observed in LNCaP cells but not so markedly (Figure VI.8f). Since the results obtained in LNCaP and C4-2B cells are overall similar in all experiments, LNCaP cells were chosen to explore the proliferative and apoptotic pathways underlying the effects of chemotherapeutic drugs when the STEAP1 gene is knocked down.

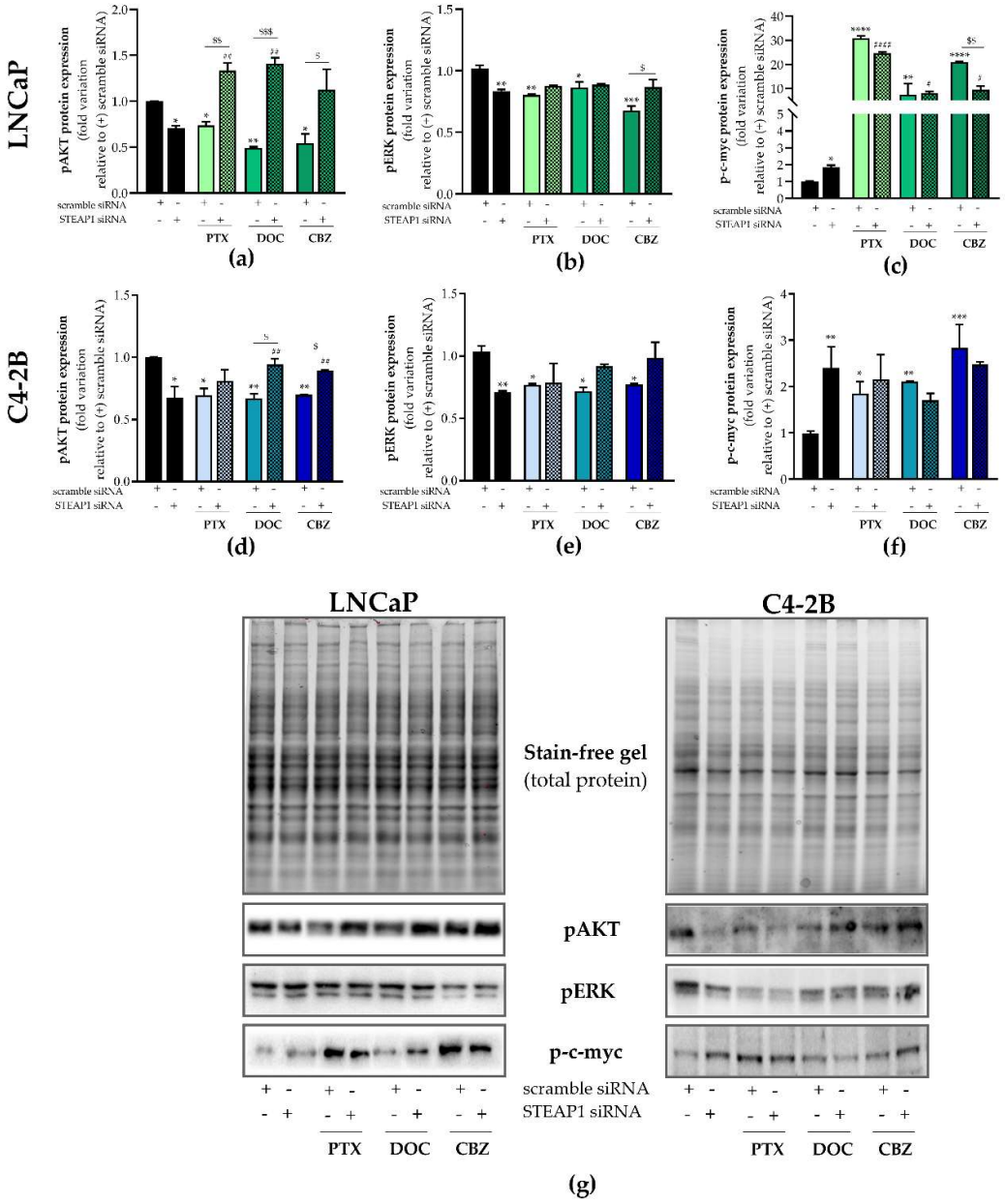

Figure VI.8. The effect of STEAP1 knockdown and paclitaxel, docetaxel, or cabazitaxel on the protein expression levels of the survival pathway regulators of PCa cells. Human neoplastic LNCaP and C4-2B prostate cells were transfected with scramble or STEAP1 small interfering RNA (siRNA) for 24 h and treated with 5 nM paclitaxel (PTX), 20 nM docetaxel (DOC) or 1 nM cabazitaxel (CBZ) for an additional 24 h. The symbol "+"or "-"means presence or absence of the type of siRNA used in each experimental group. Expression of (a,d) phosphorylated AKT, (b,e) ERK and (c,f) c-myc proteins was determined by Western blot analysis after normalization with total protein. (g) SDS-PAGE gel and representative immunoblots. Error bars indicate mean ± S.E.M (n ≥ 2). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ when compared with the scramble siRNA group; # $p < 0.05$, ## $p < 0.01$ and #### $p < 0.0001$ when compared with the STEAP1 siRNA group; and $ $p < 0.05$, $$ $p < 0.01$ and $$$ $p < 0.001$.

6.8.4. Effect of STEAP1 Knockdown and Chemotherapeutic Drugs in Proliferative Activity

Immunofluorescence staining of the nuclear proliferation marker ki-67 was used to evaluate the proliferation of LNCaP cells in different experimental conditions. The number of Ki-67-stained cells was significantly decreased in LNCaP cells knocked down for STEAP1, being 54.5 ± 1.01% when compared to scramble siRNA transfected cells (Figure VI.9a). Also, the results of fluorescent immunocytochemistry showed that the number of Ki-67-positive cells was significantly decreased in scramble siRNA-transfected LNCaP cells after treatment with 5 nM paclitaxel, 20 nM docetaxel or 1 nM cabazitaxel (50.5 ± 0.91%, 62.2 ± 3.6%, and 64.0 ± 1.54%, respectively, Figure VI.9a). The inhibitory effect on cell proliferation caused by paclitaxel, docetaxel, and cabazitaxel treatment was reversed in LNCaP cells knocked down for STEAP1. In addition, the chemotherapeutic drugs increased the number of Ki-67- positive cells in LNCaP cells knocked down for STEAP1 relative to LNCaP cells silenced for STEAP1 (Figure VI.9a). The p21 protein, a well-established cyclin-dependent kinase inhibitor, it is described as having an important role in controlling cell cycle progression [36]. Our results showed that STEAP1 knockdown induced p21 mRNA expression levels (2.161 ± 0.16- vs. 0.965 ± 0.026-fold variation, Figure VI.9b). Treatment with paclitaxel, docetaxel, or cabazitaxel also significantly increased p21 mRNA levels in scramble siRNA-transfected LNCaP cells (1.955 ± 0.05- vs. 0.965 ± 0.03-, 1.958 ± 0.03- vs. 0.965 ± 0.03-, and 2.390 ± 0.02- vs. 0.965 ± 0.03-fold variation, respectively, Figure VI.9b). The knockdown of the STEAP1 gene decreased the expression of p21 induced by chemotherapeutic drugs (1.331 ± 0.08- vs. 1.955 ± 0.05-fold variation to paclitaxel, 1.174 ± 0.09- vs. 1.958 ± 0.03-fold variation to docetaxel and 1.339 ± 0.05- vs. 2.390 ± 0.02-fold variation to cabazitaxel, Figure VI.9b). In addition, a significant reduction in p21 expression was triggered by chemotherapeutic drugs in LNCaP cells knocked down for STEAP1 in comparison with the respective control group (Figure VI.9b).

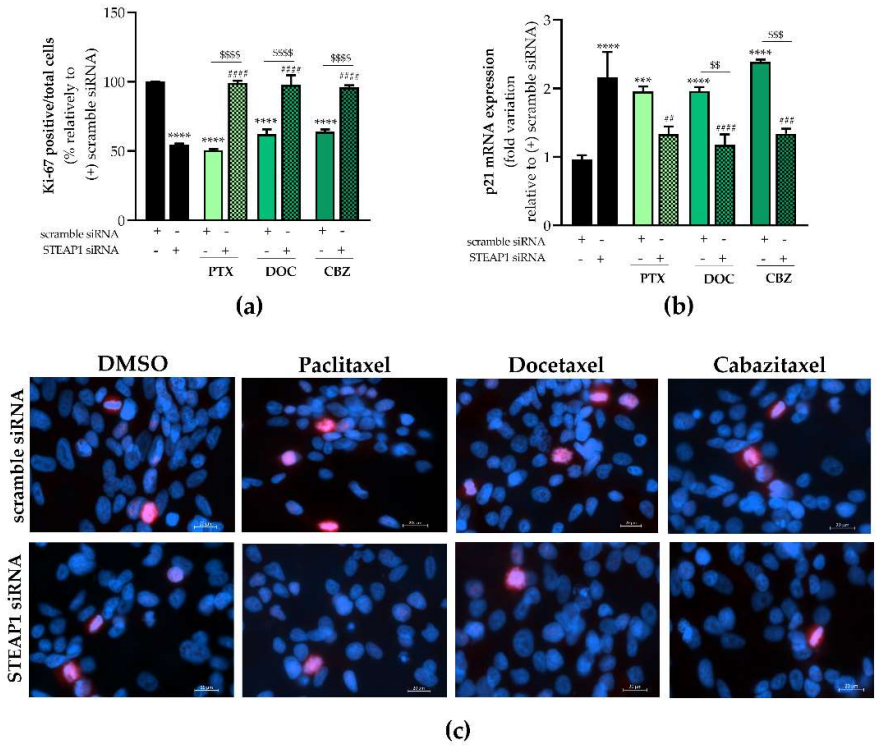

Figure VI.9. Figure 5. Effect of STEAP1 knockdown and paclitaxel, docetaxel or cabazitaxel on the proliferation activity on LNCaP cells. Human neoplastic LNCaP prostate cells were transfected with scramble or STEAP1 small interfering RNA (siRNA) for 24 h and treated with 5 nM paclitaxel (PTX), 20 nM docetaxel (DOC) or 1 nM cabazitaxel (CBZ) for an additional 24 h. The symbol "+" and "-" means presence or absence of the type of siRNA used in each experimental group. (a) Results of Ki67 immunofluorescence analysis, representing the number of Ki-67-positive cells out of total cell number. Expression of (b) p21 mRNA; expression levels were determined by RT-qPCR analysis after normalization with β2M housekeeping gene. (c) Representative fluorescent immunocytochemistry images of Ki-67-labeled cells (red) and Hoechst 33342 stained nuclei (blue) obtained in the AxioImager Z2 microscope under 400× magnification. Ten randomly selected fields per microscope cover glass were assessed. Results are expressed as percentage or fold-variation relative to scramble siRNA (control group). Error bars indicate mean ± S.E.M (n ≥ 2). *** $p < 0.001$ and **** $p < 0.0001$ when compared with the scramble siRNA group; ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$ when compared with the STEAP1 siRNA group; and $$ $p < 0.01$, $$$ $p < 0.001$ and $$$$ $p < 0.0001$.

6.8.5. Effect of STEAP1 Knockdown and Chemotherapeutic Drugs in Apoptosis

The apoptotic status of LNCaP cells knocked down for STEAP1 and exposed to paclitaxel, docetaxel, and cabazitaxel was also evaluated. The results of Figure VI.10 showed that the pro-/antiapoptotic Bax/Bcl-2 ratio increased after STEAP1 knockdown in LNCaP cells (1.670 ± 0.04- vs. 0.993 ± 0.02-fold variation, Figure VI.10a). The ratio of Bax/Bcl-2 was also increased in scramble siRNA-transfected LNCaP cells treated with chemotherapeutic drugs (1.671 ± 0.02- vs. 0.993 ± 0.02-fold variation to paclitaxel, 1.512 ± 0.08- vs. 0.993 ± 0.02-fold variation to docetaxel and 1.444 ± 0.04- vs. 0.993 ± 0.02-fold variation to cabazitaxel, Figure VI.10a). The silencing of STEAP1 in

LNCaP cells significantly reversed the effect of paclitaxel (1.413 ± 0.01- vs. 1.671 ± 0.02-fold variation, Figure VI.10a) and cabazitaxel (1.444 ± 0.04- vs. 0.837 ± 0.02-fold variation, Figure VI.10a) treatment in increasing Bax/Bcl-2 ratio. Also, a significant decrease in the Bax-Bcl-2 ratio was observed in STEAP1 siRNA LNCaP cells treated with chemotherapeutic drugs when compared to the STEAP1 siRNA group (Figure VI.10a). The tumor suppressor protein p53 was also evaluated. As shown in Figure 6b, a strong increase in p53 expression levels was detected in LNCaP cells silenced for STEAP1 (2.004 ± 0.08- vs. 1.001 ± 0.001-fold variation). Similar effects were seen in scramble siRNA mock-transfected LNCaP cells, upon paclitaxel, docetaxel, or cabazitaxel treatment, with the induced expression of p53 (2.452 ± 0.05- vs. 1.001 ± 0.001-, 2.164 ± 0.302- vs. 1.001 ± 0.001-, and 2.535 ± 0.04- vs. 1.001 ± 0.001-fold variation, respectively, Figure VI.10b). STEAP1 knockdown abolished the effect of chemotherapeutic drugs, decreasing p53 expression compared to the respective scramble siRNA drug-treated group (1.715 ± 0.05- vs. 2.452 ± 0.05-fold variation to paclitaxel, 1.744 ± 0.17- vs. 2.164 ± 0.30-fold variation to docetaxel, and 1.762 ± 0.13- vs. 2.535 ± 0.04-fold variation to cabazitaxel, Figure VI.10b).

Apoptosis is triggered by the caspase enzymes, and intrinsic and extrinsic pathways converge at the activation of caspase-3, which is considered a remarkable endpoint of apoptosis [37]. Caspase-3-like activity significantly increased in response to STEAP1 knockdown (~94% ± 0.04 relative to scramble siRNA, Figure VI.10c). With similar magnitude effects, paclitaxel, docetaxel or cabazitaxel significantly increased caspase-3-like activity in LNCaP cells knocked down for STEAP1 in comparison with the scramble siRNA control group (~86% ± 0.11, ~66% ± 0.13 and ~83% ± 0.12 increase, respectively, Figure VI.10c). In STEAP1- knockdown LNCaP cells, the increased effect of chemotherapeutic drugs in caspase-3-like activity was abolished (Figure VI.10c). Moreover, the chemotherapeutic drugs reduced the activity of caspase-3 in LNCaP cells knocked down for STEAP1 when compared to the respective control group (Figure VI.10c). Considering apoptosis based on the TUNEL assay, STEAP1-knockdown significantly increased the number of TUNEL-stained LNCaP cells compared to the scramble siRNA transfected cells (1.951 ± 0.12- vs. 1.002 ± 0.004-fold variation, Figure VI.10d). It was also found that in LNCaP cells transfected with scramble siRNA, the number of TUNEL-positive cells was significantly increased after treatment with paclitaxel, docetaxel, or cabazitaxel (1.872 ± 0.16- vs. 1.002 ± 0.004-fold variation, 1.867 ± 0.10- vs. 1.002 ± 0.004-fold variation and 2.081 ± 0.29- vs. 1.002 ± 0.004-fold variation, respectively, Figure VI.10d). The effect of chemotherapeutics was reversed when STEAP1 was knocked down in LNCaP cells (Figure VI.10d). Furthermore, the number of TUNEL-stained LNCaP cells was significantly decreased in cells silenced for STEAP1 and treated with paclitaxel or docetaxel drugs relative to the respective control group (Figure VI.10d).

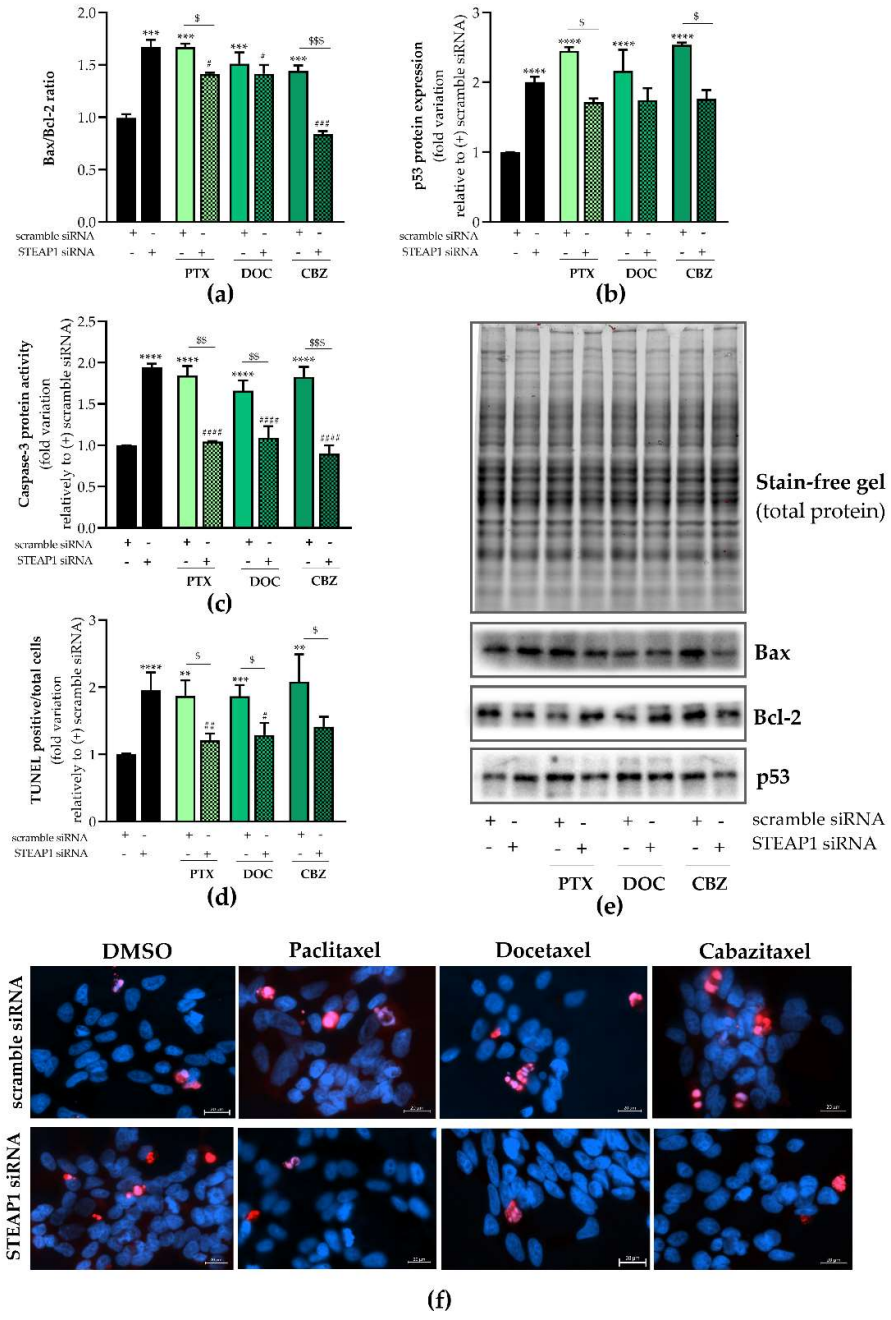

Figure VI.10. Effect STEAP1 knockdown and paclitaxel, docetaxel, or cabazitaxel on LNCaP cells apoptosis. Human neoplastic LNCaP prostate cells were transfected with scramble or STEAP1 small interfering RNA (siRNA) for 24 h and treated with 5 nM paclitaxel (PTX), 20 nM docetaxel (DOC) or 1 nM cabazitaxel (CBZ) for anadditional 24 h. The symbol "+" and "-" means presence or absence of the type of siRNA used in each experimental group. (a) Bax/Bcl-2 protein ratio and (b) p53 protein was determined by Western blot analysis, after normalization with total protein. (e) Representative SDS-PAGE gels and immunoblots. (c) Caspase-3-like activity measured spectrophotometrically through the detection of p-NA. (d) TUNEL-positive LNCaP cell relatively to total cell number. (f) Representative fluorescent immunocytochemistry images of TUNEL-labeled cells (red) and Hoechst 33342 stained nuclei (blue) obtained in the AxioImager Z2 microscope under 400× magnification. Ten randomly selected fields per microscope cover glass were assessed. Results are shown as fold change relative to the scramble siRNA group (control). Error bars shows mean ± S.E.M (n ≥ 2). ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ when compared with the scramble siRNA group; # $p < 0.05$, ## $p < 0.01$, ### $p <$

0.001 and #### $p < 0.0001$ when compared with the STEAP1 siRNA group; $ $p < 0.05$, $$ $p < 0.01$ and $$$ $p < 0.001$

6.9. Discussion

In the last few years, several pieces of evidence have associated STEAP1 with being an oncogenic protein driving the progression of several human cancers, particularly PCa [4, 10, 12, 13, 15, 16]. Some strategies have been developed targeting the STEAP1 protein as a potential treatment of PCa. In fact, it has been shown that monoclonal antibodies designed against STEAP1 can inhibit PCa in mice models [2]. Our research group established that the STEAP1 knockdown reduced PCa cell growth accompanied by the enhanced rate of apoptosis [11]. One of the main strategies in cancer therapy is the use of chemotherapeutic drugs, such as paclitaxel, docetaxel and cabazitaxel, which have emerged as the treatment of choice in PCa patients [22, 26]. However, most patients develop resistance to these drugs due to changes in the expression of oncogenes [38]. Recently, it was shown that STEAP1 did not alter the response of PCa cells to anti-androgen treatment [39]. Taking into account that there are no studies evaluating the relationship between STEAP1 and taxane-based chemotherapeutics, this study intended to explore the effect of chemotherapeutic drugs in modulating the expression of STEAP1, as well as to evaluate the role of STEAP1 in influencing the response of PCa cells to chemotherapeutic drugs.

As a first approach, it was investigated whether treatment with paclitaxel, docetaxel or cabazitaxel would modify the expression of STEAP1 protein in PCa cells (Figure VI.6). In LNCaP cells, it was found that both paclitaxel and cabazitaxel treatment promoted a significant increase in STEAP1 protein expression, whereas no significant effect was observed in response to docetaxel. Currently, no definitive conclusion can be drawn, but the increase in STEAP1 expression may be a way for cells to overcome the effects of paclitaxel and cabazitaxel. Curiously, the STEAP1 knockdown in LNCaP cells upon transfection with the STEAP1 siRNA was reversed in the presence of docetaxel, but not paclitaxel or cabazitaxel, which suggests that STEAP1 may also be a mediator counteracting the docetaxel effects in LNCaP cells. Regarding the C4-2B cells, no differences were observed in STEAP1 expression upon treatment with chemotherapeutic drugs. Altogether, these results suggest that the effect of chemotherapeutic drugs may be dependent on the characteristics of PCa cells.

Next, it was analyzed whether silencing STEAP1 may affect the action of chemotherapeutic drugs in controlling the cell viability. The STEAP1 gene silencing decreased the viability of LNCaP and C4-2B cells, as indicated by MTT assay (Figure Vi.7). These results are in agreement with previous studies performed by our research group [11]. As expected, LNCaP and C4-2B cell viability decreased with a taxane-based drug treatment (Figure VI.7). Similar studies showed that treatment of PCa cells with paclitaxel, docetaxel, and cabazitaxel triggered cytotoxic effects inducing apoptosis [40–42]. Noteworthy, STEAP1 knockdown abolished the effect of taxane-based chemotherapeutics increasing LNCaP and C4-2B cell viability (Figure VI.7). In addition, it should be highlighted that these chemotherapeutic drugs increased the cell viability of PCa cells knocked down for STEAP1. These results are very interesting and are the first report indicating that the use of taxane-based drugs combined with STEAP1 knockdown may not only be ineffective but even deleterious in PCa with

reduced levels of STEAP1. This was an unexpected finding since a previous study showed that the downregulation of STEAP1 significantly increased the chemosensitivity of gastric cancer cells to docetaxel treatment [43]. However, considering that the STEAP1 protein seems to act as a channel for small molecules [2], it is plausible that these drugs may also enter cells through the STEAP1 protein, or the molecules exchange across the cell membranes through the STEAP1 protein result in better uptake of these drugs (or are less extruded from the PCa cells). This hypothesis is supported by other studies involving channel proteins with a similar structure to STEAP1, such as the TRPM7 protein, which is also overexpressed in PCa and act as an oncoprotein. The knockdown of TRPM7 suppressed the migration, invasion, and proliferation of PCa cells [44–46]. On the other hand, the suppression of TRPM7 increased the cell viability in response to doxorubicin, indicating that reduced expression of TRPM7 may be associated with resistance to doxorubicin [47].

In order to deepen the knowledge underlying the role of STEAP1 and chemotherapeutic drugs in PCa progression, AKT and ERK signaling pathways were analyzed. AKT is one of the major downstream effectors of the PI3K signaling pathway to mediate cell survival, and ERK is another kinase that also regulates cell proliferation and survival of PCa cells [48, 49]. The knockdown of STEAP1 decreased the levels of pAKT and pERK isoforms in PCa cells (Figure VI.8), which underpinned the diminished cell viability of PCa cells (Figure VI.7). As expected, the proliferative activity decreased in response to treatment with chemotherapeutic agents. In addition, and supporting the results obtained with MTT assay, the anti-proliferative effect of STEAP1 knockdown or drug treatment alone was abolished when both treatments were applied simultaneously. Altogether, our results indicate that these chemotherapeutic drugs may induce cell growth and proliferation in PCa cells with low levels of STEAP1. It is well-established that AKT is associated with cell survival due to the inhibition of pro-apoptotic proteins (e.g., Bax) and the activation of anti-apoptotic ones (e.g., Bcl-2) [50]. Also, the increase of the Bax/Bcl-2 ratio may induce apoptosis through the activation of caspase-3 [51]. Therefore, we have explored the role of STEAP1 and chemotherapeutic drugs in the apoptotic process. The STEAP1 knockdown or treatment with chemotherapeutic drugs significantly increased the Bax/Bcl-2 ratio and caspase-3-like activity (Figure VI.10), suggesting that the inhibition of apoptosis due to overexpression of STEAP1 in LNCaP cells may be mediated by the activation of AKT. Also, the AKT signaling may be linked with the inactivation of the tumor suppressor p53 protein [52, 53], which is involved in cell cycle arrest and apoptosis activation. Our results, which showed an increase in p53 expression when PCa cells were knocked down for STEAP1 or treated with chemotherapeutic drugs (Figure VI.10), support this connection. The role of p53 in cell cycle arrest is supported by the increased expression of p21 mRNA (Figure VI.9), a p53 responsive-gene that encodes an inhibitor protein of cyclin-dependent kinase at the G1 phase of the cell cycle [54]. These results are also supported by the diminished levels of pERK in response to STEAP1 knockdown or treatment with chemotherapeutic drugs. Similar results have been reported for other oncoproteins in cancer cells [55–58]. Altogether, the results obtained herein suggest that the STEAP1 knockdown and the effects of chemotherapeutic drugs in cell proliferation and apoptosis may be mediated by the MAPK and PI3K/AKT signaling pathways. However, the STEAP1 silencing combined with taxane-based drugs considerably increased the levels of pAKT in

PCa cells, and that was concomitant with the increased expression of pERK levels, indicating that these drugs may cause harmful effects in PCa with a low expression of STEAP1.

The transcription factor c-myc is essential for cell proliferation and is one of the most frequently activated oncogenes, important for cancer growth and invasion [59]. Moreover, c-myc can also induce apoptosis in several cell types and appears to be a major regulator of apoptotic responses induced by a variety of stimuli, such as hypoxia, glucose deprivation, and DNA damage induced by cancer chemotherapeutics [59, 60]. Noteworthy, p-c-myc expression increased in response to STEAP1 knockdown and was also drastically increased in LNCaP cells after treatment with paclitaxel, docetaxel and cabazitaxel (Figure VI.8). This increase was maintained when PCa cells were silenced for STEAP1 and treated with chemotherapeutic drugs, except for cabazitaxel where there was a significant reversal of the increase in p-c-myc expression levels. This increase in p-c-myc expression in PCa cells knocked down for STEAP1 may be a molecular mechanism to counteract the anti-proliferative action of STEAP1 knockdown. However, and considering the dual role of c-myc in cells, additional studies are required to clarify the biological significance of increased levels of c-myc upon STEAP1 knock-down in PCa cells. Nevertheless, some studies have described mechanisms that allow increased levels of c-myc in PCa due to inhibition of ubiquitin-mediated proteasomal degradation [59, 61]. This mechanism of c-myc stabilization may be linked to the dysregulation of ERK and GSK signaling, since for c-myc degradation an initial ERK-mediated serine 62 phosphorylation is required, followed by a phosphorylation at threonine 58 by GSK-3β [61]. As ERK protein expression levels are decreased in PCa cells silenced for STEAP1, or exposed to taxanes-based drugs, this may lead to an increase in c-myc protein stability. This premise is also supported by the cabazitaxel treatment, the effect of which is reversed by the knockdown of STEAP1. In the literature, there are contradictory results regarding the effect of chemotherapeutic drugs in the expression of p-c-myc. Various studies showed a down-regulation of c-myc expression in PCa cells treated with paclitaxel, docetaxel, and cabazitaxel [40, 62, 63]. Contrastingly, in addition to the fact that c-myc is overexpressed in CRPC and its expression correlated with poor outcomes [64], there are studies revealing the overexpression of c-myc in PCa cells after docetaxel treatment leads to tumorigenesis [65]. Others also showed that chronic paclitaxel treatment in metastatic CRPC cells promotes the development of resistance via upregulating c-myc expression [66]. Gathering together this information, it suggests that overexpression of c-myc could provide conditions to development resistance of taxanes-based chemotherapeutics.

Overall, our results revealed that taxane-based chemotherapeutics are more effective in inducing apoptosis and suppressing viability and proliferation of PCa cells that overexpress the STEAP1 protein. Furthermore, it is important to emphasize that these chemotherapeutic drugs may have a detrimental effect on PCa cells with a decreased expression of STEAP1. Overall, our results revealed that taxane-based chemotherapeutics are more effective in inducing apoptosis and suppressing viability and proliferation of PCa cells that overexpress the STEAP1 protein. Furthermore, it is important to emphasize that these chemotherapeutic drugs may have a detrimental effect on PCa cells with a decreased expression of STEAP1.

6.10. Conclusion

Taxane-based chemotherapeutics have distinct effects on PCa cells depending on STEAP1 protein expression levels. Moreover, it was addressed for the first time the effect of these drugs in combination with the overexpression or knockdown of STEAP1 oncoprotein expression in PCa cells. It allowed to conclude that the use of paclitaxel, docetaxel, and cabazitaxel is more effective in PCa cells that overexpress the STEAP1 protein. Although further studies are required, it is worth noting that PCa cells with reduced expression of STEAP1 may cause harmful effects in response to chemotherapeutic drugs.

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Chapter 7

SUMMARIZING DISCUSSION

Summarizing discussion and future perspectives

Despite the continuous research efforts contributing to improve diagnosis and treatment methods, PCa continues to be a worrying disease with high levels of prevalence and mortality for men worldwide. Basic and clinical research have contributed enormously to the understanding of the cellular and molecular basis of PCa, and leading to important improvements on the diagnosis and treatment of this pathology. However, the identification of new biomarkers and therapeutic targets, to distinguish indolent PCa from aggressive forms of disease, as well as to predict treatment responses and progression, remain an unmet clinical need. Moreover, it is known that PCa is a complex disease that requires several genetic and epigenetic modifications to occur, and responding to environmental pressure and lifestyle. The genetic/epigenetic deregulation affects the expression and/or activity of several regulators of cell survival and proliferation, leading to a set of alterations associated with PCa onset and progression, as well as determining the emergence of more aggressive phenotypes.

STEAP1 is a protein presenting a structure with six transmembrane domains connected by extracellular loops. It is commonly located at the surface of the plasma membrane, being closely associated the with intercellular communication between tumour cells. STEAP1 is overexpressed in PCa and in several other types of cancer, but its expression pattern in non-neoplastic tissues is almost restricted to the prostate gland. These features make STEAP1 a very interesting target for PCa diagnosis and treatment and a promising candidate to be explored as an immunotherapeutic target and/or biomarker. However, as STEAP1 overexpression exists since the early stages of prostate carcinogenesis, occurring in pre-neoplastic and neoplastic lesions, its usefulness as a diagnostic biomarker fails. However, its value as a prognostic biomarker needed to be better established, together with the exploitation of the clinical significance of STEAP1 expression levels in the context of progression of disease and response to treatment. In Chapter 3 of this thesis, it was explored the clinical significance of STEAP1 protein expression in PCa unravelling its usefulness as a prognostic biomarker using databases and bioinformatics platforms. Furthermore, its relationship with the expression of the other STEAP family members (STEAP2, STEAP3 and STEAP4) was also investigated. We confirmed that STEAP1 is a gene differentially expressed in PCa, being overexpressed from benign lesions to metastases. This result suggested that the deregulation of STEAP1 expression levels may be involved in the malignant transformation of prostate and increasing the risk of PCa onset and development. The expression analysis of the other three STEAP family members indicated that STEAP2 and STEAP4 also are overexpressed in prostate tumours. In contrast, STEAP3 is underexpressed in PCa. The differential expression of these proteins appears to be of prognostic value, especially in the case of STEAP1. In this chapter, it was also verified that STEAP1 overexpression is associated with poor clinical outcomes, whereas STEAP4 offers better overall survival and progression-free survival. However, additional work should be performed in large scale clinical cohorts to definitively clarify the prognostic value of the STEAP1 protein.

The regulation of STEAP1 expression is an intricate process, which may involve several molecular mechanisms. It was already demonstrated that the DHT steroid hormone down-regulate STEAP1 expression by a mechanism independent of AR, since no ARE were found in promoter region

of the STEAP1 gene by activation of nuclear AR. On the other hand, potential post-transcriptional and post-translational modifications sites may be responsible for modulating the levels of the STEAP1 mRNA and protein. However, these alterations do not justify the overexpression of STEAP1 in tumour cells, suggesting that other mechanisms may be involved. Epigenetic mechanisms have been pointed out to promote the deregulation of gene expression and, consequently, to play an important role in PCa development and progression. In Chapter 4 it was evaluated the effect of epigenetic alterations in regulating STEAP1 expression in PCa (Figure VII.1.). We observed that the CpG island at the STEAP1 gene promoter had a lower methylation level in neoplastic cells compared with non-neoplastic prostate cells. This demethylation in human PCa seems to contribute to the overexpression of STEAP1. Alteration of CpG-island methylation might occur by the direct recruiting of DNMTs and HDACs. So, we also observed that the treatment of non-neoplastic cells with DNMT and HDAC inhibitors increased the *STEAP1* gene expression by a synergistic effect of combined hypomethylation and histone hyperacetylation. The finding obtained within this chapter suggested that STEAP1 overexpression in PCa can be driven by the hypomethylation of *STEAP1* gene promoter. However, considering the complexity of the mechanisms associated with HDACs, more studies are required to clarify their role in STEAP1 regulation, as well as to elucidate this association with PCa development and progression.

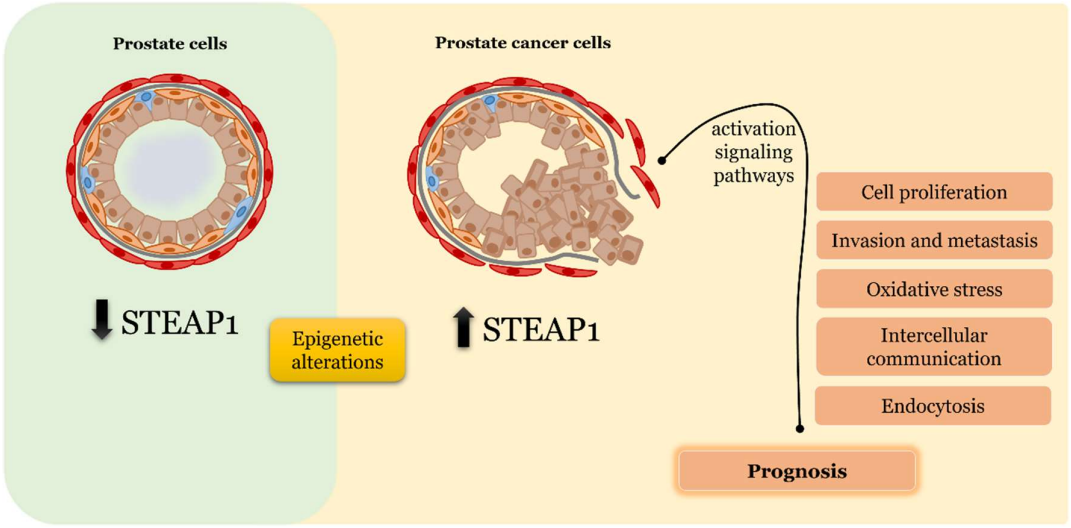

Figure VII.1. An integrative view of the regulation of STEAP1 expression in prostate cells and its potential action as an oncoprotein. In non-neoplastic prostate cells, STEAP1 is expressed at low levels. Several modifications and/or mutation of genes that regulate normal cellular function cause the deregulation of protein network and activity triggering transformation into cancer cells. Epigenetic alterations at the *STEAP1* gene promoter, among other changes, promote the overexpression of STEAP1 in PCa cells. High levels of STEAP1 in PCa tissue/cells activate several biological processes, including cell proliferation, invasion and metastization, oxidative stress and intercellular communication favouring prostate tumorigenesis and progression of disease. Overexpression of STEAP1 in human PCa samples is also directly correlated with lower survival of PCa patients meaning worse prognosis. Interestingly, the knockdown of STEAP1 counteracts these biological effects, diminishing cellular metabolism and inducing apoptosis, increasing the endocytosis of cell-surface receptors to reduce cell growth and reducing intracellular communication. These landscapes sustain STEAP1 as an interesting therapeutic protein to be used in PCa treatment in the future.

Although STEAP1 is definitely linked to PCa, there are several gaps concerning the molecular mechanisms underlying its function in prostate cells. Nowadays, the characterization of global proteome profiling is an important approach to identify signatures at cellular level in order to unravel the molecular functions and signaling pathways of proteins. Thus, Chapter 5 aimed to find expression changes in the proteome of PCa cells induced by STEAP1 knockdown. For that, the proteome-wide alteration of STEAP1 silencing-PCa cell lysates was measured using a label-free quantification combined with an Orbitrap LC-MS/MS system. Comparing the two experimental groups (STEAP1 siRNA *versus* scramble siRNA), 526 proteins were found to be differentially expressed, and systematic bioinformatical analysis of these differentially expressed proteins showed that endocytosis, RNA transport, apoptosis, aminoacyl-tRNA biosynthesis and metabolic pathways were the biological process strongly associated, suggesting that STEAP1 can play a role in these signaling pathways. The validation of key proteins of these signaling pathways in different extracts of PCa cells silenced for STEAP1, by the Western Blot technique, allowed us to reveal that STEAP1 may modulate metabolism, proliferation, apoptosis, endocytosis and intercellular communication elucidating its oncogenic role in PCa (Figure VII.1.). However, these findings that we verified should be evaluated in *in vivo* model and in human PCa samples. In a future work, it will be of interest to evaluate the influence of STEAP1 on the onset and progression of PCa using the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice knockout for the STEAP1 gene. Using human PCa samples, the expression of proteins associated with signalling pathways identified in LNCaP cells should be determined by immunohistochemistry.

The development of resistance to anti-androgens and/or chemotherapeutics is a huge constraint in PCa treatment, which demands searching for new alternative and effective treatments. Some studies have tested the efficacy of anti-androgens in combination with chemotherapeutic drugs, though the benefits for patients were poor. Thus, studying how the anti-androgen and/or chemotherapeutic drugs affect oncogenes expression and action, and the identification of the best strategy for combined therapies, are essential to improve the efficacy of treatments. In Chapter 6, it was evaluated the expression of STEAP1 in response to anti-androgens and taxane-based chemotherapeutic drugs, and it was also determined if the sensitivity of PCa cells to treatment with anti-androgens and these chemotherapeutic drugs changes when STEAP1 gene is knocked-down (Figure VII.2.). Relatively to anti-androgens, the results presented in Chapter 6 showed that the regulation of STEAP1 expression is distinct for the different anti-androgen drugs, and that the inhibition of STEAP1 does not alter the response of PCa cells to anti-androgen treatment. So, the levels of STEAP1 in PCa do not seem to change the effect of anti-androgens in cell proliferation and apoptosis. On the other hand, taxanes-based chemotherapeutic drugs are more effective in inducing apoptosis and suppressing viability and proliferation of the PCa cells that overexpress the STEAP1 protein. These results also suggested that STEAP1 overexpression may be used as a putative positive predictive biomarker for chemotherapy with these anti-cancer drugs, once blocking STEAP1 in PCa cells had a great impact on the response to taxanes-based chemotherapeutics. Further *in vitro* studies should be carried out to clarify whether the development of resistance to these drugs can be related to the expression of STEAP1, and it would also be interesting to analyse the expression of

STEAP1 in biopsies of PCa patients treated with these taxanes-based chemotherapeutics in order to establish a relationship between STEAP1 levels and treatment efficacy.

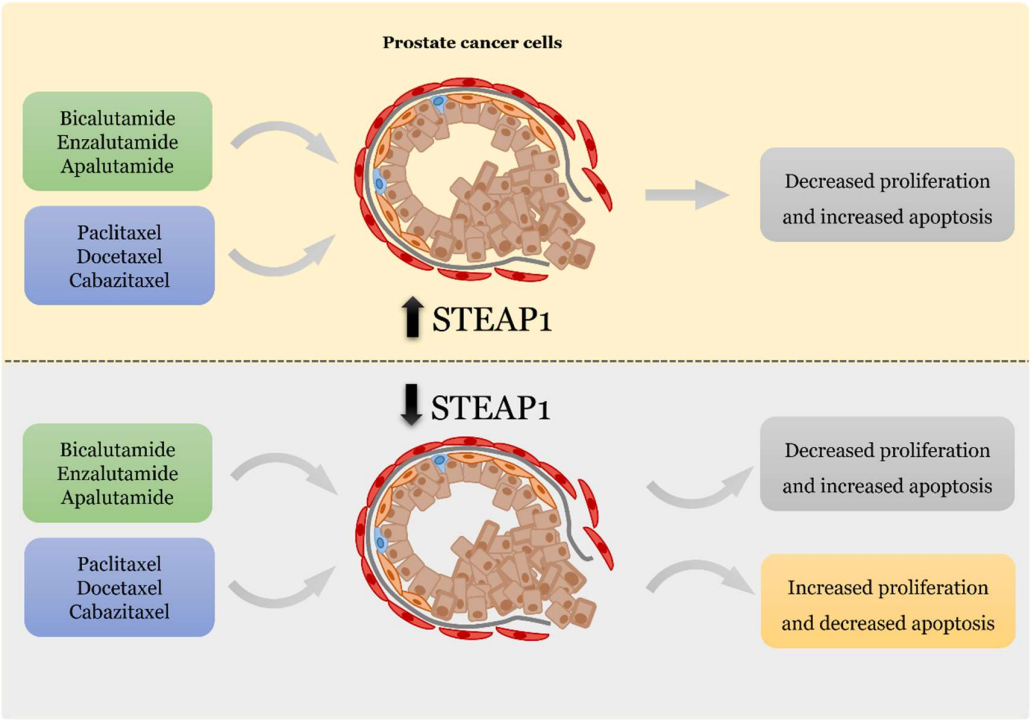

Figure VII.2. Representative scheme of the influence of STEAP1 expression levels on the response of PCa cells to antiandrogenic and chemotherapeutic drugs. Androgens promote the differentiation and growth of normal prostate, being also involved in adulthood in the stimulation of prostate oncogenic process. For this reason, androgen deprivation therapies (bicalutamide, enzalutamide and apalutamide) are widely used in PCa treatment, once it blocks androgenic action and consequently suppress PCa. Chemotherapeutic drugs (paclitaxel, docetaxel and cabazitaxel) exert anti-cancer effects by binding tubulin and affecting microtubule polymerization, leading to apoptosis of cancer cells. In PCa overexpressing STEAP1, anti-androgen and chemotherapeutic drugs promoted a decrease of proliferation and an increase of apoptosis of PCa cells. Blocking the action of STEAP1 does not alter the effect observed by anti-androgens. Interestingly, the silencing of STEAP1 counteracts the effect observed by chemotherapeutic drugs increasing proliferation and decreasing apoptosis of PCa cells. These results suggest that STEAP1 overexpression may be used as a putative positive predictive biomarker for choosing the type of treatment for the patient with PCa.

Final Remarks

The work presented in this thesis confirmed the oncogenic role of STEAP1, identifying new molecular targets of its action and further highlighting the usefulness of this protein in the definition of effective PCa treatments. It is well established that the overexpression of STEAP1 modulates cell proliferation, apoptosis, invasion and metastasis promoting development and progression of PCa. On the relationship between PCa and STEAP1, the outcomes of this thesis revealed new pathways by which STEAP1 could lead to prostate carcinogenesis. STEAP1 silencing increased the endocytosis of cell-surface growth factor receptors in order to reduce cell growth, and also decreased the intracellular communication by vesicles. These are two important pathways in the promotion of

tumour growth, invasion, and metastasis. Therefore, blocking of STEAP1 expression may act as a protective effect preventing cells from accelerated proliferation. The findings obtained herein contributed to a better understanding of the STEAP1 role in prostate pathophysiology. Moreover, the new and interesting results highlighted the diversity of the molecular mechanisms underlying proliferation, apoptosis and intercellular communication of PCa cells.

The work developed in this thesis also showed a new mechanism in the regulation of STEAP1 expression. It was found that epigenetic alterations, namely demethylation of the promoter region, can sustain the overexpression of STEAP1 in PCa.

STEAP1 is consistently overexpressed in neoplastic and pre-neoplastic prostate tissue, namely adenocarcinoma and PIN lesions, and its overexpression is positively associated with higher Gleason scores. This scenario strongly suggests that STEAP1 may be involved in tumor initiation and progression. The present dissertation was crucial to start exploring the role of STEAP1 as a prognostic biomarker in PCa. Although further studies are required, it was found that PCa patients with high levels of STEAP1 have a poor prognosis, and reduced survival time. At this point, STEAP1 can be considered as a putative prognostic biomarker for patients with PCa, and the inhibition of STEAP1 in earlier stages of the disease may be a strategy to slow tumour progression.

Another rationale of this work was the use of combined therapies as an attempt at defining effective treatment of PCa, namely of CRPC. The present dissertation showed that the efficacy of taxane-based chemotherapeutic drugs seems to be dependent on the STEAP1 expression levels, which could be of maximum relevance in the clinical context. Indeed, the results obtained alert for the particular attention clinicians should have when deciding the type of drug used for treatment of PCa in patients with low STEAP1 levels. Importantly, the present thesis contributed to the advance of knowledge on the biological functions of STEAP1, opening new avenues of research with likely clinical impact by envisaging the development of PCa therapies targeting STEAP1.