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MASTER'S THESIS

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ANKARA YILDIRIM BEYAZIT UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

THE EFFECT OF THYMOQUINONE ON THE EXPRESSION OF PLA2G7, UCP2, AND NEDD4L GENES ASSOCIATED WITH OIL DROPLET FORMATION IN PROSTATE CANCER

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I certify that this thesis meets all the requirements for the master’s degree.

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Abdelhak HADJ MIMOUNE

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ÖZET

Prostat kanserinde Timokinon'un yağ damlacığı oluşumu ile ilişkili PLA2G7, UCP2 ve NEDDL4 genlerinin ekspresyonları üzerine etkisinin araştırılması.

Prostat kanseri (PCa) tüm dünyada ve ülkemizde erkeklerde en sık görülen kanser türüdür. Bu kanser türü, erkeklerde kanserlerin %7,1'ini oluşturur. Androjen yoksunluğu (AD) tedavisinin etkinliğine rağmen, hastalar kastrasyona dirençli tipe doğru gelişime ek olarak ciddi yan etkilere maruz kalabilirler. PCa hücrelerinin esas olarak artmış lipogenez ile karakterize olduğu bilinmektedir. lipid metabolizmasının ve lipid damlacığı (LD) oluşumunun düzenlenmesinde çeşitli genler rol oynar. Timokinonun (TQ) hücre proliferasyonunu, metastazı ve invazyonu inhibe ettiği gösterilmiştir. Bununla birlikte, literatürde TQ'nun lipid damlacık oluşumu ile ilişkili NEDDL4, PLA2G7 ve UCP2 genlerinin ekspresyonu üzerine bir çalışma bulunmamaktadır.

Bu çalışmanın temel amacı, TQ'nun PLA2G7, UCP2 ve NEDD4L proteinleri üzerindeki DU 145 ve PC3 hücre hatları üzerindeki etkisini araştırmaktır. Çalışmanın ilk aşamasında, hücreler çoğaldı ve DU 145 ve PC3 için sırasıyla 60 μM ve 80 μM IC50 ile TQ ile tedavi edildi. Kuluçkadan sonra, PLA2G7, UCP2 ve NEDD4L seviyeleri ELISA yöntemi kullanılarak ölçüldü. TQ, her iki hücre hattında da NEDD4L seviyesini önemli ölçüde artırmıştır. Ayrıca, TQ'nun UCP2 proteinlerinin seviyesi üzerinde azalan bir etkiye sahip olduğu da gösterilmiştir. ($p<0,001$)

Çalışmamızın ön sonucu, TQ'nun NEDD4L ve UCP2 üzerinde bir etkisi olduğunu göstermektedir. Bu, TQ'nun LD üzerinde inhibitör bir etkiye sahip olabileceğini ve daha ileri araştırmalarla TQ'nun prostat kanseri tedavisi için potansiyel bir ajan olabileceğini düşündürmektedir.

Keywords: Prostat kanseri, Lipit damlacık oluşumu, Timokinon; NEDD4L, PLA2G7, UCP2.

ABSTRACT

The effect of Thymoquinone on the expression of PLA2G7, UCP2, and NEDD4L genes associated with oil droplet formation in prostate cancer

Prostate cancer (PCa) is the most common type of cancer in men globally. This type of cancer accounts for 7.1% of cancers in men. Despite the effectiveness of Androgen deprivation (AD) treatment, patients can be subject of severe side effects in addition to the development toward castration resistant type. It is known that PCa cells are characterized mainly by increased lipogenesis. several genes are involved in regulating lipid metabolism and lipid droplet (LD) formation. Thymoquinone (TQ) has been reported to impede cell proliferation, metastasis and invasion. However, there was no study in the literature on the expression of NEDDL4, PLA2G7, and UCP2 genes associated with lipid droplet formation of TQ.

The main objective of this study is to investigate the effect of TQ on PLA2G7, UCP2, and NEDD4L proteins on DU 145 and PC3 cell lines. In the first phase of the study, cells were proliferated and treated with TQ with IC50 of 60 µM and 80 µM for DU 145 and PC3 respectively. After incubation, levels of PLA2G7, UCP2, and NEDD4L were measured by using the ELISA method. TQ has significantly increased the level of NEDD4L in both cell lines. Furthermore, it was also shown that TQ had a decreasing effect on the level of UCP2 proteins. ($p<0.001$)

Preliminary result of our study indicates that TQ has an effect on NEDD4L and UCP2. This suggest that TQ might have an inhibitory effect on LD and, by further investigations, TQ can be a potential agent for prostate cancer treatment.

Keywords: Prostate cancer, Lipid droplet formation, Thymoquinone, NEDD4L, PLA2G7, UCP2.

LIST OF ABBREVIATIONS

ACLY	:	ATP citrate lyase
AD	:	Androgen Deprivation
ADRP	:	Adipose differentiation-related protein
ADT	:	Androgen deprivation therapy
AJCC	:	American Joint Commission on Cancer
AMPK	:	AMP-activated protein kinase
AR	:	Androgen receptor
ASTRO	:	American Society for Radiation Oncology
AUA	:	American Urological Association
BPH-1	:	Benign Prostatic Hyperplasia
CEs	:	cholesteryl esters
CoA	:	coenzyme A
cPLA2	:	Cytosolic Phosphate lipase
CPT-1	:	Carnitine palmitoyl-transferase 1
CTC	:	Circulating Tumour Cell
DAG	:	Diacyl Glycerol
DEG	:	Differentially Expressed Genes
DGAT	:	diacylglycerol acyltransferase
DMEM	:	Dulbecco's Modified Eagle's Medium- high glucose
DMSO	:	Dimethyl sulfoxide
DRE	:	digital rectal examination
DTX	:	Docetaxel
DWI	:	Diffusion-weighted imaging
EGFR	:	epidermal growth factor receptor
ELISA	:	enzyme-linked immunosorbent assay
EMT	:	epithelial to mesenchymal transition

ER	:	endoplasmic reticulum
FABP	:	Fatty acids binding protein
FABP5	:	Fatty acids binding protein 5
FABPs	:	Fatty acids binding proteins
FAO	:	Fatty acids oxidation
FA	:	Fatty acid
FASN	:	Fatty acid synthase
FBS	:	Fetal Bovine Serum
GnRH	:	gonadotropin-releasing hormone
GS	:	Gleason score
HIFU	:	high-intensity focused ultrasound
IC50	:	inhibitory concentration-50
IL-6	:	interleukin 6
ISUP	:	International Society of Urological Pathology
LD	:	lipid droplets
Lp-PLA2	:	Lipoprotein-associated phospholipase A2
mCRPC	:	metastatic castration resistant
mHSPC	:	metastatic hormone-sensitive prostate cancer
MS	:	mass spectrometry
MSI	:	MS-based imaging
MUFA	:	monounsaturated FAs
NEDDL4	:	Neural precursor expressed developmentally downregulated 4-like
NF-Kb	:	nuclear factor kappa B
OD	:	optical density
PBS	:	Phosphate-Buffered Saline
PCa	:	Prostate cancer
PET	:	positron-emission tomography

PHI	:	Prostate Health Index
PLA2	:	Phospholipase A2
PLA2G7	:	Phospholipase A2 Group VII
PSA	:	prostate-specific antigen
ROS	:	reactive oxygen species
SCDs	:	stearoyl-CoA desaturases
SFAs	:	saturated fatty acids
SUO	:	Society of Urologic Oncology
TAG	:	triacylglycerol
TCA	:	tricarboxylic acid
TQ	:	Thymoquinone
TME	:	tumor microenvironment
TNM	:	Tumor-Node-Metastasis
UCP2	:	Uncoupling Protein 2

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1. INTRODUCTION

Prostate cancer (PCa) is the most widespread cancer among males worldwide with a prevalence of 7.1% of cancers in men (1). The increasing incidence of PCa over the past century has resulted in about substantial changes in the diagnosis and treatment (2).

Due to advances in detection and prognosis coupled with improvements in surgical and medical oncology, PCa treatment now can be, to some extent, tailored based on the characteristics of the patient's disease (3).

Notwithstanding these advances, complete cure from the PCa remains an illusion and significant numbers of men will continue to suffer from the metastatic disease and their treatment will require a combination of systemic and local therapies (4).

Androgen deprivation therapy (ADT) is regarded as the primary strategy for metastatic PCa treatment. Nevertheless, besides being only palliative and curative, all prostate cancers will, sooner or later, progress toward castration resistant after ADT (3).

Plus, despite its primordial indication, patients under ADT are subject of severe side effects such as hot flashes, reduced libido, loss of bone density resulting in osteopenia or osteoporosis, increase clotting risk and insulin resistance (3).

Moreover, when PCa becomes metastatic castration resistant (mCRPC) the first line usually consists of chemotherapeutic agents initially Docetaxel. But still, this option comes with the burden of a clinically significant febrile neutropenia and diarrhea (5).

Thus far, PCa therapy induced side effects throw light on the necessity of considering novel treatments alternatives in order to enhance the life quality of patients as well as the overall survival.

Thymoquinone (TQ)’s anticancer characteristics allow it to be an intriguing biopharmaceutical agent to enhance the effectiveness and reduce toxicity of cancer treatment (6). As a matter of fact, TQ has exhibited higher effectiveness in combination with available drugs (7).

Furthermore, TQ was reported to impede DNA synthesis and proliferation in various PCa cell lines. Hence it was suggested by the same study that TQ may be efficient in treating various PCa malignancies (8).

Recent decades have altered attention of the role of lipids in cancer development and progression toward malignancy. The plasticity of lipid metabolism reprograming permits PCa cells to thrive in aggressive and nutrient-deprived environments and to spread to distant tissues. As a result, new mechanisms of therapy resistance and disease progression associated with lipid metabolism rewiring have recently emerged (9). For that matter, arising evidence suggest that accumulation of LDs is positively associated with higher Gleason score (GS) grade of human PCa (10).

As targeting specific cancer therapeutics is a major focus in present anticancer treatment, specific molecular targets for thymoquinone, like enzymes, receptors, DNA or RNA materials should also be kept under investigation. Our study aligns tightly with these needs when we are aiming to investigate the effect of TQ on the levels of PLA2G7, UCP2, and NEDD4L proteins which are associated to the formation of LD. the following research will be an important attempt in determining whether TQ will be a potential agent for the treatment of prostate cancer. Moreover, our study will fill the gap in the literature and pave the way for further research in this area.

In the upcoming chapter, we first present a brief description of prostate anatomy and histology. Also, we introduce prostate cancer and discuss its screening, staging and grading. Afterward, we provide a brief description of the specific hallmarks of prostate cancer especially the lipogenic phenotype of prostate cancer and its correlation with increasing malignancy and pathogenesis. Later, we discuss currently available treatment options for different stages of prostate cancer and their limitations in the hormone refractory stage of the prostate cancer. In the end, we present our rationale for the anticancer or chemo-preventive role and mechanism of action of thymoquinone on different cancer types both in vivo and in vitro.

2. GENERAL INFORMATION

2.1. Prostate Cancer Physiopathology and Diagnosis:

2.1.1. Anatomy and Histology

The prostate gland is a male reproductive accessory organ situated beneath the bladder and nearby the urethra. The key role of the prostate is to provide essential secretions to semen which maintain sperm viability (11). The prostate consists of five anatomical regions as illustrated in the figure below when most tumors originate in the peripheral zone (Figure 2.1) (12). Each zone contains various cell types one of which are fibroblasts that have significant roles in prostate development (Figure 2.2) (13).

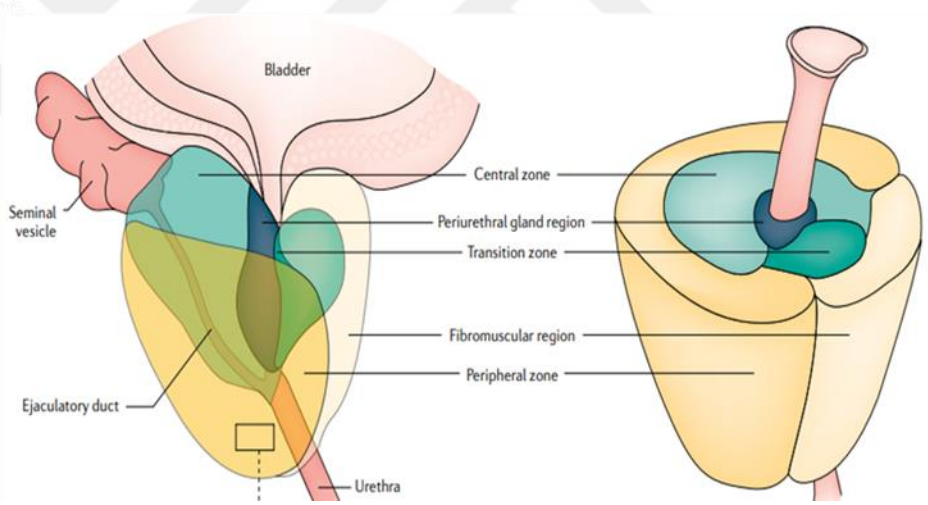


Figure 2.1. Anatomy of the human Prostate gland (14).

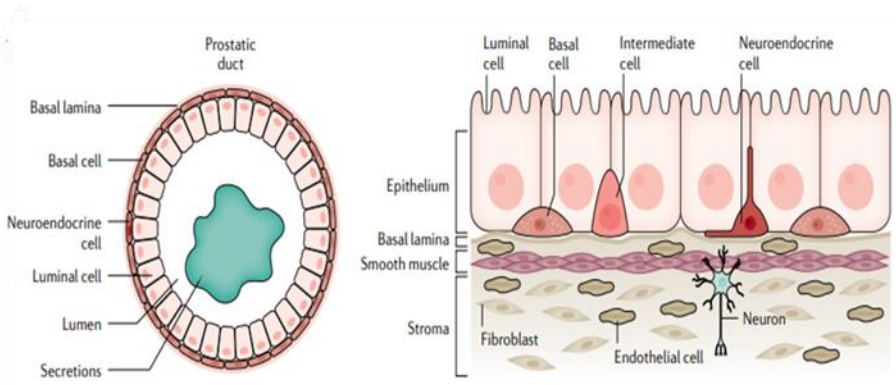


Figure 2.2. Histology of the human Prostate gland (14).

2.1.2. Pathology and Epidemiology

Cancers in the prostate gland are mostly adenocarcinomas that arise from the prostate epithelial cells (15). The development of prostate cancer is considered to be a multistep process, whereby the accumulation of somatic mutations over a patient's lifetime (14,16). This has been confirmed by multiple autopsy studies that have demonstrated the presence of microscopic adenocarcinomas in the prostate in 30% of men between 30 and 40 years of age in the US, while most men are diagnosed with clinical cancer after the age of 60 (17).

Furthermore, due to the multistep development feature of prostate cancer, its risk increases strongly with age and >85% of newly diagnosed individuals are >60 years of age (1). These epidemiological records could be further used in quantifying geographical disparities, identifying high-risk populations, and more importantly, outlining the extent of using screening tools in order to ameliorate the prognosis and overall survival.

2.1.3. Prostate cancer Screening

The Prostate cancer is notoriously difficult to treat in the advanced stages and so its early detection is crucial for disease management and reduction of mortality rates. Current early detection methods enable diagnosis at an early stage (18) (Figure 2.3).

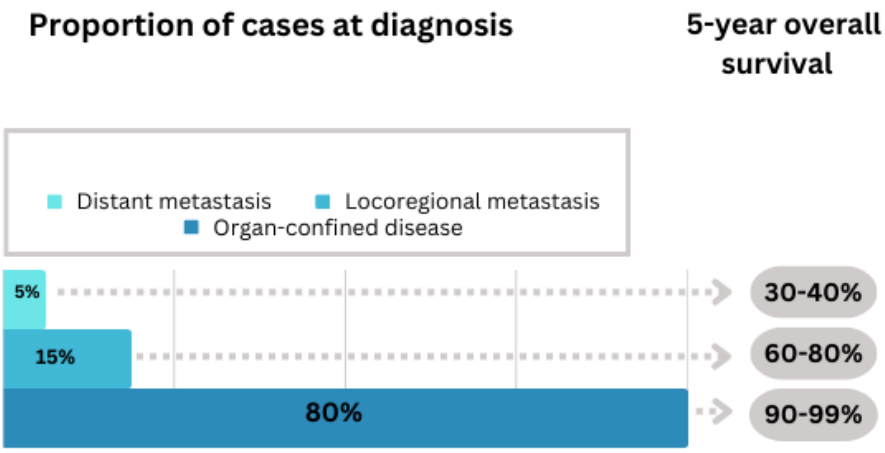


Figure 2.3 Relative prostate cancer stage distribution at the time of diagnosis (14).

Patients with confined PCa may have a life expectancy as high as 99% over a decade if diagnosed at an early stage (18). This considerably long survival can be ascribed to improvements in diagnosis through PSA screening. In general, Screening tends to identify cancers at an early and treatable stage. Hence, increasing the chances of effective therapy while also improving a patient's quality of life (19).

2.1.4. Staging and Grading

Identifying the stage of PCa is an important component in determining the potential treatment options. As new technologies have emerged, it has been possible to more accurately diagnose and establish personalized treatment options (20,21).

The Tumor-Node-Metastasis (TNM) staging system separately describes the characteristics of cancer within the prostate (T), the state of cancer in the regional lymph node (N), and further spread to other organs (M) (22,23).

Furthermore, the GS for grading has largely been used and it remains the typical method of histologic grading of PCa (24). This score is mostly based on morphology and contrasts the amount of cancer progression to normal tissue (Figure 2.4) (Table 2.1) (25)(26).

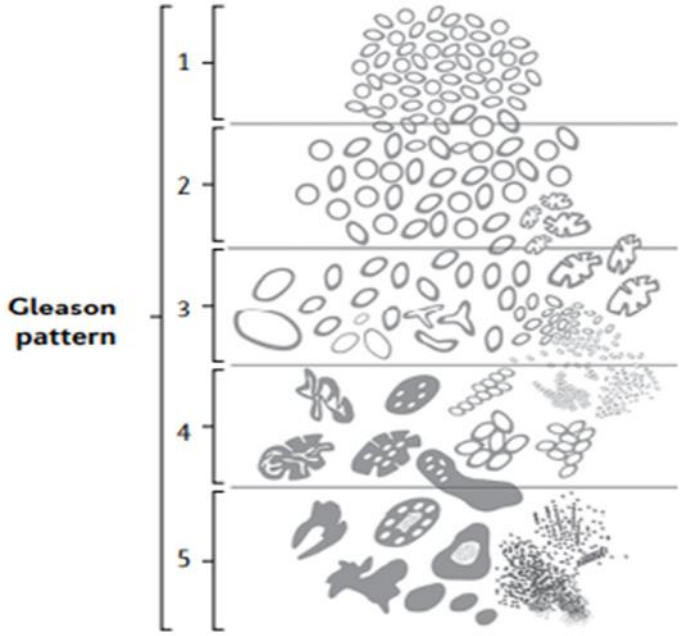


Figure 2.4. Gleason patterns to score prostate cancer severity (26).

Table 2.1. Histological Definition of New Grading System (44).

	Gleason Score	Anatomic Description
Group 1	(Gleason score <= 6)	Only individual discrete well-formed glands
Group 2	(Gleason score 3+4= 7)	Mostly well-formed glands.
Group 3	(Gleason score 4+3= 7)	Mostly poorly formed.
Group 4	(Gleason score 4+4= 8; 3+5= 8; 5+3= 8)	Only poorly formed.
Group 5	(Gleason scores 9-10)	Lacks gland formation (or with necrosis)

In order to improve patient stratification for management and prognosis, subsequently to the effort of the American Joint Commission on Cancer (AJCC) and the Union for International Cancer Control (UICC), the TNM staging system was frequently updated and modified and, more importantly, combined with the Gleason score grading system.

The UICC 8th has published only an anatomic stage grouping although the AJCC 8th has contained the prognostic stage groups with the incorporation of TNM classification, PSA and grade group (Table 2.2) (27).

Table 2.2. The prognostic stage groups of prostate cancer in the AJCC 8th (47).

Stage group	T categories	N categories	M categories	PSA (ng/ml)	Grade Group
I	cT1a-c, T2a	N0	M0	<10	1
I	pT2	N0	M0	<10	1
IIA	cT1a-c, T2a	N0	M0	≥10, <20	1
IIA	cT2b-c	N0	M0	<20	1
IIB	T1-2	N0	M0	<20	2
IIC	T1-2	N0	M0	<20	3
IIC	T1-2	N0	M0	<20	4
IIIA	T1-2	N0	M0	≥20	1–4
IIIB	T3-4	N0	M0	Any	1–4
IIIC	Any T	N0	M0	Any	5
IVA	Any T	N1	M0	Any	Any
IVB	Any T	N0	M1	Any	Any

2.2. Specific hallmarks of Prostate cancer

The cancer conceptual framework's key characteristics are a heuristic tool for breaking down the large complexity of cancer phenotypes and genotypes into a preliminary set of fundamental principles. Increasingly powerful experimental and computational tools and technologies are generating a flood of "big data" about the numerous manifestations of cancer-related diseases. Thus, the emergence conceptualization of cancer hallmarks has an unquestionable advantage in terms of lowering the complexity of various manifestations of cancer disease.

The Hallmarks of Cancer were proposed as a set of functional capabilities acquired by human cells as they progress toward neo-plasticity, more specifically capabilities essential for the formation of malignant tumors. the six hallmarks proposed at first includes: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (28).

Decades later of a deep biological and molecular understanding of the underlying mechanisms of malignancy, the former model of Cancer hallmark was revised, and the acquisition of the existing hallmarks was thought to be made possible by two enabling and two emerging characteristics (29).

Still, other features of cancer have been suggested to be crucial for the development of cancer and therefore might be added to the list of cancer hallmarks. One of them involves major rewiring of metabolic features to sustain continuous cell growth and proliferation (30) (Figure 2.5).

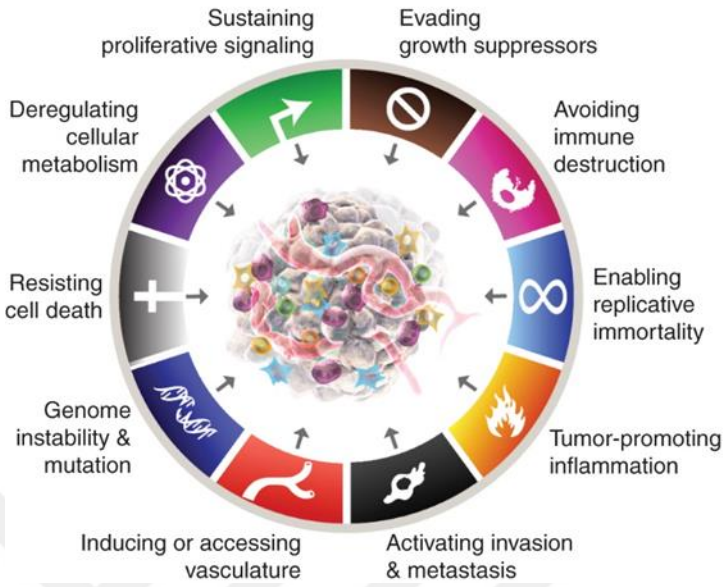


Figure 2.5. The current Hallmarks of Cancer (31).

As for the rest of this chapter, we will only developpe numerous hallmarks that are specific to prostate cancer. These Prostate Cancer unique hallmarks will include metabolic reprogramming, androgen receptor-mediated signaling, and the interplay between genomics and metabolism pathways alterations. Throughout the following chapters, we will focus on the hallmark concerning metabolic reprogramming and genomic instability.

2.2.1. Metabolic reprogramming

Cellular metabolism entails a variety of metabolic processes that allow certain nutrients to be consumed. Major nutrients, namely carbohydrates, fatty acids and amino acids, are the primary substrates of anabolic and catabolic pathways (Figure 2.6) (32).

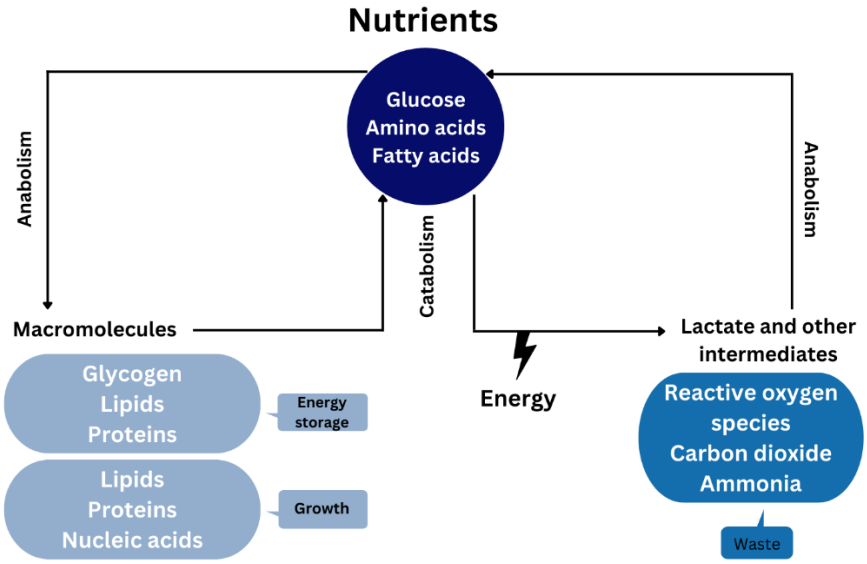


Figure 2.6. Core metabolic pathways (32).

PCa will exhibit this hallmark that fundamentally promotes the disruption of normal metabolic regulation, leading to an augmentation of prostate tumorigenesis. PCa metabolic rewiring is initiated by several crucial factors, including the Warburg effect, reverse Warburg effect, cholesterol accumulation, mitochondrial biogenesis, intra-tumoral steroidogenesis, androgen receptor activation etc. (33).

Although many cancers rely on glucose to support cells proliferation, Prostate cancer, however, is considered not glycolytic-especially during the first stages of tumor progression. Alterations in lipid and particularly fatty acid metabolism necessary for energy production, and for membrane synthesis and post-translational modification of signaling molecules, are increasingly being recognized as vital for the early phases of malignant transformation and in tumor progression (34).

Given the rising corpus of data focusing on the link between the lipogenic phenotype and prostate cancer development, and for the aim to fulfill the main objective of the following thesis (35), we will only consider the upregulated lipogenesis feature of PCa and its implications in pathogenesis with the necessity, sometimes to draw links between the rest of metabolic reprogramming aspects.

2.2.2. Lipid Metabolism

The plasticity of lipid metabolism rewiring allows PCa cells to thrive in hostile and nutrient-deprived environments and to spread to distant tissues. As a result, new mechanisms of therapy resistance and disease progression associated with lipid metabolism rewiring have recently emerged (9). We will discuss how lipid metabolism reprogramming, which includes changes in both de novo lipid synthesis, uptake, transport, storage, and utilization, contributes to PCa progression and therapy resistance, and how these mechanisms can be therapeutically exploited.

Despite circulating lipids, PCa cells exhibit a new feature of FA biogenesis from glucose or glutamine, (36) (Figure 2.7) (37).

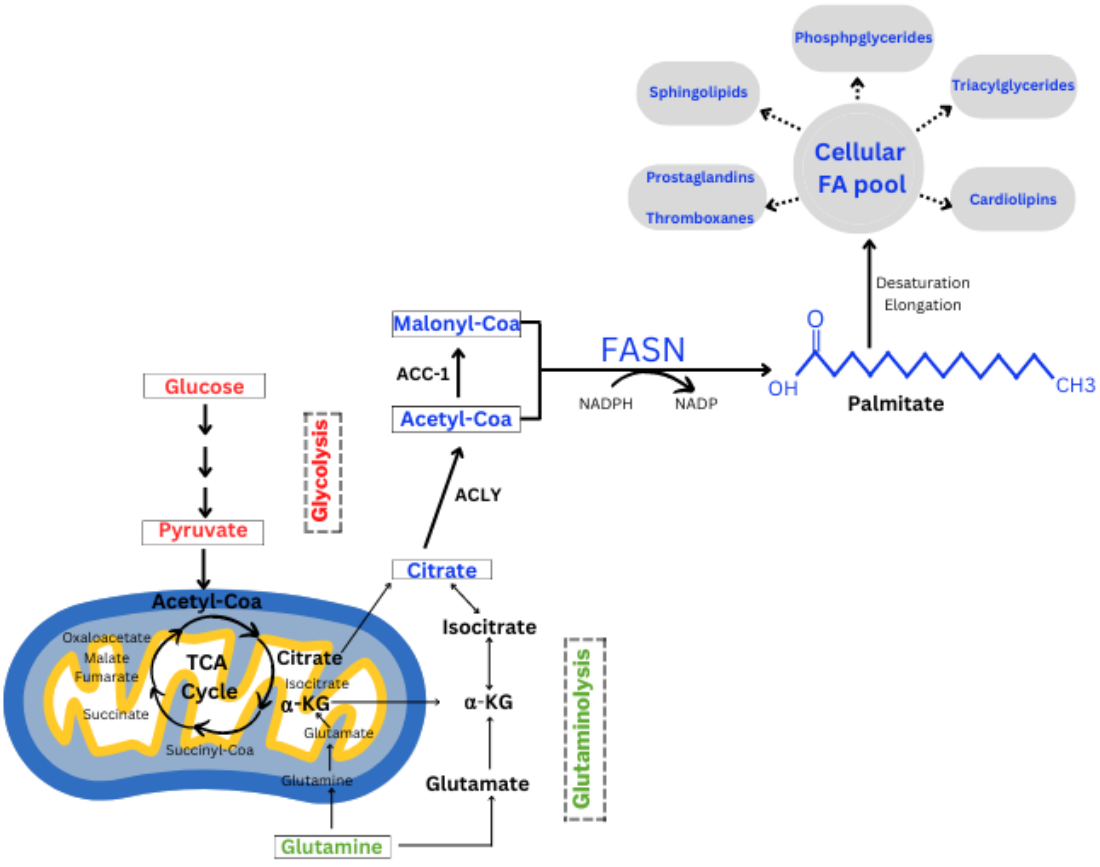


Figure 2.7. De novo FA synthesis in PCa progression (9).

2.2.3. Lipid Droplets and PCa hallmarks

In times of lipids abundance, de novo or acquired FAs are incorporated in Triacylglycerols (TAGs) and accumulates as LDs, organelles that consist of englomerate of TAGs and cholesterol esters and enclosed by a monolayer of phospholipids. It was reported that cancerous cells, including PCs, are characterized by a higher number of LDs compared to healthy cells and tissues (38). Arising evidence suggest that accumulation of LDs is positively associated with higher Gleason score (GS) grade of human PCa (10). Recently, due to an accumulating document on their role in malignancy and cancer progression, LDs have been suggested to be involved in cancer outlined hallmarks that characterized the capabilities acquired during tumor development (Figure 2.8) (31)(39).

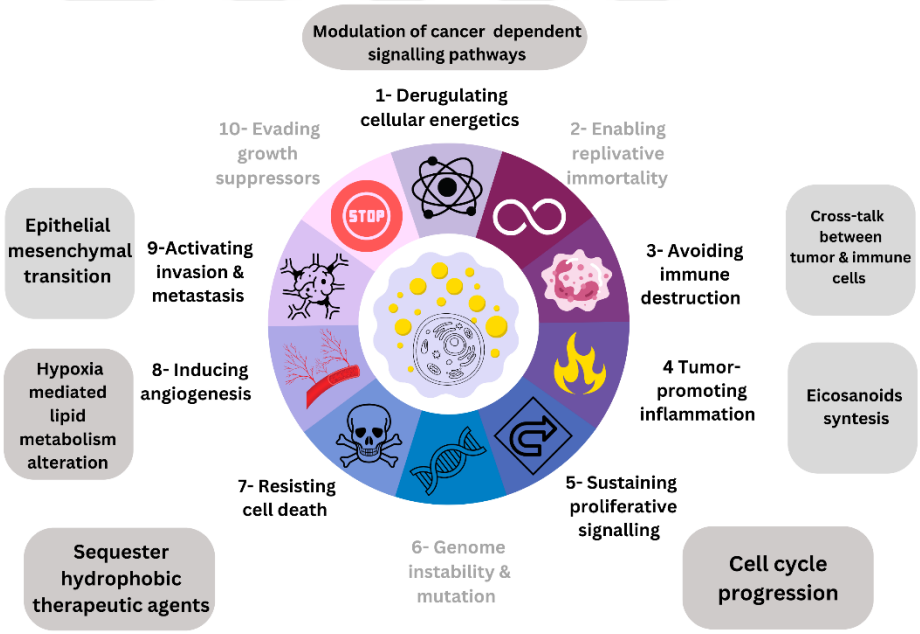


Figure 2.8. Lipid droplets as players in hallmarks of cancer (39).

The following is a presentation of the main implications of LDs in PCa:

Lipid droplets in cell proliferation and apoptosis:

Number of studies have shown that an increase in LD number occurs in cells undergoing proliferation, which is a common feature in many neoplastic processes, indicating that LDs may play a role in cell proliferation (40). Furthermore, various number of tumorigeneses signaling-associated proteins have been found within LDs such as PI3K and caveolin, suggesting a key role for these organelles as a cytoplasmic supply favoring rapid proliferative signaling (41,42). Nevertheless, the studies available are only a subject of suggestion and further investigations should be conducted.

2.2.4. Androgen Receptors and PCa metabolism

The androgen receptor (AR) signaling axis plays a critical role in the development, function and homeostasis of the prostate (43). Indeed, AR transcriptional program supports PCa viability during the course from primary tumor formation to progression to metastasis by regulating all aspects of cellular metabolism (44) since PCa exhibits specified metabolic and energetic phenotypes depending on the stage of disease progression. For instance, while AR signaling persists, the shift from oxidative to glycolytic metabolism occurs during the progression to advanced PCa (45). Lipogenesis is constantly maintained by AR during the development of PCa (46). It had been made evident that AR regulates FA metabolism by controlling expression of more than 20 enzymes involved in many aspects of lipid metabolism, including uptake, trafficking, de novo synthesis, and degradation (47). Understanding how these distinct metabolic characteristics relate to AR signaling in PCa may help identify metabolic vulnerabilities that present opportunities for both diagnosis and treatment.

2.2.5. Interplay between genomics and metabolism

Deciphering the functional impact of prostate cancer genomics on disease progression has been a challenge in comparison to other cancer types for many reasons such as sample accessibility and the limited availability of model systems (48). The figure below depicts the underlying impact of Myc overexpression, PTEN loss, p53 loss,

mutations, and other tumor suppressors on lipid metabolism reprogramming and the progression of the disease toward castration resistance state (Figure 2.9) (48).

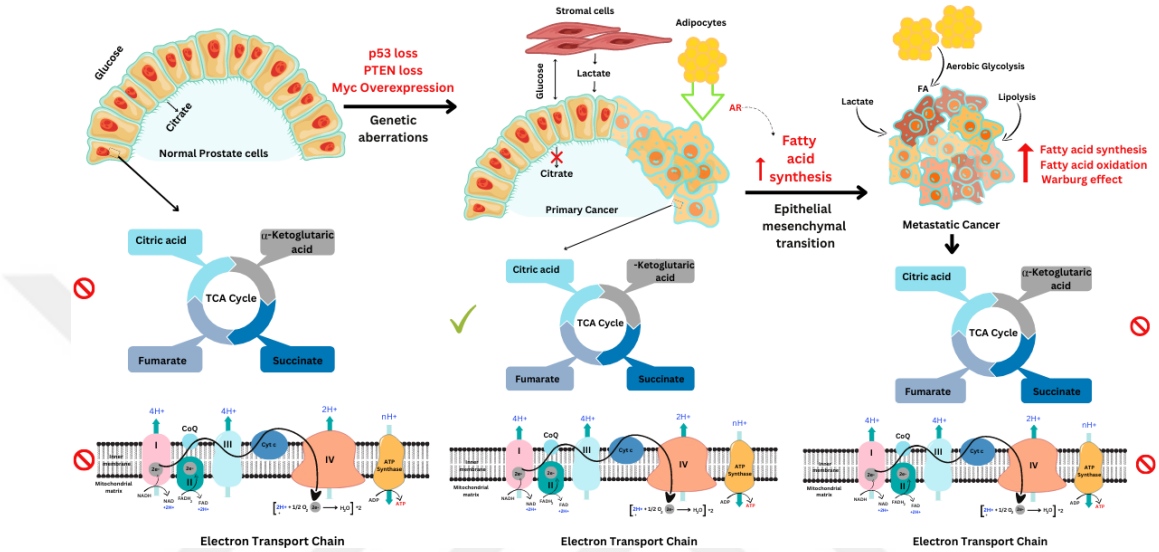


Figure 2.9. The metabolic alterations during prostate cancer metastasis (48).

Advances in microarrays and sequencing technologies play an essential role in the study of the occurrence and development of cancer and provide effective tools and have been broadly used in screening biomarkers for cancer diagnosis, treatment, and prognosis. This, all along with novel bioinformatics methods, allowed the integration data from different independent studies and obtain more clinical samples for data mining, in order to obtain more robust and accurate analysis to connect the dots between the genomic makeup and the metabolism rewiring features (49). The following table illustrates the Differentially Expressed Genes (DEG) Analysis of a recent study aiming to explore the relationship between metabolic reprogramming and GS in PCa (Table 2.3) (50).

Table 2.3. Remarkable Metabolic Rewiring Genes in Prostate Cancer (the genes of interest in our study are in bold) (139).

Well-differentiated prostate cancer	
Up-regulated	**Down-Regulated**
ACSM1, AMACR, **PLA2G7**, OR51E2, OR51E1	PDK4, ALAD, **UCP2**, PTGS2, SLC2A5, INSIG1, MSMO1, FDFT1,
Poorly differentiated prostate cancer	
Up-Regulated	**Down-Regulated**
APOE, HSD17B4, **PLA2G7**, IMPA1, TWIST1 ACSM1, AMACR, FABP5, **NEDD4L**, SLC25A6, MAPK9, OR51E2, OR51E1	PDK4, **UCP2**, SLC2A5, SLC16A5, INSIG1, ALOX15B, SEC14L2

For our study, we arbitrarily selected 3 genes of interest, including PLA2G7, NEDD4L and Ucp2, which will be the subject of the following:

Upregulated metabolic reprogramming genes:

Phospholipase A2 (PLA2) hydrolyze phospholipids into fatty acids and is encoded by the PLA2G7 gene. It was found that the PLA2G7 gene is upregulated in well-differentiated prostate cancer and is significantly linked to the altered phospholipid metabolism of prostate cancer and played a key role in pathogenesis (51). Aside from the role of PLA2s in LD synthesis from the cytosolic face of the smooth endoplasmic reticulum membranes depicted in (Figure 2.10), they may act as providers of free fatty acids, generators of metabolite that may control LD formation and direct controllers of LDs (52).

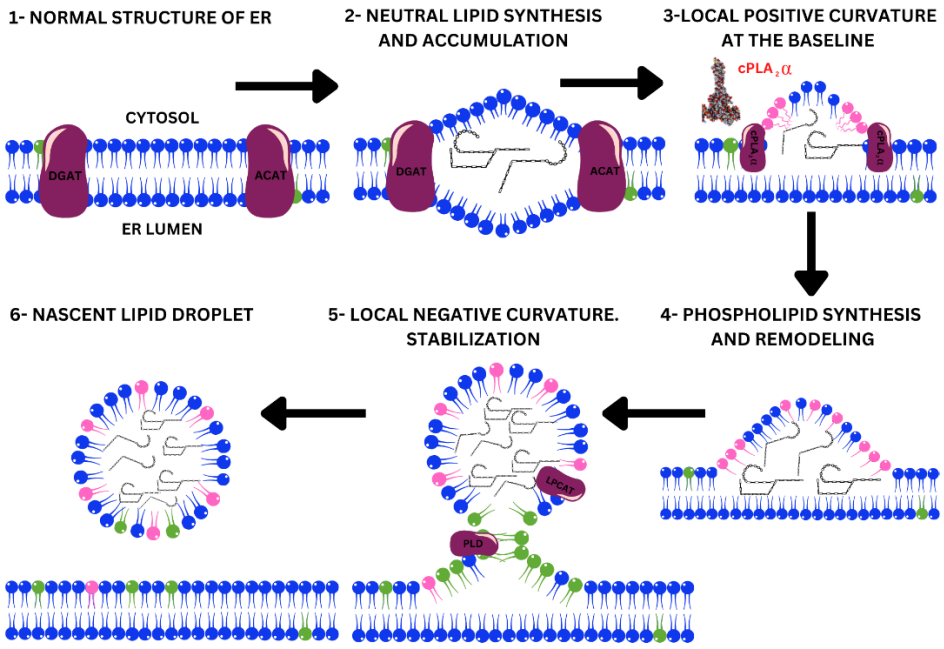


Figure 2.6. The role of PLA2 in Lipid Droplet synthesis (52).

Nedd4L (neural precursor cell expressed,developmentally down-regulated 4-2) is an E3 ubiquitin-protein ligase that contains a HECT domain. As shown in the (Figure 2.11), Spartin is a multifunctional protein that associates with many cellular organelles, including lipid droplets. Recent studies showed that spartin interacts with E3 ubiquitin ligases that belong to (Nedd4) family, spartin acts as a linker for ubiquitination of adipophilin on lipid droplets, likely by sequestering Nedd4-E3 ubiquitin ligases and ubiquitination machinery (53).

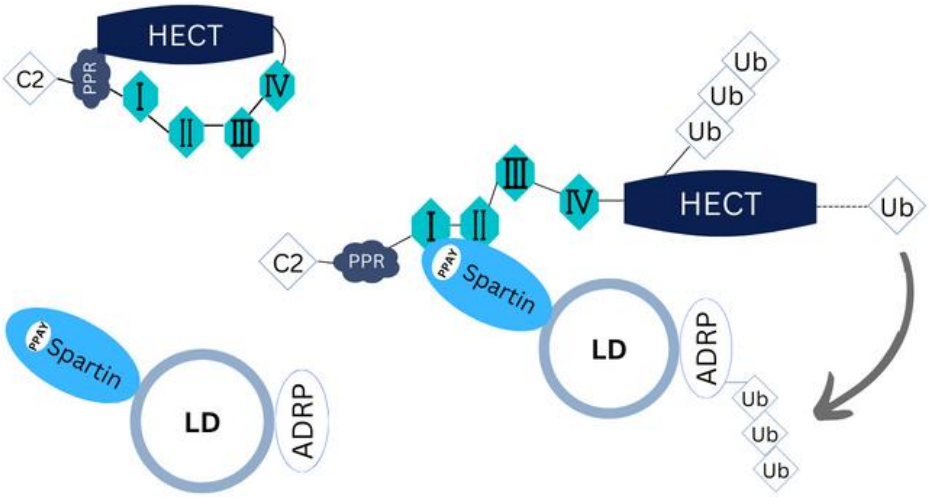


Figure 2.7. The role of Nedd4 in Lipid Droplet ubiquitination (53)

The expression of Nedd4L is decreased in the progression to prostatic malignancy and the loss of Nedd4L expression correlating with increasing GS (54). The discrepancy with the data provided in the previous may be explained by the fact that level of expression of Nedd4L differs according to the stage of malignancy which is directly proportional to Gleason score. Thus, Nedd4L expression is decreased in well differentiated PCa (54) and upregulated in poor differentiated PCa (50). These data suggested that regulation of Nedd4L expression might play a role in the development of prostate cancers and may give insights on new therapeutic strategies (55).

Downregulated metabolic reprogramming genes:

Uncoupling protein 2 (UCP2) is a mitochondrial carrier whose protein expression is strongly related to changes in cell proliferation, thus it is a crucial player in the cascade of mitochondrial molecular events related to carcinogenesis. It has been shown that UCP2 protein expression level correlates closely and negatively with tumor proliferation in vitro and in vivo (56). Indeed, the in-silico analysis states that UCP2 expression is downregulated in PCa (50). Furthermore, another study came to confirm the role of UCP2 downregulation in metabolic rewiring when it has been shown that UCP2 Loss Promotes Phospholipid Synthesis and Increases Expression of Lipogenic Enzymes, thus lipid

accumulations and a potential promotion of LDs formation (57). In the figure below, green arrows indicate upregulated reactions, and red arrows indicate downregulated reactions. UCP2 loss in colon tumors redirects glucose metabolism toward phospholipid synthesis by promoting the use of pyruvate to sustain lipogenesis, which is required for cell growth (Figure 2.12) (57).

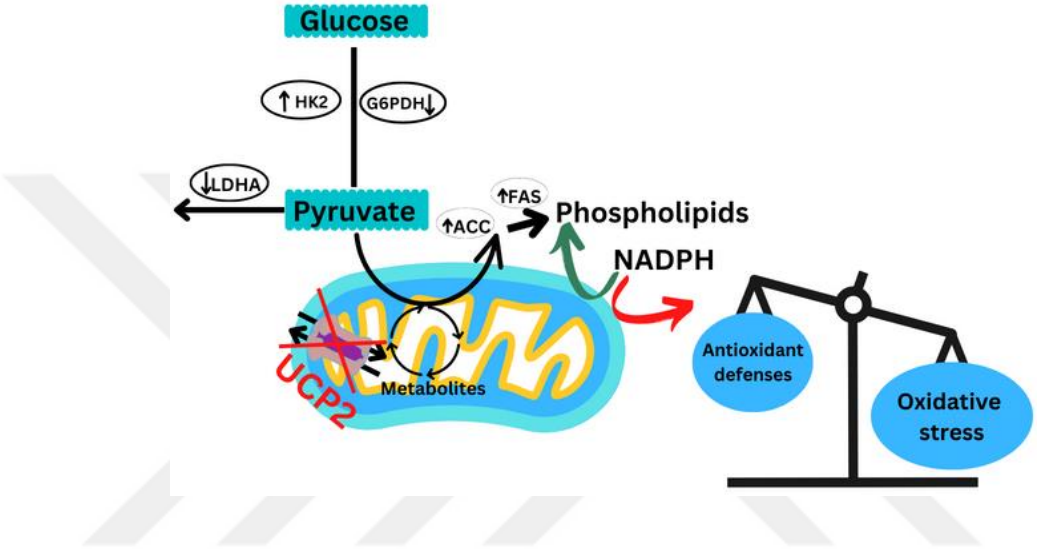


Figure 2.8. UCP2 Downregulation Stimulates Phospholipid Synthesis and Increases Expression of Lipogenic Enzymes (57).

2.3 Prostate Cancer Treatment

The first decision in treating PCa is determining whether treatment is required. Low-grade tumors often grows so slowly that frequently no treatment is needed, mainly in elderly patients and those with comorbidities that would reasonably limit life expectancy to 10 additional years or less. According to whether the cancer has spread away from original cite, we can either classify it as localized or metastatic PCa. Despite that, the best option depends on the cancer stage, Gleason score, and PSA level, as well as individual patient preferences, overall health, comorbidities, quality of life, and age (3).

Throughout this section we are to focus on the drawbacks of each treatment option of both localized and metastatic PCa including castration resistant type. The reason behind

that is to shed light on the weaknesses areas of treatment which we are trying to improve by our study using Thymoquinone molecule.

2.3.1 Localized Prostate Cancer

Radiation therapy, radical prostatectomy, and cryotherapy are now considered definitive treatments for localized disease. Radiation therapy tends to have much fewer side effects (about 50% less) than radical prostatectomy surgery, with very similar overall survival (3).

In general, Surgical therapy is associated with immediate operative risks such as pain and infections, erectile dysfunction, and persistent incontinence (58). Radiation therapy, on the other hand, is uniquely associated with a higher risk of persistent fecal urgency, hemorrhagic cystitis and, more importantly, secondary malignancy in the radiation field including the bladder, colon, and rectum (59). Furthermore, in order to improve survival in men with localized disease who are treated with external radiation, the follow up with androgen ablation therapy has shown promising outcomes. A study has shown that, among intermediate risk patients, the 10-year rate of overall survival was 62% with combination therapy, versus 57% with radiation therapy alone (60). Recently several treatments options have emerged in the management of localized PCa such as cryoablation, high-intensity focused ultrasound (HIFU), and photodynamic therapy. Besides being utilized for only low-risk patients, Long-term safety and efficacy data remain elusive (61).

2.3.2 Advanced and Metastatic Prostate Cancer

Besides being only palliative and curative, all PCas will eventually escape and regrow after ADT (3). Thus, mHSPC remains incurable. Plus, despite its primordial indication, ADT are the subject of severe side effects such as hot flashes, reduced libido, loss of bone density resulting in osteopenia or osteoporosis, increase clotting risk and

insulin resistance (3). On top of that, long-term androgen blockade for prostate cancer may also increase a patient's risk for colorectal cancer (62).

Furthermore, in case of mHSPC or the disease progresses toward a non-metastatic castration resistant (nmCRPC) form, further drug combination can take place. Multiple studies have shown that additional therapy with a chemotherapy molecule especially docetaxel (63) or androgen pathway–directed therapy (abiraterone acetate plus prednisone(64) , enzalutamide(65)) significantly extends overall and progression-free survival.

Moreover, when PCa become metastatic castration resistant (mCRPC) the first line usually consists of docetaxel which, despite its toxicity including gastrointestinal and cardiovascular side effects (66), remain the first choice in association with ADT.

In order to expand the list of mCRPC treatment options, the indication of Abiraterone was expanded in 2012 by the FDA for mCRPC. This was based on a study in which patients who received abiraterone had a median overall survival period of 35.3 months, compared with 30.1 months for patients receiving placebo (67).Later on in 2018, Enzalutamide indication has been also extended to take place for nmCRPC management (68). Nevertheless, according to a meta-analysis study, Abiraterone was associated with an increased risk of cardiovascular events, while enzalutamide was associated with an increased risk of fatigue (69).

Besides, after failure of response to Docetaxel, Cabazitaxel, another taxane that acts as a microtubular inhibitor, is indicated as a second line treatment for mCRPC. But still, this option comes with the burden of a clinically significant febrile neutropenia and diarrhea (5).

To put it in a nutshell, the previous walk-through of the diverse treatment options along with the associated side effects throws light on the necessity of considering novel treatments alternatives in order to both enhance the life quality of patients as well as the overall survival.

2.4. Thymoquinone

Throughout the course of the previous years, it has been reported that TQ has been used as an antineoplastic, antioxidant, analgesic, and anti-inflammatory agent (70). TQ selectively inhibits the cancer cells' proliferation in leukemia, breast, lungs, larynx ,colon , and osteosarcoma (71). However, yet amazingly, its toxicity to healthy cells is very low compared to cancer cells (72).

2.4.1. Anticancer and Pharmacological Features of TQ

Over the past decade, several studies have taken place to evaluate the anticancer or chemo-preventive role and mechanism of action of thymoquinone on different cancer types both in vivo and in vitro. The following figure illustrates the various mechanism of actions of TQ as an anticancer agent (Figure 2.13) (6).

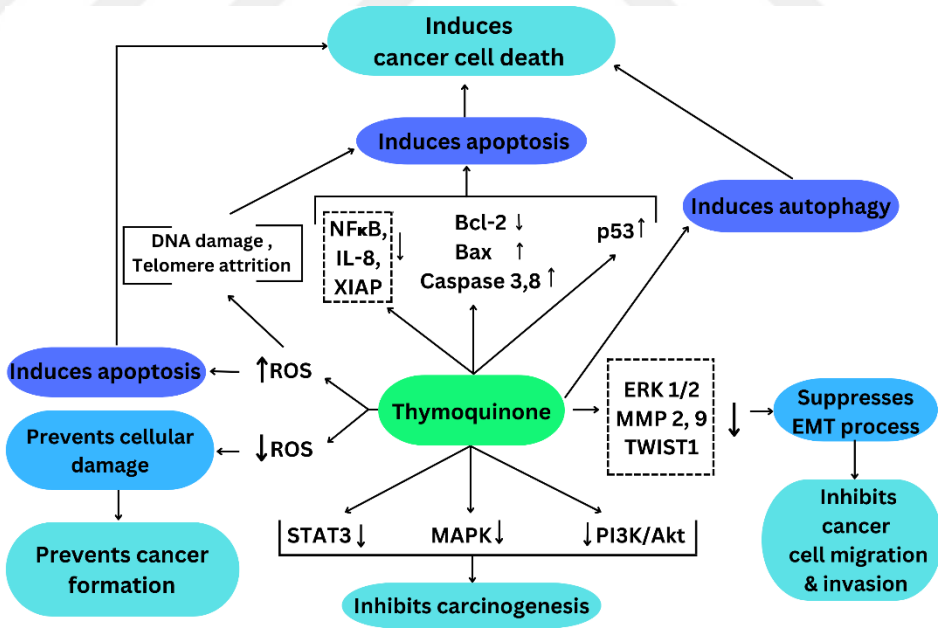


Figure 2.9. Important mechanisms of thymoquinone's anticancer actions (6).

The promising effect of TQ was further extended to be proven in several in vivo studies. The results of a study showed TQ's ability to reduce prostate weight/body weight ratio, epithelial hyperplasia, serum interleukin 6 (IL-6) levels, and the expressions of TGF-β1 and VEGF-A in the treated group (73).

There is not any clinical trial for thymoquinone registered by the U.S government yet (6). Nevertheless, On the basis of a phase I study, thymoquinone was well tolerated at a dose ranging from 75mg/day to 2600mg/day among various patient's cancer profiles. Neither toxicities nor therapeutic responses were reported (74). The use of thymoquinone in humans is limited due to its hydrophobicity, poor aqueous solubility, and photosensitive nature causing poor membrane penetration capacity and rapid elimination (75) which results in limitations in the bioavailability and drug formulation of TQ (76). To overcome these pharmacokinetics obstacles, scientists are looking to use TQ-based nanotechnology and synthesize novel TQ analogs with more effectiveness and bioavailability. Thus, a report describes the first successful encapsulation of thymoquinone into liposome, which maintains stability, improves bioavailability and maintains its anticancer activity (77). In the context of TQ's pharmacological properties, it is important to note that thymoquinone shows anticancer activity in small concentrations, approximately < 10 mg/kg (78). Therefore, safety issue might not pose a big problem.

2.4.2. TQ combination with chemotherapeutic drugs

The effective anticancer properties of TQ made it an interesting therapeutic candidate for combination therapy with standard therapeutic agents already used clinically in order to improve cancer treatment efficacy and safety. As a matter of fact, TQ has been proved to be very effective in synergistic anticancer activity with available drugs.

While 5-fluorouracil is regarded as the chemotherapeutic gold-standard for certain cancers, low concentrations of TQ was found to inhibit the proliferation of several chemo-resistant cancer cells and induce apoptosis in colon CSCs that are resistant to the conventional 5-FU (79). Furthermore, A combination of TQ and 5-FU showed more vigorous anticancer activity against nasopharyngeal carcinoma cells (80).

Likewise, Docetaxel, though considered as 1st line chemo-drug after the progress toward hormone refractory PCa, low water solubility, treatment related toxicities and drug resistance limit its application in clinical practice (81,82). TQ was found to enhance the apoptotic activity of DTX in prostate cancer cells by inducing the suppression of the signaling pathway PI3K/Akt compared to DTX alone (7).

This suggest that the combination of DTX and TQ might allow a reduction in docetaxel doses and minimize adverse effects of docetaxel while maintaining the therapeutic effect in patients with CRPC. Additionally, in mice bearing Ehrlich ascites carcinoma, the co-delivery of TQ and DTX in lipid nano-capsules produced more effective apoptotic and a higher tumor growth inhibition. Interestingly, these dual drugs loaded lipid nano-capsules prevented the development of DTX-induced hematological side effects (83). Similar observation has been made when TQ was combined with Cabazitaxel causing a synergistic anticancer potential (84).

Another molecule that was studied in combination with TQ is Zoledronic acid which has a preventive effect of the progression of skeletal complications associated with bone metastasis from solid tumors including prostate cancer plus an anti-tumor effect in different types of cancer (85)(86). TQ intensified the apoptotic activity of zoledronic acid in PC-3 and DU-145 prostate cancer cell lines through a synergistic increase in DNA fragmentation and activation of caspases 3 (87).

To put it altogether, contemplating the various molecular mechanism of Thymoquinone action, its effectiveness in small concentrations, in vivo success, enhanced bioavailability, promising results in combinations with other drugs, it is more convenient now to move a step further and focus on clinical trials for TQ. Moreover, basic biochemical or molecular biological investigations should be continued for the better understanding of the molecular mechanisms of thymoquinone. As targeting specific cancer therapeutics is a major focus in present anticancer treatment, specific molecular targets for thymoquinone, like enzymes, receptors, DNA or RNA materials should also be kept under investigation.

3. MATERIAL AND METHODS

3.1. Cell lines specification

The Human Prostate Carcinoma Cell lines used in the study, PC-3 and Du-145, were supplied by Mugla Sıtkı Kocman University and Yeditepe University respectively.

PC-3 cells were isolated from bone metastasis of grade IV prostatic adenocarcinoma from a 62-year-old man (88). Du-145 is a cell line with epithelial morphology derived from the brain metastasis of a 69-year-old man with prostate cancer (89).

3.2. Chemicals and Apparatus

The chemicals used in the study with the company name and product code all along with the apparatus and equipment are shown in Table 3.1 and Table 3.2 respectively.

Table 3.1. The chemicals used in the study.

Chemicals	Brand and reference code
Dulbecco's Modified Eagle's Medium- high glucose, DMEM	Capricorn Scientific
Fetal Bovine Serum (FBS)	Capricorn, CP17-1756
Penicillin-streptomycin	Capricorn Scientific
Phosphate-Buffered Saline (PBS)	Gibco, 1720774
Trypsin-EDTA	Gibco, 25200-056
Dimethyl sulfoxide (DMSO)	Fisher Chemical, 1414511
Aquaguard-1 Solution	Sartorius 01-867-1B
Thymoquinone	BLD pharm
Human NEDD4-L ELISA kit	Shanghai Coon Koon Biotech (20522)
Human UCP2 ELISA kit	Shanghai Coon Koon Biotech (13901)
Human Lipoprotein-associated phospholipase A2 (Lp-PLA2) ELISA kit	Shanghai Coon Koon Biotech (12314)

Table 3.2 The apparatus used in the study.

Apparatus	Brand
Distilled water device	ELGA
Microscope	Euromex
Refrigerator (+4)	Klimasan
Deep Freezer (-20)	Uğur
Deep Freezer (-80)	Haier
Nitrogen Tank	Thermo
Laminar flow hood	Telstar
CO2 incubator	Panasonic
Inverted Microscope	Euromex
Centrifugal	Hettich
Water bath	Memmert

3.3. Cell culture conditions

Before and after proceeding with the cell culture, the workplace and laminar flow hood were sterilized with UV rays and cleaned with 70% ethanol. The parameters of 37 °C and 5% CO2 were maintained throughout the cell culture. Distilled water at the bottom of the incubator was periodically changed and sanitized with Aquaguard-1 Solution in accordance with the instructions for use of the incubator.

3.4. Media content

Human prostate cancer cell lines, PC-3 and DU-145, were cultivated in a complete growth media containing % 10 FBS, % 1 Penicillin-Streptomycin, % 1 L-Glutamine in Dulbecco's Modified Eagle's Medium- high glucose (DMEM). The prepared medium was stored at a temperature of +4°C.

3.5. Cell Thawing and culture.

The cryovials containing the frozen cells were kept at 37 °C in the water bath for 2 min to thaw the cells. The cells were transferred into a 50 ml centrifuge tube and 7 ml of pre-warmed growth medium were slowly added in order to maintain the viability of the culture and enable the culture to recover more quickly, then centrifuged for 7 min at 200 g. The supernatant was aspirated, the pellet resuspended, transferred into a T-75 cell culture flask, supplemented with 10 ml complete growth medium and incubated at 37°C and 5% CO_2.

3.6. Cell subculture

During the period of incubation, the culture viability, confluency, and contamination were regularly checked with the inverted microscope. The medium was changed every 2 days to preserve the viability of the cells. In order to avoid depletion in nutrients and an increase in toxic metabolites, which will affect the cells viability, subculture took place when cells reached a confluence of 80-90 %.

In the first step, medium is removed from the cell culture flask with an aspiration pipette. The cells are washed once with 7 mL of PBS, before 2 ml of the dissociation reagent Trypsin-EDTA is added.

After adding the dissociation reagent, the cell culture flask was incubated at 37 °C and 5% CO2 for approximately 5 minutes. Gently tapping after the incubation period helped to expedite the cell detachment, before pre-warmed 8 mL of cell culture medium

was added to rinse off detached cells and to stop the enzymatic reaction. Cells were then transferred to a 15-mL centrifuge tube and centrifuged at 200 g for 7 min at room temperature.

After centrifugation, the supernatant is removed, and the remaining cell pellet is resuspended in 7 mL of pre-warmed cell culture medium. The cell suspension was then transferred into new T-75 flasks. Afterwards the cells were returned to the incubator at 37°C and 5% CO2 until the next passage.

3.7. Cryopreservation

Long-term cultivation might alter important genotypic and phenotypic properties and increase the risk of loss of culture due to contamination leading to variations in results. For that matter, after each subculture cryopreservation of cells is needed to supply a backup for losses due to contamination or accidents.

The procedure of detachment has already been described in the previous section. The cells were then resuspended in the freezing medium containing 20% DMSO, 20% FBS, and 60% DMEM highly glucose in appropriate cryovials. On the cryo-tubes were written the cell line, the freezing date and the number of passages already taken place. To preserve the cells, cryovials were first transferred into -20 °C for an hour, then into -80 °C overnight. The final storage temperature took place in a liquid nitrogen tank at a temperature between -186 °C and -176 °C.

3.8. Thymoquinone Preparation and application

For this study, 14 mg of Thymoquinone powder (Molar mass =164.201 g/mol) was measured using an analytical balance and dissolved into 1 ml of DMSO to get the stock solution with a final concentration of 85 mM. This stock was later diluted as in DMEM to produce the concentrations of 60 µM and 80 µM, to be used to treat DU145 and PC3 cell lines respectively. After the dilutions, the concentration of DMSO was

brought down to less than 0.1 % which is favorable since DMSO Concentrations up to 1% are considered to be safe and non-cytotoxic (90) .

Prior the treatment phase, cells were transferred into 6-well plates and incubated at 37°C and 5% CO2 until 80-90% of confluency. The subculture in 6-well plates took place in triplicate for both the treatment group, labeled as TQ+, and the control group, labeled as TQ-. After that, TQ was added to the treatment groups and the plates were left for incubations for 24 hours.

3.9. Freeze-thaw cells lysis

At first, for each well, spent medium was removed and the cells were washed with 500 µl of PBS before 300 µl of the dissociation reagent Trypsin-EDTA was added and left for incubation for 5 min at 37°C and 5% CO2. After adding 600 µl of culture media, each well contents were transferred into sterile 36 Eppendorf tubes with respective labeling then centrifuged at +4°C, at 1000 rpm for 5 min. Next, the supernatant was removed, and three cycles of PBS washing took place along with centrifugation at +4°C, at 1000 rpm for 5 min.

In order to prepare cells lysates, freeze-thaw procedure was followed by incubating the cells at -80 °C for 5 min, then at 37°C for the same period for 3 cycles. This process was followed by a centrifugation, at +4°C at 1000 rpm for 5 min, after which the supernatant was collected into sterile and labeled Eppendorf tubes.

3.10. ELISA

ELISA (Enzyme Linked Immunosorbent Assay) method was used to determine the effect of Thymoquinone on the levels of NEDD4L, UCP2 and PLA2G7 proteins in both Du145 and PC3 cell lines (Figure 3.1). The three kits used in this study were based on the principle of double antibody sandwich technology ELISA that has the advantage of a high sensitivity and specificity.

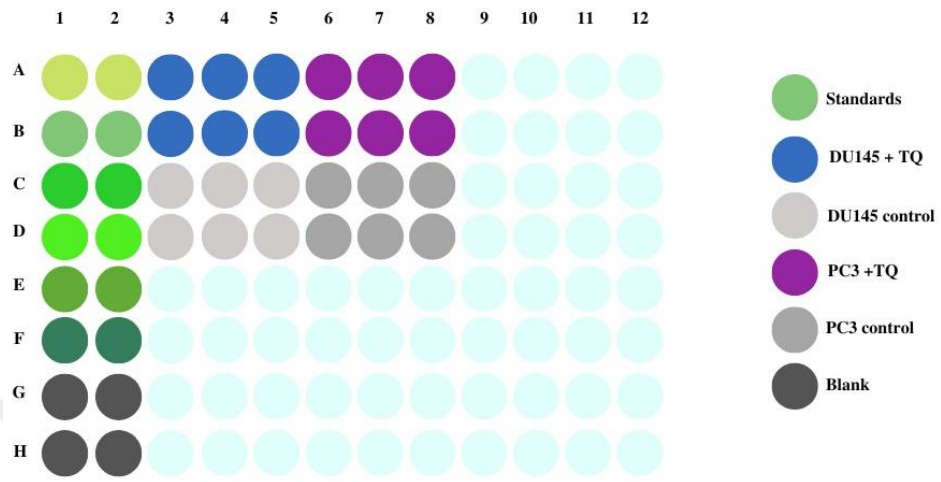


Figure 3.1. ELISA plate design.

The ELISA protocol was initiated by adding 50 µL of standard successive solutions to the standard wells, followed by adding 10 µL of sample and 40 µL of sample diluent to the testing sample well, with the blanks left empty. After that, 100µl of HRP-conjugate reagent was added to each well (Standard wells and testing sample wells), covered with seal plate membrane, and incubated for 60 minutes at 37 ° C. After 5 times of washing, 50µl chromogen solution A was added to each wells, followed by an addition of 50µl chromogen solution B, Shaken gently to mix up, and incubate for 15 minutes at 37℃, away from light for color developing. Finally, 50µl of Stop Solution was added to each well to stop the reaction, when color change from blue into yellow was immediately observed, and the optical density (OD) was evaluated using Biotek Epoch plate Reader at 450 nm.

3.11. Data Analysis

The statistical analyses were conducted with GraphPad Prism software. The testing for normality was done with the Shapiro-Wilk-normality test. Statistical differences

between each group of values and its control group were evaluated using a two-tailed Student's t test and differences with a $p < 0.05$ were considered significant.

4. RESULTS

4.1. The effect of TQ on PLA2G7 protein level in Pc-3 and Du-145 cell lines

ELISA was performed to determine the effect of thymoquinone on the level of PLA2G7 proteins in both cell lines. According to standards' concentrations and the corresponding OD values, the linear regression equation as well as the PLA2G7 proteins standard curve were generated using Microsoft Excel® software (Figure 4.1). Then, by plotting the OD value of samples on PLA2G7 proteins standard curve y-axis, the corresponding concentrations were determined.

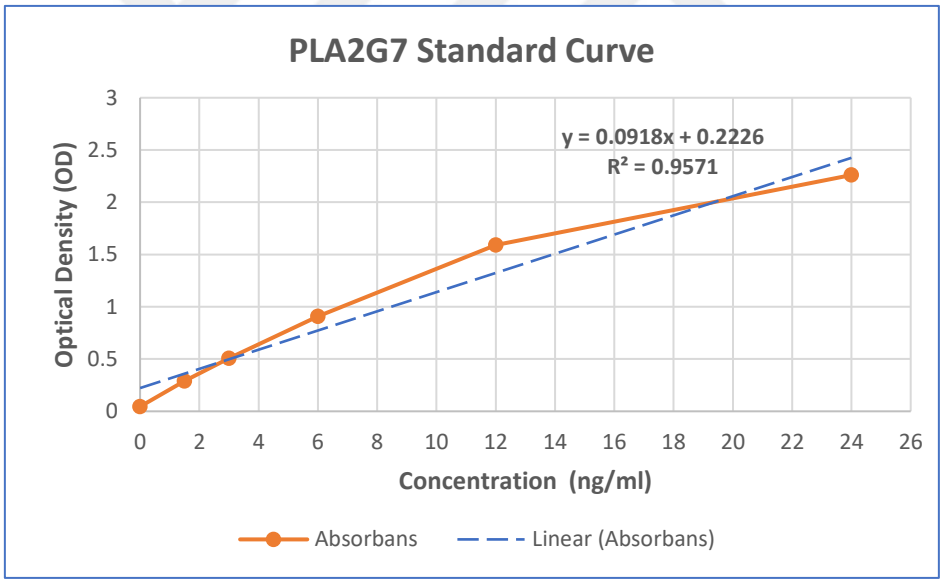


Figure 4.1. PLA2G7 Concentration Standard Curve.

T-tests were used to determine the connection between the treated group versus the control group in terms of PLA2G7 protein level. As shown in (Figure 4.2), no significant effect on PLA2G7 protein in the treated group was found compared with the control group in Du-145 cell lines. Similarly, TQ has shown a slight yet no significant reduction of the protein of interest in PC3 cells as well (Figure 4.3). Together, these results

imply that TQ hasn’t exhibited a statistically significant effect on the level of PLA2G7 protein in both prostate cancer cell lines.

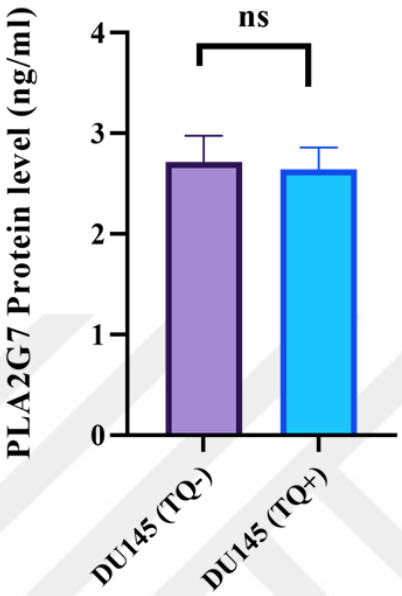


Figure 4.2. The effect of TQ on PLA2G7 levels in DU145 compared to the control group.

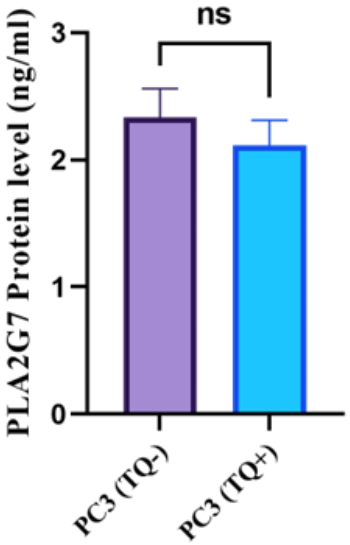


Figure 4.3. The effect of TQ on PLA2G7 levels in PC3 compared to the control group.

4.2. The effect of TQ on UCP2 protein level in Pc-3 and Du-145 cell lines

ELISA was performed to determine the effect of thymoquinone on the level of UCP2 proteins in both cell lines. According to standards' concentrations and the corresponding OD values, the linear regression equation as well as the UCP2 proteins standard curve were generated using Microsoft Excel® software (Figure 4.4). Then, by plotting the OD value of samples on UCP2 proteins standard curve y-axis, the corresponding concentrations were determined.

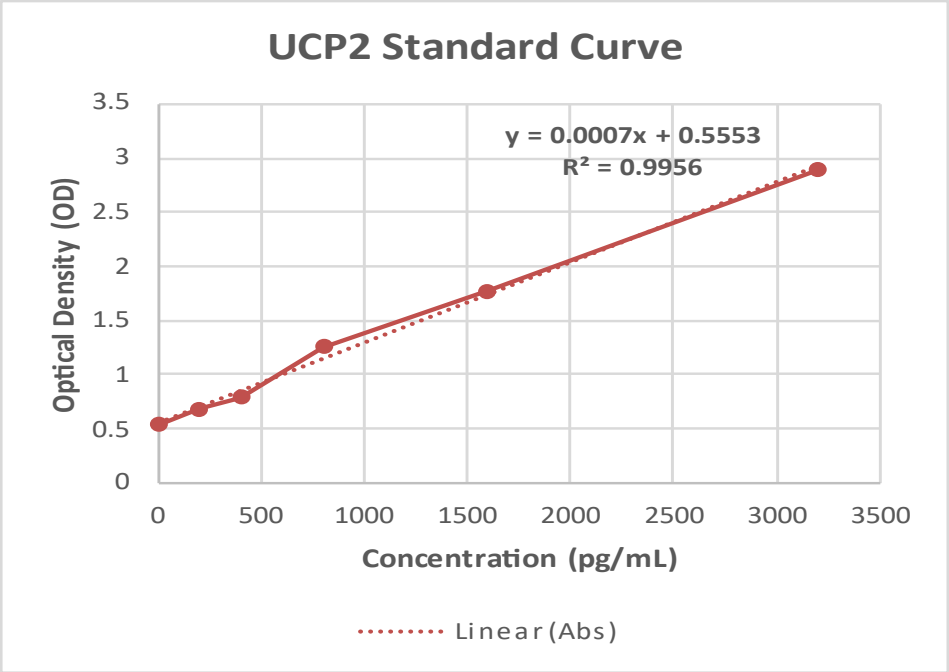


Figure 4.4. UCP2 Concentration Standard Curve.

T-tests were used to determine the connection between the treated group versus the control group in terms of UCP2 protein level. The analysis results, shown in (Figure 4.5), indicate that TQ has significantly decreased the level of UCP2 protein compared to the untreated group in DU-145 cells. Likewise, TQ has also exhibited a statistically significant decrease of UCP2 protein in PC-3 cells (Figure 4.6). Overall, TQ has a significant decreasing effect on UCP2 proteins on both cell lines with a p value of 0.03 and 0.017 respectively.

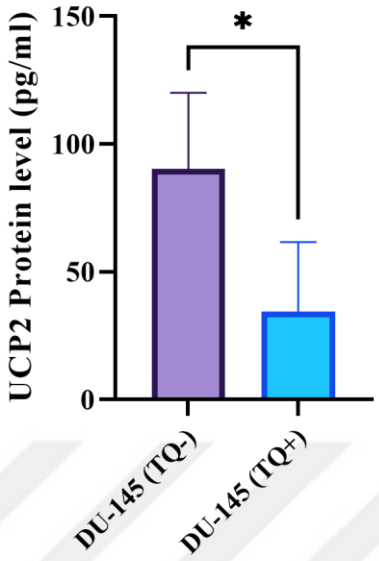


Figure 4.5. The effect of TQ on UCP2 levels in DU145 compared to the control group.

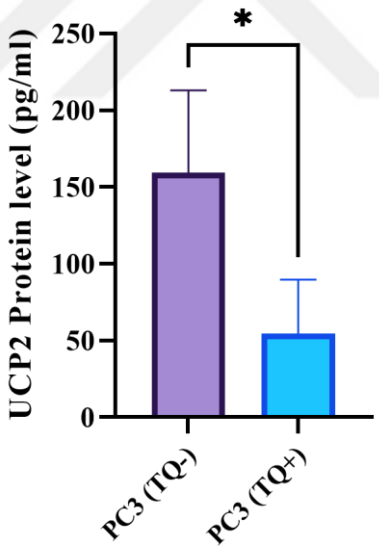


Figure 4.6. The effect of TQ on UCP2 levels in PC3 compared to the control group.

4.3. The effect of TQ on NEDD4L protein level in Pc-3 and Du-145 cell lines

ELISA was performed to determine the effect of thymoquinone on the level of NEDD4L proteins in both cell lines. According to standards' concentrations and the

corresponding OD values, the linear regression equation as well as the NEDD4L proteins standard curve were generated using Microsoft Excel® software (Figure 4.7). Then, by plotting the OD value of samples on NEDD4L proteins standard curve y-axis, the corresponding concentrations were determined.

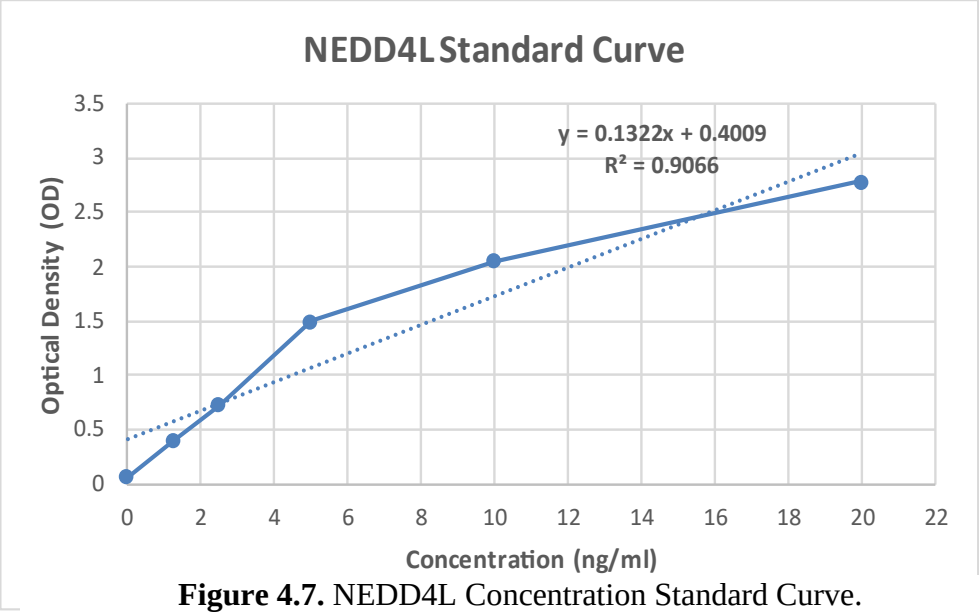


Figure 4.7. NEDD4L Concentration Standard Curve.

Two tails t-tests were conducted to determine the connection between the treated group versus the control group in terms of NEDD4L protein level. Surprisingly, as provided in (Figure 4.8), TQ has significantly increased the level of NEDD4L protein compared to the untreated group in DU-145 cells. Interestingly, TQ has also exhibited an even more statistically significant increase, p=0.0011, of NEDD4L protein in PC-3 cells (Figure 4.9).

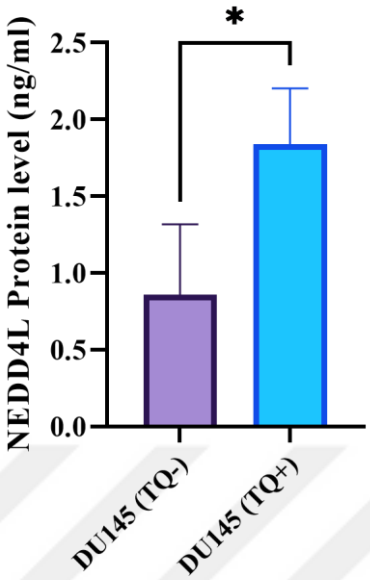


Figure 4.8. The effect of TQ on NEDD4L levels in DU145 compared to the control group.

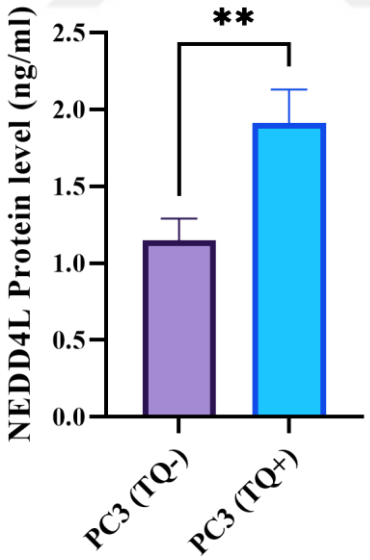


Figure 4.9. The effect of TQ on NEDD4L levels in PC3 compared to the control group.

Altogether, these findings provide crucial insights into the potential effect of Thymoquinone on decreasing the level of UCP2 protein in two different prostate cancer cells. Additionally, TQ has exhibited an increasing effect on NEDD4L proteins in Du-145 cells yet more significative in Pc-3 cells. Therefore, the next chapter will be dedicated for discussing our findings and draw meaningful conclusions referring to the existing literature as well as the contribution of our findings in unraveling more of the effect of TQ on lipid droplets in Prostate cancer cells.

5. DISCUSSION

Cancer cells are characterized by metabolic changes that sustain tumor growth and facilitate the process of coping with new growth rate demands during malignancy. Interventions aimed at modifying metabolic rewiring of cancer cells are currently being investigated in several tumor types, including prostate cancer.

With regards to metabolic alterations intended to maximizing energy intake and generating biomass, cancerous cells display a distinct metabolism compared to normal cells that provides them with a proliferative advantage (91) .

In many cancer cells, in order to sustain cell proliferation, glucose is used as a primary source for the anabolic processes which is known as the Warburg effect (33). PCa, however, is often not glycolytic. A growing body of literature has demonstrated a strong positive correlation between upregulated lipogenesis, known as lipogenic phenotype, and PC pathogenesis (35). PCa development and progression are associated with increased lipogenesis and de novo FA synthesis is currently considered as a hallmark of PCa (92).

In times of lipids abundance, de novo or acquired FAs are incorporated in Triacylglycerols (TAGs) and build up as LDs serving as a reservoir and source of lipids particularly under stress conditions (93). Interestingly, it was reported that LDs may contribute to chemoresistance by sequestering harmful molecules such as ROS eliciting a cytoprotective response (94). This was further supported by Cotte et al. 2018, when lipid droplet accumulation was evidenced to support colorectal cancer resistance to 5-fluorouracil (5-Fu) and oxaliplatin by inhibiting ER stress (95). In concordance with that increased abundance of LDs is a feature of many aggressive cancers, including PCa (96). Additionally, arising evidence suggest that accumulation of LDs is positively associated with higher Gleason score (GS) grade of human PCa hence the contribution of LD into more aggressiveness and malignancy (10).

These observations open up new avenues for the exploitation of lipid metabolomics as a source of new therapeutic targets. The usage of both natural and synthetic agents that affect lipogenic phenotype in cancer is now a rapidly growing field

of research (97). In accordance with that, we have selected Thymoquinone, one of the bioactive constituents in Nigella sativa (N. sativa), black seed, to investigate possible effect on LDs in PCa.

Throughout previous years, it has been reported that TQ has been used as an antineoplastic, antioxidant, analgesic, and anti-inflammatory agent (70). By Acting on different targets and enzymes, TQ has shown various anticancer activities through cell proliferation inhibition, apoptosis induction, or interference with other tumorigenic processes, such as cell migration, invasion, and altering epigenetic events alteration in cancer cells (6).

As targeting specific cancer therapeutics is a major focus in present anticancer treatments, specific molecular targets for thymoquinone, like enzymes, receptors, DNA or RNA materials should also be kept under investigation. Our study aligns with these needs as we hypothesized that TQ has an effect on the amount of PLA2G7, UCP2, and NEDD4L proteins which are associated with Lipid droplets formation. Given the fact that no such study is found in the literature, our research is original, unprecedented, and it will unravel more of the mechanisms of TQ and fill in the gap in the literature.

As the ultimate aim is to find new treatments of men with high volume hormone refractory PC, the selection of the cells lines was performed to allow to cover an extended range of PCa with different features, namely different aggressiveness and hormonal dependency (98).

It was found that the PLA2G7 gene is upregulated in well-differentiated prostate cancer and is significantly linked to the altered phospholipid metabolism of prostate cancer and played a key role in pathogenesis (51). Aside from the role of PLA2s in LD synthesis, they also act as providers of free fatty acids, generators of metabolite that may control LD formation (52).

Dong et al. study has demonstrated that PLA2 is overexpressed in androgen-independent prostate cancer cells relative to their parental androgen-dependent counterparts. More importantly, high levels of PLA2s were shown to be significantly associated with high GS and advanced disease stage (99). Our choice of cell lines aligns

with this study since we have selected androgen-independent prostate cancer cell lines, PC3 and DU145, to be the subject of our research.

Furthermore, PLA2s has been the subject of research in several types of cancer including Prostate cancer where both synthetic and plant derived PLA2 inhibitors showed antitumoral effects on cancer cells in vitro as well as in in vitro. As such, Patel et al. study has shown increased levels of PLA2 in DU145 and PC3 cell lines and that siRNA knockdown or specific inhibition of PLA2 by Wyeth-1 resulted in a decrease in cell growth and proliferation both in vitro and in vivo. More interestingly, the immunohistochemical analyses of human prostate tissues showed that PLA2 is present and even higher in some men reaching hormone refractory state (100). Similarly, a significant cytostatic effect has been observed after treating PC3 cell lines with Bromoenol Lactone, a synthetic PLA2 selective inhibitor, in comparison with LNCaP, androgen-sensitive cell lines (101).

Our study is the first to investigate the effect of TQ on PLA2s, yet very few studies have addressed the PLA2s using natural products. Thus, the following study has contributed to filling the gap in the literature and providing new insights on TQ anticancer effect. Contrary to expectations, our study located no significant differences between the treated group versus the control in both cell lines thus TQ has shown no statistically significant reduction on PLA2 proteins level. There are several possible explanations for such a result. One is that the IC50 could be below the threshold required to see a positive effect on the protein of interest. Hence, using a value of IC50 based on existing data from the literature might be one of the leading limitations of our study that need to be addressed in the future. Another possible explanation for this might be the possibility that TQ may exert an effect in a timely-dependent manner, so the incubation time was not sufficient to reach an inhibitory effect. As such, further investigations should be held in order to address these limitations.

In cancer cells, UCP2 is generally overexpressed to attenuate ROS formation and to trigger anti-apoptotic properties and resistance to therapies (102). Besides, UCP2 amplification has been detected in several human cancers, including Prostate cancer (103). A study has shown that the protein levels of UCP2 were significantly higher in

human head and neck, skin, prostate, and pancreas tumor tissues compared to that in normal tissue (103).

On the other side, it has been shown that UCP2 protein expression level correlates closely and negatively with tumor proliferation in vitro and in vivo (56). Indeed, Emre in-silico analysis states that UCP2 expression is downregulated in PCa (50). Likewise, another study came to confirm the role of UCP2 downregulation in metabolic rewiring when it has been shown that UCP2 loss promotes phospholipid synthesis and increases expression of lipogenic enzymes, thus lipid accumulations and a potential promotion of LDs formation (57).

The hypothesis today for this discrepancy of data is that UCP2 would be repressed in early phases of tumorigenesis to allow the accumulation of ROS and promote genomic instability (104), while on the contrary the protein would be overexpressed in the late phase to maintain the proliferative capacities of the cells in the tumor microenvironment (TME) that are already under stress conditions (105).

The cell lines used in our study, PC3 and DU145, are of an advanced phase of prostate cancer and derived from bone and brain metastasis respectively. Given the late stage of carcinogenesis of our cells, we were expecting UCP2 to be upregulated and TQ to reduce the amount of UCP2 proteins. As expected, TQ has demonstrated a significant decrease of UCP2 in both cell lines. The effect of TQ on UCP2 has not previously been described in the literature; hence, the originality of our study vastly contributes into opening new insights on further investigations to demonstrate the underlying mechanism.

Furthermore, our finding match those of a recent study where Genipin, a plant chemical extract, by decreasing UCP2 expression in MCF7 breast cancer lines, inhibited tumor promotion characterized by lower cell proliferation and migration compared to control (106). Likewise, Genipin was shown to inhibit proliferation of androgen-independent PC-3 prostate cells by suppressing UCP2 (107), which, as hypothesized, supports the idea TQ could be also a potential inhibitor of UCP2.

Additionally, a growing body of literature shows that cells expressing higher levels of UCP2 are more resistant to chemotherapy which is mediated by inhibition of ROS

generation and Stat3 activation (108); thus, the idea of lowering the UCP2 levels as an attempt to increase chemosensitivity sounds valid. In fact, the combined use of trastuzumab, a cytotoxic molecule, with Genipin, a UCP2 inhibitor, synergized their action and created a similar effect on the inhibition of cell viability to the use of UCP2 siRNA and trastuzumab (109). Our findings seem to be consistent with previous studies and will definitely open a new fresh area of investigation about the effect of TQ on UCP2 in terms of enhancing the chemosensitivity of prostate cancer cells.

In a nutshell, based on all the studies cited above, the inhibition or activation of UCP2 according to the metabolic status of the tumor represents a therapeutic opportunity to improve cancer remission for which TQ can be a suitable candidate. Targeting UCP2 in combination with conventional treatments (chemotherapy, radiotherapy, immunotherapy) could promote a beneficial response in patients.

To date, several reports have described Nedd4 expression in cancer cell lines. Although Nedd4 is widely expressed in the cancer cell lines, the intensity of Nedd4 expression was cell-type specific (110). It was reported that the expression of NEDD4L was decreased in prostate cancer specimens compared with benign prostatic hyperplasia (54). In concordance with that, another study has also demonstrated that NEDD4L was downregulated in androgen-independent prostate cancer cells (111).

Unexpectedly, Hellwinkel et al. reported that the level of NEDD4L was higher in prostate cancer tissues than in adjacent normal tissues (112). Similarly, the expression of three NEDD4L transcripts, was upregulated in LNCaP human prostate cancer cells prostate cancer cells after androgen administration (113).

The discrepancy with the data previously provided may be explained by the fact that level of expression of Nedd4L differs according to the stage of malignancy which is directly proportional to Gleason score. Thus, Nedd4L expression is decreased in well differentiated PCa (54) and upregulated in poor differentiated PCa (50). These data suggested that regulation of Nedd4L expression might play a role in the development of prostate cancers and may give insights on new therapeutic strategies (55).

Moreover, In order to draw links to the objective of our research, we point out that it was shown in recent studies that spartin, which interacts with E3 ubiquitin ligases that belong to (Nedd4) family, acts as a linker for ubiquitination of adipophilin on lipid droplets, likely by sequestering Nedd4-E3 ubiquitin ligases and ubiquitination machinery (53).

Therefore, given the fact that the cell lines we used are of an advanced aggressive phenotype, we projected a downregulation of NEDD4L before treatment and TQ to increase the levels of NEDD4L which is a potential effect on the ubiquitination of lipid droplets.

As expected, TQ has significantly increased the levels of NEDD4L in both cell lines. What is interesting is that the effect of TQ on NEDD4L was even more significant on PC3 cell line, suggesting the potential efficacy of TQ on even more aggressive type of PCa.

In a similar fashion but on Lung Adenocarcinoma cells, β,β-dimethyl-acryl-alkannin (ALCAP2), a natural small-molecule compound separated from the root of Lithospermum erythrorhizon, could increase the expression of NEDD4L inhibiting cell proliferation and metastasis as well as inducing apoptosis and cell cycle arrest (114). Additionally, Wogonin, a natural flavonoid agent, was revealed to upregulate the expression of NEDD4L, the ubiquitin E3 ligase of Pik3ca, inducing the degradation of Pik3ca which reverses the over-activation of the PI3K/Akt pathway (115). Indeed, inferring from the findings described above, we suggest that TQ is more likely to induce lipid droplets degradation with the same way.

Our research is one the very few published studies conducted to investigate a potential effect on NEDD4L level, and it is regarded as the first of its kind in terms of targeting NEDD4L proteins, in a context of prostate cancer cell lines, using one of *nigella sativa*'s active component.

By combining studies altogether with our findings, due to its role in tumorigenesis, NEDD4 can be a potential target in drug discovery against prostate cancer. As yet there is a paucity of information as to what is happening at a cellular level to Nedd4L signaling, a

better understanding of the mechanisms underlying the association of Nedd4L with its interplay molecular in prostate cancer progression could potentially lead to more effective clinical management, as well as provide new target pathways for prostate cancer therapy.

Finally, additional investigations should take place to unravel the TQ mechanism of action and take it even a step further toward more in vivo and in vitro studies.

6. CONCLUSION

✓ Increasing knowledge of metabolic rewiring is revealing novel metabolic signatures in PC. These signatures could be utilized for PC diagnosis, as well as for the discovery of novel therapeutic interventions to overcome castration resistance.

✓ Natural and synthetic agents that affect lipid metabolism in cancer is a rapidly growing field that was recently reviewed elsewhere. In the present study, we demonstrated that Thymoquinone has a significant effect on UCP2 and NEDD4L proteins which are thought to be correlated with lipid droplets genesis.

✓ Given the fact that no such study is found in the literature, our research is indeed unprecedented, and it undoubtedly gives more insight on the effectiveness of TQ toward various targets, including the proteins related to LD such as NEDD4L which may contribute into reversing cancer cell resistance to chemotherapeutic agents.

✓ For future perspectives, the flexibility and the complexity of metabolic networks which poses a issue in cancer therapy, additional investigations should take place to unravel TQ various mechanisms of action and take it even a step further toward more in vivo and in vitro studies. Thus, targeting UCP2 and NEDD4L with TQ in combination with conventional treatments (chemotherapy, radiotherapy, immunotherapy) could promote a beneficial response in patients.

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