THE IMPACT OF CAFFEINE ON PROSTATE CANCER PROGRESSION: AN IN VITRO STUDY

A Thesis

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In Partial Fulfillment

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Master of Science in Biology

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by

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Notre Dame University - Louaize
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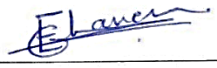

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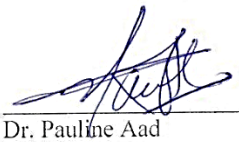

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The impact of caffeine on prostate cancer progression: an *in vitro* study

Table of contents

ABSTRACT

Prostate cancer (PCa) is the most frequent cancer malignancy among men and the third leading cause of cancer death in the developed world. To date, the exact molecular mechanism underlying the role of caffeine one of the major coffee constituents on PCa prognosis is not yet unraveled. Caffeine has many antioxidant, anti-inflammatory and anti carcinogenic properties through the modulation of certain inflammatory markers such as IL-6 and TNF-α.

The present study was carried out to evaluate the effect of caffeine on PCa cell lines viability, namely PC3 and PC3-PSMA and assessing as well their inflammatory status by quantifying the gene expression of IL6 and IκB-α. For the viability, WST1 assay was performed and indicated a significant decrease in growth up to 50% with increased caffeine doses depending on the cell type and incubation period ($p<0.05$). Interestingly, IL-6 concentration increased after 24 hrs incubation with caffeine reaching ELISA levels of 321pg/ml for PC3 cells and 88 pg/ml for PC3-PSMA cells ($p<0.05$). After 48 hrs IL-6 concentration dropped to 34 pg/ml for PC3 cells and it was undetectable in PC3-PSMA cells. The expression of IL-6 and IκB-α genes were assessed using qRT-PCR. Variation in either caffeine dose or incubation time period had no effect on IκB-α mRNA abundance for both cell lines ($p>0.05$). High IL-6 expression was found at high caffeine concentration (10 and 20 mM) for PC3 and PC3-PSMA cells ($p<0.05$). These results demonstrate that caffeine increased inflammation but decreased viability through the stabilization of IκB-α which is perfectly correlated with the levels of NF-κB responsible of cell proliferation and survival.

List of tables

List of figures

List of abbreviations

Activator protein-2: AP-2

Androgen deprivation therapy: ADT

Aldo-keto reductase family 1 member C3: AKR1C3

Androgen receptor: AR

Avian reticuloendotheliosis: RelA

Avian reticuloendotheliosis viral oncogene homolog B: RelB

Cancer stem cells: CSC

Castration-resistant prostate cancer: CRPCa

Complete androgen blockade: CAB

Cyclic adenosine monophosphate: cAMP

Cytotoxic T cells: CTL

Dihydrotestosterone: DHT

Fas ligand: FasL

Folate hydrolase: FOLH

Gamma-aminobutyric acid: GABA

Gamma activated sequence: GAS

Glucocorticoid receptors: GR

Heterodimeric T cell receptor: TCR

Histone deacetylase 1: HDAC 1

Human prostate cancer line: PC3

Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2: HSD3B2

Inhibitor of NF-κB: IκB-α

Interferon: IFN

Interleukin 6: IL-6

Interleukin 1: IL-1

Janus kinase: JAK

Monoclonal antibody: mAb

N-acetylated α-linked acidic dipeptidase: NAALADase

N-acetyl-aspartyl-glutamate: NAAG

Nitric oxide: NO

Nitric oxide synthase: NOS

Nuclear factor-kappa B: NF-κB

Ovariectomized: OVX

Peripheral mononuclear blood cells: PMBC

Prostaglandin E2: PGE2

Prostate cancer: PCa

Prostate-specific membrane antigen: PSMA

Protein inhibitor of activated STAT: PIAS

Reactive oxygen species: ROS

Signal transducer and activator of transcription 3: STAT 3

Suppressor of cytokine signaling: SOCS

Tumor necrosis factor: TNF

Tyrosine kinase 2: TYR

I. INTRODUCTION

Coffee is one of the most popular beverages worldwide and the most commercialized for decades. Increasing consumption of coffee is related to the pleasing taste and aroma, as well as its physiological effects including cardiovascular, respiratory, renal, and smooth muscle effects, as well as effects on the mood, memory, alertness, and physical and cognitive performance [1].

Since the opening of the first coffee house in Mecca at the end of the fifteenth century, coffee consumption has greatly increased all around the world. In2010, coffee production reached 8.1 million tons worldwide. This represents more than500 billion cups, with the United States, Brazil, Germany, Japan, and Italy being the major consumer countries[2].

Coffee contains various concentrations of biologically active compounds such as caffeine, chlorogenic acids, melanoidins, trigonelline and diterpenes which include cafestol and kahweol as represented in figure 1.The intake of each compound depends on the variety of coffee species, roasting degree, type of brewing method and serving size [3].

Caffeine is a methylxanthine with bitter characteristics; however, it is responsible for no more than 10% of the perceived bitterness of the coffee beverage[2].

The mean half-life of caffeine in plasma of healthy individuals is about 5 hours. However, caffeine's elimination half-life may range between 1.5 and 9.5 hours, while the total plasma clearance rate for caffeine is estimated to be 0.078 L/h/kg. This wide range in the plasma mean half-life of caffeine is due to both innate individual variation, and a variety of physiological and environmental characteristics that influence caffeine metabolism (e.g., pregnancy, obesity, use of oral contraceptives, smoking).Caffeine is rapidly and completely absorbed in humans, with 99 percent being absorbed within 45 minutes of ingestion and is also sufficiently lipophilic to pass through all biological membranes and readily crosses the blood-brain barrier.

When it is consumed in beverages (most commonly coffee, tea, or soft drinks) caffeine is absorbed rapidly from the gastrointestinal tract and distributed throughout body water. Peak plasma concentrations occur between 15 and 120 minutes after oral ingestion[4].

The effects of caffeine on the heart are primarily stimulatory and are accompanied by increased coronary blood flow and blood pressure [3,5].In the lungs caffeine can cause

smooth muscle relaxation and bronchial dilatation, possibly accounting for its antiasthmatic effects. On the kidney it leads to diuresis, increased blood flow and rennin secretion[6].In the central nervous system caffeine stimulates the action of dopamine, norepinephrine, serotonin, acetylcholine, glutamate, and GABA neurons because of its ability to block the action of adenosine receptors [4].The stimulatory effects of caffeine include enhanced perception, increased capacity to remain awake for longer periods and reduced fatigue [5]. Caffeine has pharmacological effects towards diseases of the central nervous system especially Alzheimer and Parkinson being able to cross the blood brain barrier because of its low molecular weight and good lipid solubility [5, 7].

Coffee is a rich source of antioxidants that may contribute to prevention of oxidative stress-related diseases such as cancer, diabetes cardiovascular diseases and inflammation[5]. Oxidative stress is caused by reactive oxygen species (ROS) which are likely to damage several cellular components (lipids, proteins, nucleic acids, and DNA) through oxidation (figure 2)[1].

Caffeine induces apoptosis and cell cycle suppression in many cell lines which results in the inhibition of carcinogenesis [8].*In vitro*, caffeine enhances the cytotoxicity in human glioma cell and decreases the activity of HDAC1(Histone deacetylase 1)[9].Caffeine blocks glioblastoma proliferation and increases caspase-dependent apoptosis of glioma cells by activating caspase 3 [7]. It inhibits the proliferation and metastasis, and induce *in vitro* apoptosis of melanoma cells at physiologically applicable concentration which is 80 µg/ml equivalent to 412 µM. Even at low concentration ranging from 200 to 600µM, caffeine reduces melanoma cancer [10].

In vivo studies demonstrate that moderate (2-4 cups /day) to high consumption of caffeine suppresses the proliferation of breast cancer cells [11].Epidemiological studies have also shown that consumption of caffeinated beverages, including coffee and tea, reduces the risk of prostate cancer (PCa)[12].

The following literature review covers the general health effects of coffee on PCa progression and the specific effect of caffeine on certain inflammatory markers, such as IL-6, TNF-α and NFκ-B.

Figure 1: Chemical composition of coffee

Various concentrations of active compounds are present in coffee that varies based on coffee species, roasting degree and serving size.

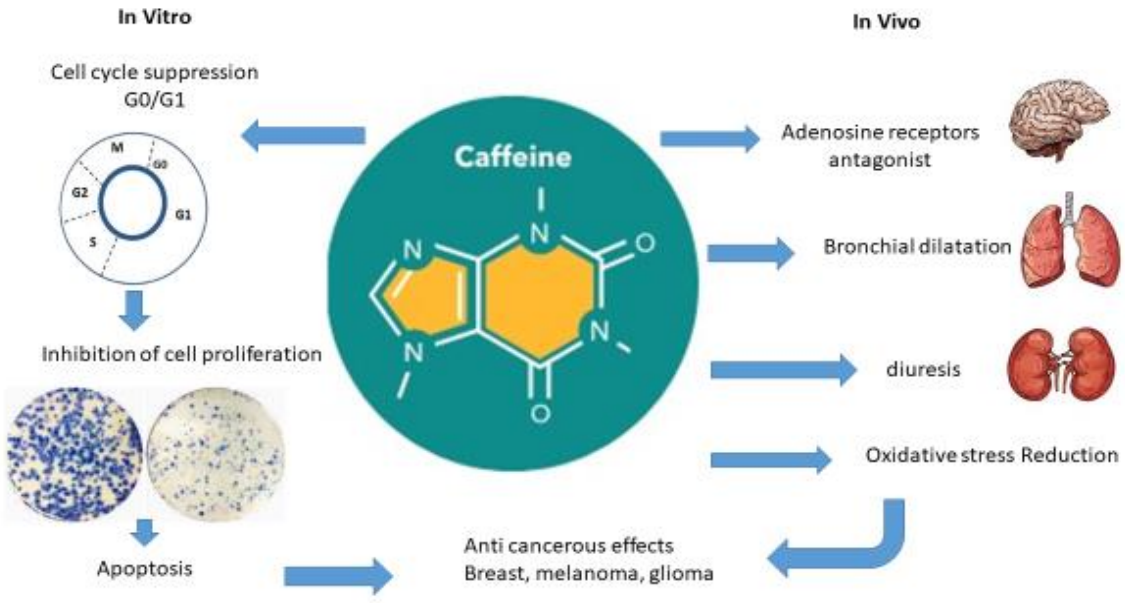


Figure 2: Physiological effects of caffeine. Caffeine suppresses cell proliferation *in vitro* and causes bronchial dilatation, diuresis, oxidative stress reduction and AR antagonism in *vivo*.

II. LITERATURE REVIEW

1- Coffee and prostate cancer

PCa is known as the second most frequently diagnosed cancer and the sixth leading cause of cancer death in men all over the world. Race, age, and family history have been established as strong risk factors associated with PCa. Coffee is one of the most popular beverages worldwide and has been investigated for its influence on the risk of cancers as it contains components with antioxidant, antiinflammatory, and anticarcinogenic properties. Coffee consumption is associated with a reduced risk of PCa and it also has an inverse association with non-advanced PCa[13],[14]. Interestingly, not only does the type of coffee and number of cups uptake affect the rate of PCa, but also the geographical location of the study investigation. A Japanese study proved that37% of PCa was estimated to be prevented in men consuming three or more cups of coffee per day, compared with non-coffee drinkers[15].Therefore, high consumption of coffee decreases aggressive PCa by 55% in a UK study [16] and by 53% in an Italian study [17].A Swedish study also confirmed that high consumption decreases5% the advanced PCa and 11% the fatal PCa.

An epidemiological study over 20 years on 47911 health professional men in the USA of which 5035 developed PCa revealed an inverse association between coffee intake and the risk of lethal and advanced cancers[18] . Men who consumed six or more cups of caffeinated coffee per day had an 18% lower risk of PCa compared with men who did not. Furthermore, men who drank four or more decaffeinated cups of coffee per day had lower risk of advanced cancer (table 1)[18].

Though most of the *in vivo* studies support the incidence decrease of PCa, a UK study on 46155 cancer cases showed that there was no relationship between coffee intake and cancer risks including breast, ovarian, lung and prostate [19].

Based on our literature search, the direct association of coffee or any of its anti- oxidant constituents on specific inflammatory markers was not properly screened. Moreover, most studies as summarized in table 1 are addressed *in vivo* on a cohort of participants with a small group or a meta- analysis based on the type of coffee, its dose, cup size and other epidemiological factors. Thus, more *in vitro* studies are needed to unravel the association of coffee with the prognosis of PCa.

To date the exact molecular mechanism underlying the role of coffee consumption on PCa is not yet unraveled. However, growing evidence supports a protective role of coffee as an anti-inflammatory agent. Most *in vitro* studies are conducted on caffeine and its effects on cell proliferation apoptosis and inflammatory markers. In the next sections, the effects of caffeine on cell viability and inflammatory markers will be described.

Table 1: *In vitro* effects of coffee on PCa. The effect of coffee on the prognosis of PCa is based on the serving coffee size and epidemiology in specific populations.

Country	Study Period	Case/Control	Type of coffee	Dose	Application	Result	Reference
USA	1986-2006	5035/47911	Caffeinated Decaffeinated	< 1cup/day ≥ 6cups/day ≥ 4cups/day	Non advanced Pca Advanced Pca Lethal prostate cancer	No effect 18 % Lower Pca 18 % Lower Pca	[18]
Japan	1995-2005	318/18853	Caffeinated	1-2cups/day ≥ 3cups/day	Total Pca	Decrease in Pca 37% decrease	[15]
Sweden	1998-2010	3801/44613	Caffeinated	3 cups/day ≥ 3cups/day 3 cups/day ≥ 3cups/day ≥ 3cups/day	Nonaggressive Low grade Pca Nonaggressive localised Pca High grade aggressive Pca Advanced Pca Fatal Pca	No effect 3% decrease No effect 5% decrease 11% decrease	[14]
UK	1970-2007	380/6017	Caffeinated	1-2 cups ≥ 3cups/day	Aggressive Pca	10% low risk 55%low risk	[16]
Italy	2005-2010	100/6989	Various kind of Italian coffee	> 3cups/day	Total Pca	53% low risk	[17]
Uk	2006-2010	46155/270342	Caffeinated	≥1cup/day	Breast,ovarian,lung PCa	No association between coffee intake and cancer risk	[19]

2- Effects of caffeine on cell viability

In vitro studies demonstrated that caffeine induces cell cycle arrest at G0/G1 phase apoptosis and decreases cell proliferation when incubated with different caffeine concentrations ranging from 0.05 mM to 20mM.Studies on human epithelial lung cells A549 showed that after incubation with 1mM caffeine for 72 hours the viability decreases and the number of cells in G1 increases [20].In addition incubation of murine epidermal, and glioma cells with different caffeine concentrations decreases viability and induces apoptosis [8, 9].Even at very low concentrations ranging from 50 to 600 μM the viability is decreased(Table 2) [10].

Table 2: The effects of caffeine on cell viability and proliferation. Overall *in vitro* studies support the apoptosis of cells from different cell lines in the presence of various caffeine concentrations.

Cell type	Caffeine dose	Application	Result	Reference
Mouse epidermal cell line JB6C1 41	025mM, 0.5mM, 0.75 mM, 1mM 0.5mM,1mM,5mM	*In vitro*	Inhibition of cell proliferation Cell cycle arrest at G0/G1 phase.	[8]
Human epithelial lung cells A 549 Mouse epithelial cells MLE 12SV 40	1 mM 0.05mM 1mM	*In vitro*	Viability decrease ROS (reactive oxygen species) decrease Increase the number of cells in G1 and reduce the number of cells in G2	[20]
RT2 rat glioma cells	0.5mM,1mM,2mM	*In vitro*	Decrease in cell viability	[9]
B16F10 murine melanoma cells	50, 100, 200, 400, 600 μM.	*In vitro*	Decrease in cell viability and proliferation. Increase in cell apoptosis	[10]
C6 and human 487MG glioblastoma cells	0.05, 0.1, 0.5, 1, 2.5, 5,10, 20 mM	*In vitro*	Decrease in cell viability and proliferation. Cell cycle arrest at G0/G1 phase.	[7]

3- Caffeine and inflammatory markers

In addition to its role in cell proliferation, caffeine modulates the levels of inflammatory markers such as IL-6 and tumor necrosis factor (TNF).The immunoregulatory role of caffeine does not possess a unifying model of action on inflammatory markers such as IL-6 and TNF-α [21]. *In vivo* and *in vitro* studies on male rats, human blood cells and human adipose tissue cells demonstrated that caffeine administration decrease IL-6, and TNF-α as depicted in table 3 [22], [23], [24], [25].However, *in vitro* studies on male mice, OVX female rats, male and female human subjects demonstrated that caffeine increases serum levels of C-reactive protein, IL-6, and TNF-α [26],[27],[28].

In conclusion, the studies are controversial in increasing or decreasing inflammatory markers. In our thesis we studied the effects of caffeine on the expression of IL-6 and NF-κB on PC3 and PC3-PSMA prostate cancer cells using ELISA and RT-PCR. In addition to its effects on cell toxicity in an attempt to define the cellular and molecular mechanism of caffeine action on PCa cells.

Caffeine → TNF-α deactivation → NF-κB decrease →IL-6/ PSMA decrease→ Apoptosis
Or activation increase increase Angiogenesis

Table 3: Effect of caffeine on inflammatory markers. The effect of caffeine on inflammatory markers is inconsistent depending on the doses and the application.

Study Type/Subjects	Caffeine Dose	Application	Markers	Result	References
Male rats n=40	37.5mg/kg/day Equivalent to 3cups of coffee	Liver injury induced by TAA (thioacetamide)	IL-6 IL-1 TNF-α	Decrease	[23]
PMBC human cells Peripheral mononuclear blood cells	100 µM	Whole blood	TNF-α	Decrease 47-69%	[25]
Human adipose tissue	0.5µg/ml 5µg/ml 505µg/ml	Stromavascular fraction cells Adipocytes	TNF-α IL-6 IL-6	Downregulation Downregulation Increase	[24]
Male rats N=50	25mg/kg/day	Myocardial cells Induced injury	TNF-α IL-6 NF-κB	Decrease Decrease Decrease	[22]
Male mice C57BL/6J	0.5 mg/ml	Blood Muscles Liver	Circulating IL-6 IL-6 mRNA IL-6	10 fold increase Increase No Increase	[26]
Ovariectomized (OVX) Female rats	9.6 mg/ml 19.2 mg/ml 38.4 mg/ml	Postmenopausal osteoporosis	IL-6 IL-1β	Increase Increase	[28]
Healthy men 1514	>200ml of coffee /day	Serum inflammatory markers	IL-6 C-reactive protein (CRP) TNF-α	50% increase 30% increase 28% increase	[27]
Healthy women 1528	>200ml of coffee /day	Serum inflammatory markers	IL-6 C-reactive protein (CRP) TNF-α	54% increase 38% increase 28% increase	[27]

4- Interleukin 6 (IL-6)

4.1 Role of IL-6 in cancer

The signal transduction of IL-6 which is produced by inflammatory cells and osteoblasts involves the activation of JAK tyrosine kinase family members, resulting in the activation of transcription factors of the signal transducers and activators of transcription 3 STAT 3 [29],[30]. A variety of events take place downstream of gp130 activation through the ligand, including the activation of cytoplasmic tyrosine kinases and the modification of transcription factors. Although gp130 has no intrinsic kinase domain, the JAK1, JAK2 and tyrosine kinase2 (TYK2) of the JAK family are found to be associated constitutively with gp130 and are activated in response to IL-6 family members. The activation of these kinases, in turn, leads to tyrosine phosphorylation of the STAT 3[30]. Following phosphorylation and acetylation, STAT 3 forms a dimer. The pSTAT3 dimer translocates from the cytoplasm to the nucleus. Within the nucleus, pSTAT3 dimers recognize and bind a canonical 8–10 base pair inverted repeat DNA element that is commonly referred to as an interferon (IFN)-gamma activated sequence (GAS) element. The engagement of pSTAT3 dimers then initiates a change in the transcription of a number of genes including the apoptotic regulatory genes. The termination and modulation of the IL-6-Jak-Stat3 signaling pathway is mediated by the SOCS (suppressor of cytokine signaling) which prevents the phosphorylation of STAT 3 and feedback inhibitors and PIAS (protein inhibitor of activated STAT) proteins(figure 3)[31],[30].

Blocking IL6/STAT 3 pathway is still being investigated to improve treatments [29].

IL-6 pathway activation is observed in a variety of human cancer cell lines and solid tumors, including PCa. It has been found to play an important role in various tumor behaviors including the development, cell migration, invasion, growth of malignancies, proliferation, apoptosis, progression, angiogenesis and differentiation of tumor cells[29].IL-6 aids tumor growth by inhibiting cancer cell apoptosis and inducting tumor angiogenesis and contributes to the advanced stage of cancer development. It has also been shown to enhance endothelial cell migration, a key step in angiogenesis, and dissemination of solid tumors[31].

4.2 IL-6 and inflammation

The influence of proinflammatory cytokines on cancer development is complex and context-dependent. Tumor-derived factors are capable of inducing local production of proinflammatory cytokines to generate an inflammatory microenvironment that promotes cell growth and invasion. Inflammation has been implicated as an etiological factor in several human cancers, including PCa. Chronic inflammation, alongside the intrinsic properties of pre-malignant cells and other determinants, may therefore be one of the driving forces of prostate malignant transformation. The final outcome of prostate cancer depends in part on the transcription factors that are activated by cytokines synthesized by tumor cells or cells in the tumor microenvironment, and on the genes that are regulated by these transcription factors[32]. Proinflammatory cytokines such as IL-6 are master regulators of prostate tumor-associated inflammation and tumorigenesis [33]. IL-6 acts as a key mediator in initiation, progression and metastases and the development of castration resistance to chemotherapy [34].

IL-6 undergoes a functional transition from paracrine growth inhibitor to autocrine growth stimulator during PCa progression [35]. *In vitro* studies on the androgen-independent prostate cancer cell lines PC3 and DU145 demonstrate that they secrete IL-6, whereas androgen-sensitive LNCaP cells do not [34].

In primary PCa, protein extracts prepared from prostate cancer tissues showed elevated IL-6 levels compared to benign tissues in approximately 50% of cases [34].IL-6 contributes to PCa progression and development [34]. The number of cells expressing IL-6 correlated positively with the presence of acute inflammation. Serum levels of IL-6 are elevated in PCa patients [30],[34].Increased levels of IL-6 are associated with increasing grade of PCa in patients. *In vitro* experiments on PC-3 and LNCaP cell lines have demonstrated that IL-6 promotes PCa cell growth and prevents chemotherapeutic-mediated cytotoxicity. *In vivo*, IL-6 has an anti-apoptotic action against murine PC3 tumors [36].IL-6 increases the expression of genes encoding many steroidogenic enzymes, including *HSD3B2* and *AKR1C3*, involved in androgen biosynthesis leading to castration-resistant prostate cancer progression [37].In addition, IL-6 enhances the secretion of testosterone with levels 2-fold higher in LNCaP-IL-6+ cells than LNCaP [37].

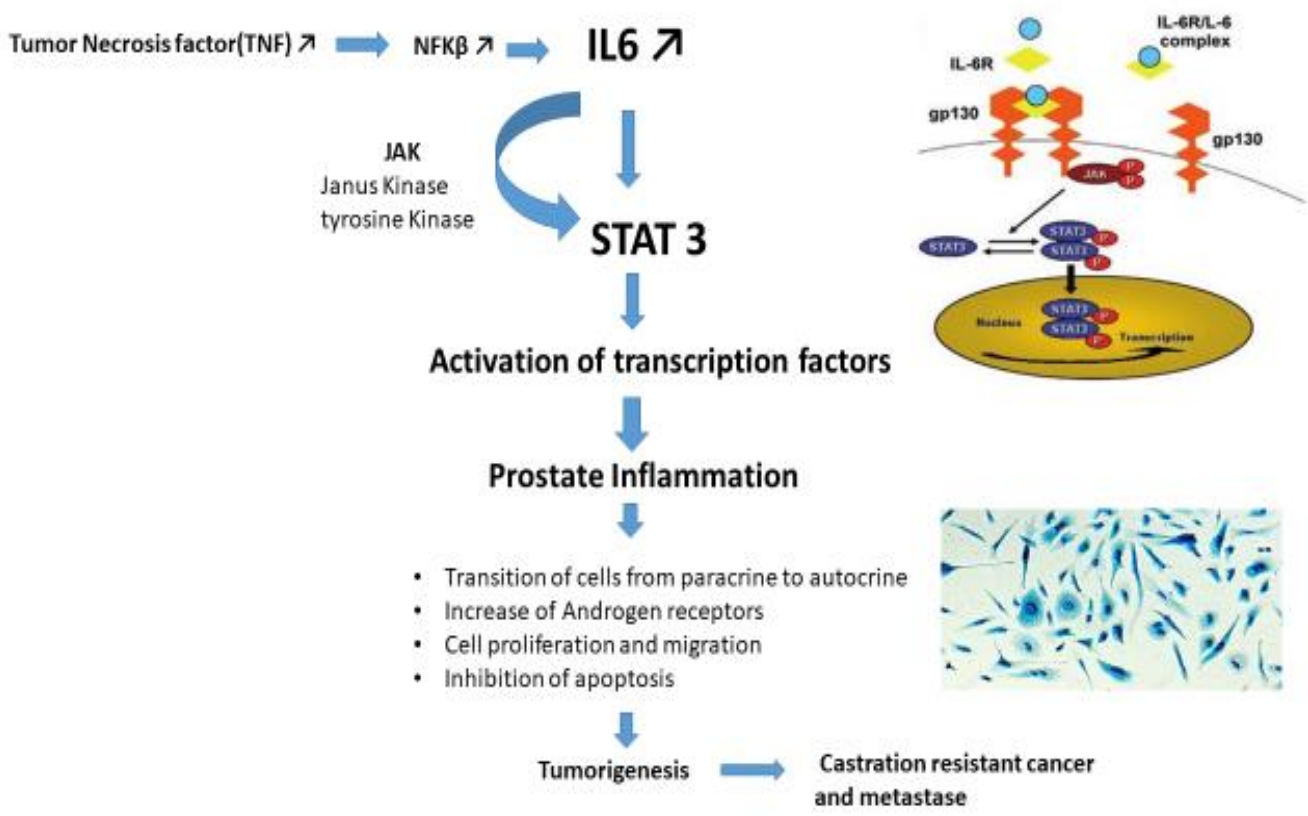


Figure 3: Effects of IL-6 on prostate cancerous cells.

IL-6 induces cell cascade resulting in the activation of JAK tyrosine kinase causing the phoshorylation of transcription factor STAT 3 which initiates a change in the transcription of a number of genes including the apoptotic regulatory genes.

5- NF-κB and PCa

The prostate gland is a sensitive organ toward inflammation with a close correlation between chronic prostatic inflammation and PCa Inflammation is thought to incite carcinogenesis by creating a tissue microenvironment rich in growth factor and cytokines that can enhance cell proliferation, angiogenesis and tissue repair. Among signal pathways leading to inflammation and cytokine production, the nuclear factor-κB (NF-κB) family proteins which are essential for inflammation, immunity, cell proliferation and apoptosis.NF-κB Rel family consists of homodimers and heterodimers formed by several subunits: (p50/p105), (p52/100), Rel A (p65), Rel B, and c-Rel proteins[38]. In most cell types, NF-κB activity is mediated by the complex p50/p65 (Rel A), which acts as a transcriptional activator. In unstimulated cells, p50/p65 is sequestered and retained in the cytoplasm where it bindsto the inhibitory IκB-α proteins which block the nuclear sequences of NF-κB [39].However, NF-κB is activated in response to a large spectrum of stimuli such as antigens, growth factors, cytokines, bacterial products, viruses, cytokines, stress and drugs that promote the dissociation of the IκB-α by polyubiquitination releasing active NF-κB into the nucleus. To sum up, quantification of the NF-κB is achieved via the measurement of IκB-α mRNA expression by real-time PCR [40].

In the nucleus NF-κB p50/p65 subunit binds to the DNA promoter region to regulate the expression of multiple target genes involved in tumor cell invasion and angiogenesis including chemokines and inflammatory cytokines such as IL-6[38, 39].

In human, prostate NF-κB is overexpressed in prostatic intraepithelial neoplasia resulting in the development and progression of Pca. *In vitro* studies on PCa human cells demonstratean increased level of NF-κBp65 and NF-κBp50 with special localization in the nucleus. Overexpression of NF-κB in benign prostatic epithelial cells leads to induce pro-inflammatory genes expression. When inflammation becomes chronic and lasts for a long period of time, the result may be pathogenic to the body, including tumorigenesis [38].

6- Prostate-specific membrane antigen and PCa

Prostate-specific membrane antigen (PSMA) is a type II membrane expressed in all forms of prostate tissue, including carcinoma. The PSMA protein has a unique 3-part structure: a 19-amino-acid internal portion, a 24-amino-acid transmembrane portion, and a 707-amino-acid external portion. The PSMA gene is located on the short arm of chromosome 11 in a region that is not commonly deleted in PCa [41][42,43].Studies have consistently demonstrated PSMA expression in all types of prostate tissue and increased PSMA expression in cancer tissue. The binding occurs in the epithelial cells of the prostate but not in the basal or stromal cells. PSMA seems to be expressed in other cancers, more specifically in the neovasculature associated with these cancers such as renal cells, transitional cells of the bladder, testicular–embryonal, neuroendocrine, colon, and breast cells[42].

Dimerization is critical to maintain the conformation and enzymatic activity of PSMA. This dimerization is apparently mediated by epitopes within the large extracellular domain because truncated versions of PSMA lacking the cytoplasmic and transmembrane domains are still capable of interacting. PSMA undergoes endocytosis from the plasma membrane. It has a dileucine motif present at its cytoplasmic tail that is responsible for the internalization. This endocytosis occurs through clathrin-coated pits and involves the first five NH2-terminal amino acids of the cytoplasmic tail[41]. This motif appears to constitute a novel endocytic targeting signal and likely interacts with the activator protein-2 (AP-2) adaptor protein complex. This endocytic trafficking allows cells to maintain homeostasis and internalize vital nutrients, lipids and proteins. In addition, it's also an established mechanism to downregulate signal transduction cascade. After endocytosis, a number of receptors are recycled back to the plasma membrane surface [44].PSMA has two unique enzymatic functions, folate hydrolase(FOLH)andN-acetylated α-linked acidic dipeptidase(NAALADase) [41, 45][43]. NAALADase activity cleaves terminal glutamate from the neurodipeptideN-acetyl-aspartyl-glutamate (NAAG) and folate hydrolase activity cleaves the terminal glutamates from g-linked polyglutamates. NAAG is concentrated in neuronal synapses while folylpoly-g-glutamates are present in dietary components and PSMA protein of the surface of the

brush border surface of small intestine enables the generation of folates and subsequent folate uptake[41].

PSMA is not accurate enough for the diagnosis and is not a predictor of disease recurrence in PCa patients. Radiographic test uses the mAb 7E11 (monoclonal antibody) anti-PSMA to produce a radiodiagnostic marker. High-risk metastatic prostate cancer and recurrent PCa have demonstrated a sensitivity rate to mAb of 60% to 80% and a specificity rate of 70% to 90%, which are better than the accuracy of current CT scans or M R Is. *In vitro* studies on mice have demonstrated that toxin – conjugated mAbs quickly internalized and killed PSMA-expressing cells and eradicated prostate tumors without toxicity[42].

7- Interaction betweenPSMA, NF-κB, IL-6

NF-κB is responsible in cell survival, cell adhesion, inflammation, differentiation and growth. The activation of NF-κB suppresses apoptosis and promotes chemoresistance and tumorigenesis. Several different tumor cell types including leukemia, lymphoma, myeloma, melanoma, prostate, colon, breast and pancreas express constitutively active NF-κB. Suppression of tumor necrosis factor (TNF) downregulates the expression of active NF-κB which inhibits the proliferation of tumor cells [39].TNF induces inflammation and activation of NF-κB which inhibits apoptosis. *In vitro*, studies on PC3 cells demonstrated that coffee inhibits TNF which induces NF-κB activity and DNA binding. Furthermore, coffee increases apoptosis and modulates transcription of genes related to PCa and inflammation [46] . *In vivo*, coffee modulates expression of inflammation and cancer related genes in PC3 xenografts [46].

PSMA leads to the phosphorylation of P65 subunit of NF-κB transcription factor. This activation of NF-κB signaling induces IL-6 production that promotes the proliferation of tumor cells [33].

8- Androgen receptors (AR)

Androgen signaling plays a critical role in the normal development, proliferation, and differentiation of the prostate gland. Androgen binding to the AR which is a nuclear hormone receptor transforms the receptor to an active conformation and initiates translocation to the nucleus, which is followed by binding to specific response elements in the promoter regions of target genes to modulate gene expression either positively or negatively. *In vitro* studies demonstrate that levels of AR mRNA in DU-145 cells were about 50% lower than the LNCaP cells, however levels of AR mRNA in PC3 cells are relatively much lower than the DU-145 and LNCaP cells. In addition, dihydrotestosterone (DHT) treatment of DU-145and PC-3 cells did not result in transcriptional activation of AR-responsive genes by AR [47].Overexpression of nuclear AR levels results in transition from androgen dependent to androgen independent prostate cancer cells [48][49].

8.1 Androgen sensitive vs androgen insensitive prostate cancer cells

Androgen sensitive cell lines such as LNCaP have an increased in total AR, increased PSMA, hormonal sensitivity and respond to androgen deprivation therapy (ADT)and to paracrine IL-6 secretions but can become insensitive to ADT (table 4). However, androgen insensitive cell lines such as PC3 and DU-145 have nuclear AR overexpression, low PSMA, autocrine IL-6 production, hormonal resistance and insensitivity to ADT (table4).

Table 4: Difference between androgen sensitive and androgen insensitive cells.

Androgen Sensitive	Androgen Insensitive/Hypersensitive
Ex: LNCaP cell lines	Ex: DU-145 and PC3 cells
Increased total AR [48]	Overexpression of nuclear AR [48]
PSMA expression[50]	Low PSMA expression [50]
Respond to paracrine IL-6 secretions [35]	Autocrine growth stimulation of IL-6 [35]
Hormone sensitive	Hormone resistant
Respond to Androgen Deprivation Therapy (ADT) [48]	No response to ADT [48]
Irreversible transition from sensitive to insensitive after ADT [48]	Survive and grow under low androgen concentrations [48]

8.2 Androgen receptors and PSMA

PSMA is repressed by androgen treatment in multiple models of AR-positive prostate cancer in an AR-dependent manner. Conversely, antiandrogens up-regulate PSMA expression. *In vitro* studies on androgen sensitive LNCaP and CWR22Rv1 cells lines demonstrate a reduction in PSMA levels but an increase of AR levels after treatment with testosterone and dihydrotestosterone. Moreover, PSMA mRNA levels were reduced by androgen treatment in LNCaP (~50% maximal reduction) and CWR22Rv1 (~80% maximal reduction).Androgen receptors downregulate PSMA in response to androgens [51].

9- Tumor escape mechanism in PCa

Numerous immunotherapy trials have been carried out in PCa patients, with induction of antigen-specific T cells in some cases, but limited success is seen in terms of tumor regression or survival. Vaccine failure may be attributed to several potential tumor escape mechanisms such as defects in antigen presentation, production of immunosuppressive substances, T cell dysfunction, and the presence of regulatory T cells.

9.1 Defects in antigen presentation

HLA (human leukocyte antigen) class I antigens are critical for the recognition and lysis of tumor cells by cytotoxic T cells (CTLs). Defects in antigen presentation could allow the tumor to escape killing by CTLs. In primary human tissue, several studies report reduced or complete loss of HLA class I in prostate tumors and lymph node metastases, compared to the normal expression in benign tissue[52].

9.2 Production of immunosuppressive substances

Many immunosuppressive cytokines are produced such as interleukin IL-4, IL-6 and IL-10. In the serum of PC patients elevated levels of these cytokines were detected when compared normal healthy patients. In prostate tumors it is postulated that IL-6 has a direct effect on tumor cell growth, and may also contribute to peripheral T cell dysfunction.

In addition, the amino acid L-arginine can be metabolized by the enzyme nitric oxide synthase (NOS), to generate the free radical nitric oxide (NO).Increased L-arginine metabolism within tumors may contribute to tumor growth, angiogenesis, metastasis, and tumor-related immunosuppression. Prostaglandin E2 (PGE2), a product of arachidonic acid metabolism , may also regulate immune function by acting as a negative feedback inhibitor for various processes, including T cell proliferation, lymphokine production and macrophage and natural-killer cell cytotoxicity[52].

9.3 T cell dysfunction

T cells recognize antigen via the heterodimeric T cell receptor (TCR) molecule, which is non covalently associated with the CD3 molecular complex. Reduced or aberrant expression of TCR-associated signal-transduction molecules is reported in many types of cancer and may contribute to tumor escape. In addition, T cells can commit suicide upon activation with antigen by secreting high amounts of Fas ligand (FasL) which is a type II transmembrane tumor necrosis factor family protein, known to trigger apoptosis in cells that bear the FasL receptor[52].

9.4 Regulatory T cells

The presence of regulatory $CD4^{+}CD25^{+}$ T cells may explain the poor clinical efficacy of immunotherapeutic protocols in human tumors. These cells inhibit immune-cell functions either directly through cell–cell contact or indirectly through the secretion of anti-inflammatory mediators, such as IL-10 and TGF-b.

$CD4^+CD25^+$T cells are increased in the peripheral blood and tumor tissue of patients with early stage PCa. Furthermore, blockade of the CD4+CD25+ T cells using an anti-CD25 antibody reduced PCa cell growth both in a prostate tumor transplant model and in the spontaneous prostate tumor model [52].

10-Therapy escape mechanisms in the malignant prostate

Luminal prostate cells are essentially dependent on androgens for survival, or more precisely, dependent on the signaling of active androgen receptors (AR). Inactivation of this signaling axis is the primary goal of androgen deprivation therapy (ADT) and complete androgen blockade (CAB) and second-generation agents.AR is the main target for prostate cancer therapy. Clinical approaches for AR inactivation include chemical castration, inhibition of androgen synthesis and AR antagonists (anti-androgens). However, treatment resistance occurs for which an important number of therapy escape mechanisms have been identified. The tumor escape mechanisms are arranged into the concepts of target modification, bypass signaling, histological transformation, cancer stem cells and miscellaneous mechanisms [53].

10.1Target modification

The most common molecular mechanism of endocrine therapy resistance is the amplification of AR gene locus. These multiple copies of the AR gene result in high expression of AR, mRNA and protein. Base substitutions in the coding sequence of the AR gene also lead to resistance but are less frequently observed than amplifications of the locus. *In vitro* studies showed that AR transcriptional activity is regulated not only by the expression levels of both ligand and the receptor, but also as a consequence of increased receptor binding to chromatin. ADT causes an amplification of the AR locus, in which a disturbed ratio of AR and its ligand results in a hypersensitive AR signaling pathway. Cells with such a high AR expression can deal with the low androgen concentrations during ADT, and give rise to new clonal outgrowth of castration resistant cells. The castrate androgen levels in castration-resistant cells are sufficient to stimulate AR activity

on enhancer elements, but not suppressor elements, resulting in an increased expression of AR which may drive progression to castration-resistant prostate cancer (CRPCa). In addition, truncated AR variants are constitutively active and can promote AR signaling without the need for a ligand[53].

10.2Bypass signaling pathway

Androgen receptors (AR) can be transactivated by growth factors, neuropeptides and cytokines such as IL-6 that leads to AR phosphorylation, and enhancing activity. Intra-tumoral upregulation of androgenic enzymes enable conversion of adrenal steroids into testosterone and dihydro-testosterone (DHT) to activate transcription and drive castration resistance. Activation of glucocorticoid receptors (GR) also lead to castration resistance.
In addition, AR transcriptional activity is controlled by numerous co-regulators which cause AR protein acetylation and increased expression of AR- regulated genes promoting survival of malignant cells[53].

10.3 Histological transformation

Neuroendocrine cells are rare secretory cells in normal prostatic tissue. Normal and malignant neuroendocrine cells do not express androgen receptors (AR) and are unlikely to be affected by AR-targeting therapies. Inactivation of AR decreases the number of luminal cells, therefore malignant neuroendocrine cancer cells may repopulate the vacant space in the prostatic tissue and thus drive tumor recurrence [53].
In addition, *in vitro* studies on mice prostate tissues and xenografts have demonstrated that epithelial to mesenchymal transition is a mechanism of castration resistance in response to androgen deprivation therapy (ADT) [53].

10.4 Cancer stem cells (CSC)

Cancer stem cells (CSC) represent a small population of cells inside a tumor which share the characteristics of normal stem cells. They are quiescent and therapy resistant due to their high DNA repair activity that protects them from DNA damage- inducing agents such as drugs, radio and chemotherapy. These therapies fail to target the small population of quiescent and AR-negative CSC, which are able to adapt to their new niche, resulting in the regrowth of a therapy-resistant and more aggressive tumor [53].

Through our search we found that the apoptotic effects of caffeine were studied *in vivo* and *in vitro* on different tissues and cells including PC3 and DU-145 metastatic PCa cell lines. However, no studies were done on PC3-PSMA cells. Moreover, the effects of caffeine on IL-6 and NF-κB gene expression were not elucidated. For this reason, our study was designed on PC3 and PC3-PSMA to determine the progression of PCa in the presence of caffeine which is a natural compound.

III.MATERIALS AND METHODS

1- Reagents

RPMI-1640 medium (R7388), fetal bovine serum (F9665), penicillin-streptomycin 100X (L0011), phosphate-buffered saline (PBS) and Trypsin were used for cell culture and obtained from Sigma-Aldrich via Ibra Haddad (Beirut, Lebanon).WST-1 cell proliferation assay kit (ab65473) for cytotoxicity and Human IL-6 Elisa Kit (ab178013) were obtained from Numelab(Beirut, Lebanon). Pure caffeine anhydrous Fluka (27602), TRI reagent Sigma (T9424), chloroform Sigma (32211), isopropanol Sigma (24137), ethanol Scharlau (ET0006), RNase, DNase free water Sigma W4502 and TE buffer Omnipur 8890 were purchased fromIbra Haddad (Beirut, Lebanon). iTaq universal SYBR Green one-step kit BIO-RAD#172-5150 was obtained via Bazilky (Beirut, Lebanon) and IL-6 and IκB-α primers were purchased from TIB (lebanese seller) with the following sequences:

IL-6 Sense (20- mer):5'- AGGGCTCTTCGGCAAATGTA-3'

IL-6 Anti-sense (23-mer):5'- GAAGGAATGCCCATTAACAACAA-3'

IκB-α Sense (21-mer): 5'-GCTGAAGAAGGAGCGGCTACT-3'

IκB-αAnti-sense (23-mer): 5'- TCGTACTCCTCGTCTTTCATGGA-3'

2- Cell culture

PC3 prostate cancer cell lines, and PC3 overexpressing PSMA (PC3-PSMA) were cultured into 75 cm^2vented cap flasks in 7ml RPMI supplemented with 1% penicillin/streptomycin and 10% FBS then incubated at 37° C in a CO_2 incubator Thermo Forma. After reaching confluency, the attached cells were washed with PBS and collected by trypsinization. Centrifugation was done and the cell pellets were cultured in new flasks.

For cytotoxicity, cells were cultured in 96-well plate. For IL-6 and RNA extraction culture was made in a 24-well plate.

3- Cytotoxicity

The premixed WST-1cell proliferation reagent provides a method to measure cell proliferation based on the enzymatic cleavage of tetrazolium salt into a water soluble

formazan dye which can be detected at a 420-480 nm absorbance. This augmentation in the enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture media.

PC3 and PC3-PSMA cells were seeded in triplicate in 96 well plates at a density of $5x10^4$ cells/well in 200µl RPMI supplemented with 10% FBS and 1% penicillin-streptomycin. When subconfluency was reached, the media in each well was aspirated and replaced with 100µl of different concentrations of caffeine dissolved in serum free media (0.5 mM; 1 mM; 2.5 mM; 5m M; 10m M; 20 mM). The plates of each cell type were incubated for 24hrs and 48hrs at 37ºC in a CO_2 incubator. After incubation, 10µl (1:10) of WST-1 reagent was added to each well. Readings were completed on an ELISA plate reader (Thermo MultisKan Go) at 450 nm after 4 hrs incubation with WST-1**.**Percent viability was calculated from the absorbance:

$$\% \text{ viability} = \frac{\text{absorbance sample} - \text{absorbance blank}}{\text{absorbance control-absorbance blank}} \times 100$$

4- IL-6 Detection

PC3 and PC3-PSMA cells were seeded in 24 well plates with 500µl RPMI, 10% FBS and 1% penicillin-streptomycin at a density of $1.9x10^5$ to reach subconfluency. The media in each well was aspirated then the cells were treated with different caffeine concentrations (0.5 mM; 1 mM; 2.5 mM; 5 mM; 10 mM) and incubated for 24 hrs and 48hrs. Supernatant from each well was collected in Eppendorf tubes and centrifuged at 7000xg for 10 minutes to remove the debris and then assayed in duplicate using the abcam human IL-6 ELISA kit.

In each well of a 96 well plate,50µl of the sample was added to 50µl antibody cocktail. The plate was then sealed and incubated for 1 hour at room temperature on a plate shaker at 200 rpm. The wells were washed 3 times with 350µl wash PT buffer (1x). After the last wash, the plate was inverted and blotted against a clean paper towel to remove excess liquid.100µl of TMB substrate from the ELISA kit was then added to each well and incubated for 20 min in the dark on a shaker at 200 rpm generating a blue color. Afterwards, 100µl of stop solution was added with 1-minute shaking to observe a shift in

color from blue to yellow. Color intensity was correlated to the number of viable cells. Readings were taken using the MultisKan Go plate reader at 450 nm.

5- RNA extraction

PC3 and PC3-PSMA cells were seeded in triplicate in a 24 well plate in 500µl RPMI, 10% FBS and 1% penicillin-streptomycin at a density of $1.9x10^5$ to reach subconfluency. The wells were then treated with 500µl of different caffeine concentrations (0.5mM; 1mM; 2.5mM; 5mM; 10mM; 20mM) and incubated for 24 hrs and 48 hrs. The media was aspirated and each well was washed with PBS. Then 330 µl of TRI reagent was added to each well and scraped to remove attached cells. Each three wells from the same caffeine concentration treatment were pooled into one 1.5ml Eppendorf tube and incubated at room temperature for 5 min. Briefly chloroform (200µl) was added to each Eppendorf and vortexed for 15 sec. prior to incubation at room temperature for 2 min. After centrifugation at 3500xg at 4˚C for 30 min, the upper aqueous layer was transferred to new 1.5 ml double autoclaved Eppendorfs. 500µl of isopropanol was then added to the aqueous layer, inverted 2 to 3 times and incubated at room temperature for 10 min. Centrifugation at 3500xg for 10 min was performed and the supernatant was poured off. The pellets were washed two times with 75% ethanol with vigorous vortexing to break the pellets before centrifugation at 3500xg for 5 min. Supernatant from each tube was poured off and the RNA was left to dry for 5 min at room temperature. 20µl of TE buffer was then added to all tubes and allowed to sit for 10min at room temperature. Finally, the tubes were quickly vortexed and centrifuged at 3500xg for 1min at 4°C. RNA concentration was determined using the nanodrop Thermo 2000c. RNA samples were stored at -80°C until real time PCR analysis.

6- Dilution of primers

Each primer received at a lyophilized content was diluted to a final concentration of 100µM by adding 150µl RNase free water to the original tubes. Then, 25µl from the diluted primer was taken and mixed with 475µl of RNase free water to get a final concentration of 5µM.The diluted primers were aliquoted into 10 eppendorf tubes of 50µl

each. At the end, all primers tubes were stored at -20° C. Aliquots were done to prevent freeze thaw cyclical damage to the diluted primers.

7- Real-time PCR amplification

The differential expression of target genes (TG) in PC3 and PC3-PSMA was quantified using one step real-time qRT-PCR with SYBR green to amplify IL6 and IκB-α genes.18S was used for all the runs as internal control to normalize samples for the variation in amounts of RNA loaded. In one step qRT-PCR, the amplification of each TG and the internal control was optimized and validated using different combinations in order to find the optimal concentration for each primer and internal control. Quantification of gene expression was done according to the Ct values of each amplicon which correspond to the SYBR green curves. Relative quantification of target mRNA was expressed using comparative threshold cycle method. The ΔCt was determined by subtracting the internal control Ct value from the target unknown value. Within each experiment, the ΔΔCt was determined by subtracting the higher ΔCt (the least expressed value) from all other ΔCt values. Relative abundance in mRNA expression (fold change) was calculated as being equal to $2^{-\Delta\Delta Ct.}$

8- Primer Optimization

In each well 2µl of RNA pool mix at 50ng/µl was added to 0.125µl reverse transcriptase, 5µl of master mix, 0.4µl reverse and forward primers for 200ng/µl concentration and 0.2 µl for 100ng/µl. The volume was completed to 10µl by nucleases free water. Two primer concentrations were added to the wells 100ng fwd/100ng reverse or 200ng fwd/200ng reverse for IL-6, IκB-α and one 100ng fwd/100ng reverse for the internal controls 18S and GAPDH. For 18S RNA samples were diluted 1/1000. PCR reactions were performed on BIO-RAD thermal cycler CFX 96.

The following program was used: Reverse transcription at 50° C for 10min, polymerase activation at 95° C for 1min, denaturation at 95° C for 20 seconds, annealing- extension for 30 seconds at gradient temperatures from 56° C to 61° C for 45 cycles. Finally, the samples were gradually heated from 65° C to 95° C at 0.5° C increment to obtain melting curves.

After the run, the optimal primer concentration is 200ng/ml for IL-6 and 100 ng/ml for IκB-α, 18S and GAPDH. The optimal annealing temperature is 60° C.

9- Parallelism of the primers and internal control

In each well, 2µl of RNA pool mix is added at different concentrations (200ng/µl , 100ng/µl, 50ng/µl,25ng/µl,5ng/µl and 1ng/µl) , 0.125µl reverse transcriptase, 5µl of master mix, 0.2µl forward and reverse primers for IκB-α, 18S, GAPDH and 0.4µl for IL-6. The volume in each well is completed to 10µl with nucleases free water. For 18S RNA samples are diluted 1/1000.PCR reactions are performed on BIO-RAD thermal cycler CFX 96.

The following program was used: Reverse transcription 50° C for 10min, polymerase activation 95° C for 1min, denaturation 95° C for 20 seconds, annealing- extension at 60° C for 30 seconds for 45 cycles. Finally, the samples were gradually heated from 65° C to 95° C at 0.5° C increment to obtain melting curves.

10- Quantification of mRNA

RNA samples extracted from PC3 and PC3-PSMA cells after 24hrs and 48hrs incubation with different concentrations of caffeine were analyzed for gene expression. In each reaction 4µl of RNA was added to 0.125µl of reverse transcriptase, 5µl of master mix, 0.2µl of forward/reverse primers for IκB-α and 18S and 0.4µl of forward/reverse primers (25µM) for IL-6. The volume in each well was completed to 10µl with nucleases free water. For each sample, the pool of 3 cultured wells was analyzed in duplicate.

For 18S RNA samples were diluted 1000 fold (1/1000). Both target gene and internal control were amplified in one step RT-PCR with the following steps of reverse transcription at 50° C for 10min, polymerase activation at 95° C for 1min, denaturation at 95° C for 20 sec and annealing- extension for 30 sec at 60° C for 45 cycles. Finally, the samples were gradually heated from 65°C to 95°C at 0.5° C increment to obtain the melting curves

11-Statistical analysis

The results are expressed as means±SD for the WST1 viability and for the Elisa IL-6 concentration as analyzed using SPSS software (22) with $p< 0.05$ considered significant. Treatment and time effects on gene expression were analyzed as a factorial treatment design in the PROC Mixed procedure of SAS 9.0 (Statistical Analysis Systems). Mean differences between treatments were determined using t-test comparison and LS means considered significantly different at $p<0.05$.

IV. Results

1- The effect of caffeine treatment on the viability of PC3 and PC3-PSMA at 24 and 48 hrs

PC3 and PC3-PSMA cells were incubated with 0.5 mM, 1 mM, 2.5 mM, 5 mM, 10 mM of caffeine for 24 and 48 hrs.

For PC3-PSMA cells, the viability decreased to 81% after 24hrs incubation with a dose of 2.5 mM and to 71% and 45% at 5mM and 10mM respectively as compared to control.

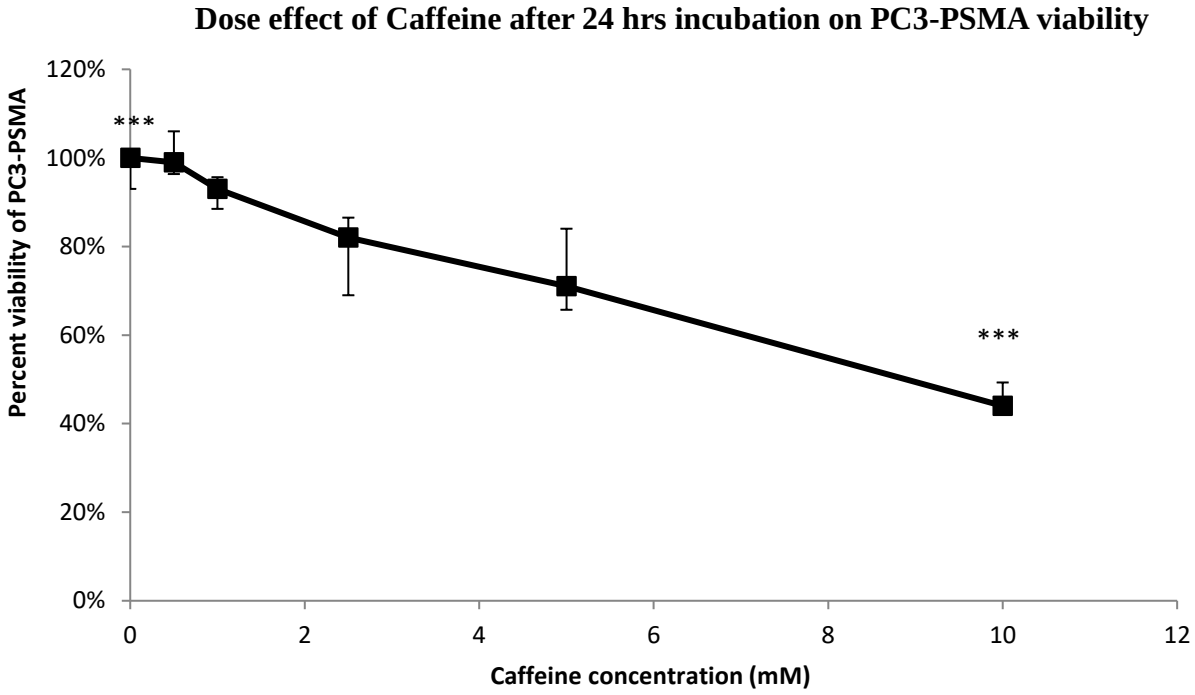
A significant difference in viability between the control and the treated groups at 10 mM (81.5± 22.5, $p = 0.0002$) is shown in figure 4A.

Similarly, the viability decreased to 75% after 48 hrs incubation with 0.5 mM of caffeine and to 59% and 19% at 1mM and 10mM, respectively. Given these values a significant difference in viability was displayed between the control and treated groups at 5 and 10 mM (52.33±32, $p= 0.01$) as displayed in figure 4B.

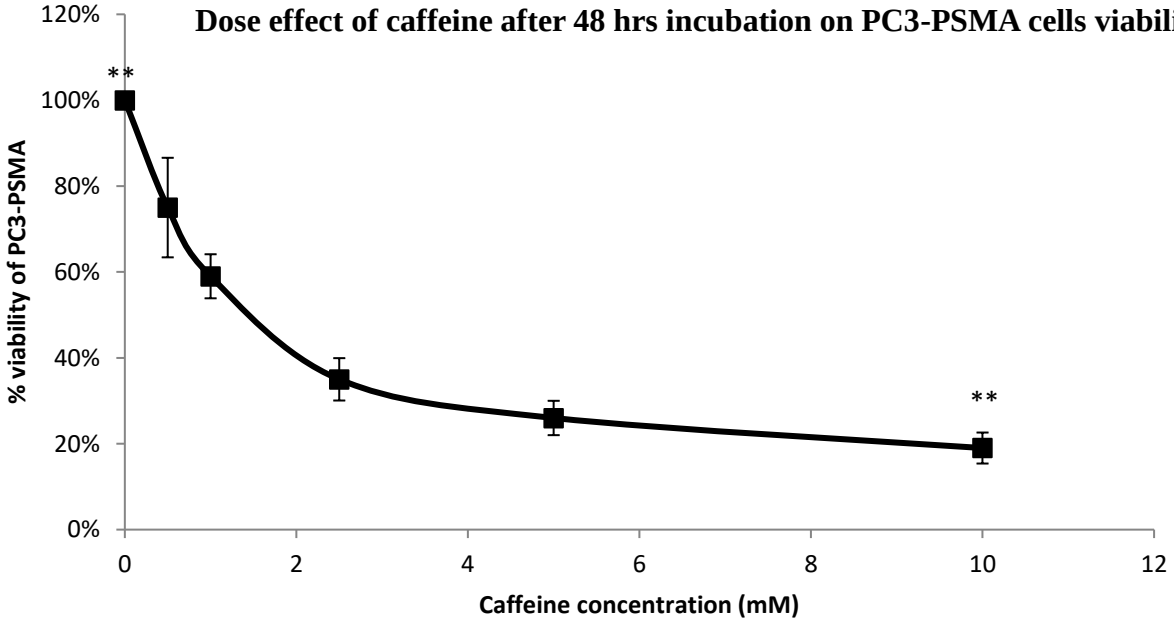
Viability was decreased for approximatively 50% at 48 hrs as compared to 24 hrs at a 20 mM dose as depicted in figure 4C.

Overall, caffeine dose dependently reduced cell viability in PC3-PSMA cells. The lowest percentage of viability was recorded at 10 mM after 24 and 48 hrs of incubation. Strong negative linear correlation exists between viability and caffeine doses (Pearson's $r = -0.99$ at 24 hrs and $r = -0.82$ at 48 hrs).

A


Dose effect of Caffeine after 24 hrs incubation on PC3-PSMA viability
Percent viability of PC3-PSMA
120%
100%
80%
60%
40%
20%
0%
***
***
0
2
4
6
8
10
12
Caffeine concentration (mM)

B


Dose effect of caffeine after 48 hrs incubation on PC3-PSMA cells viability
% viability of PC3-PSMA
120%
100%
80%
60%
40%
20%
0%
**
**
0
2
4
6
8
10
12
Caffeine concentration (mM)

C

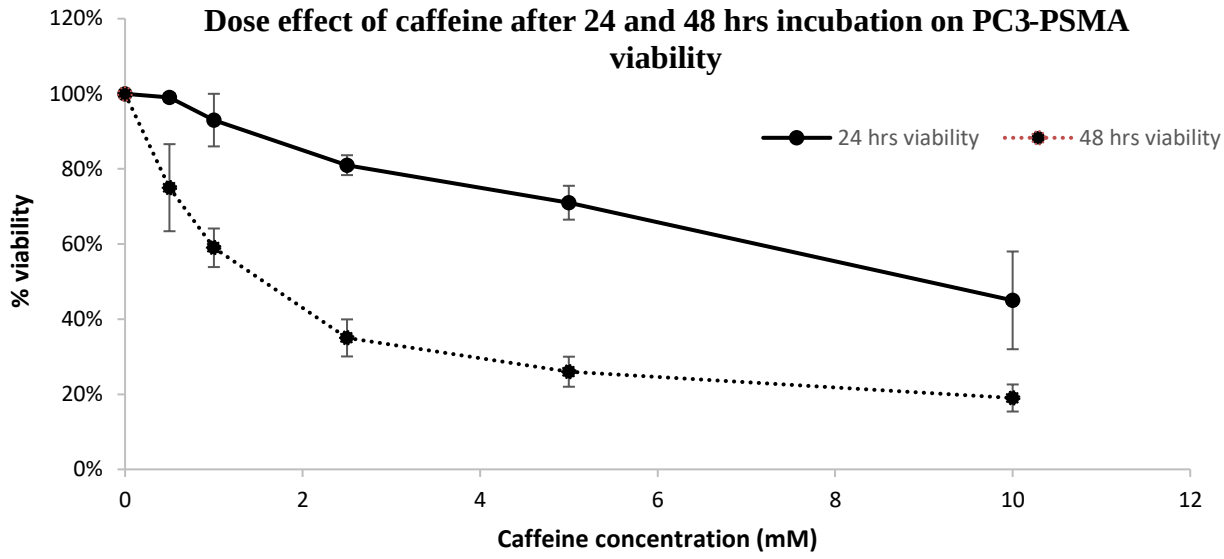


Figure 4: Dose effect of caffeine treatment of PC3-PSMA cells on viability after 24 and 48hrs. In panels A and B; cells were plated, treated with different caffeine doses (0, 0.5,1, 2.5,5 and 10mM) and incubated for 24 and 48 hrs respectively. In Panel C, the viability was decreased at 48 hrs as compared to 24 hrs. Overall, caffeine reduced human prostate cancer cell growth *in vitro*. Viability was assessed using WST1 assay. Total cell enzymatic activity was automatically counted by MultisKan Go plate reader. Values are mean ± SD of three replicas. ** $p \leq 0.01$, *** $p \leq 0.001$.

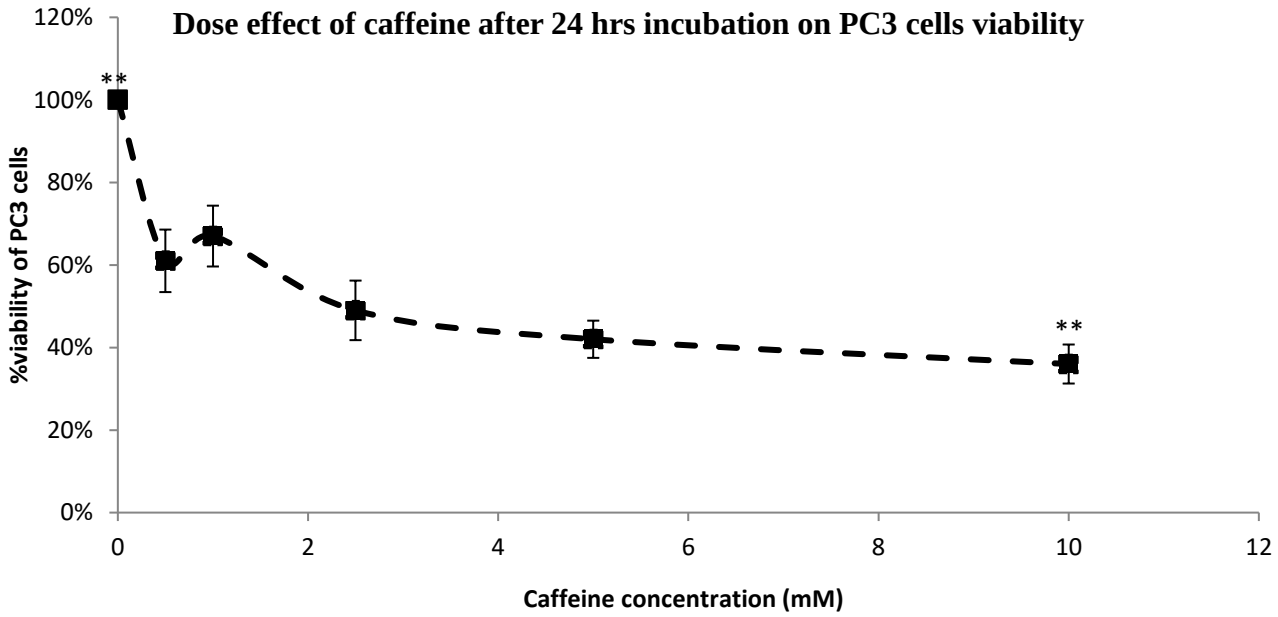
For PC3 cells, viability decreased to 61% after 24 hrs incubation with a caffeine dose of 0.5mM and to percentile ranging between 50% and 35% at 2.5 and 10mM respectively.

A significant difference in viability between the control and the treated groups at 10 mM (59 ± 24.2, p = 0.002) as shown in figure 5A.

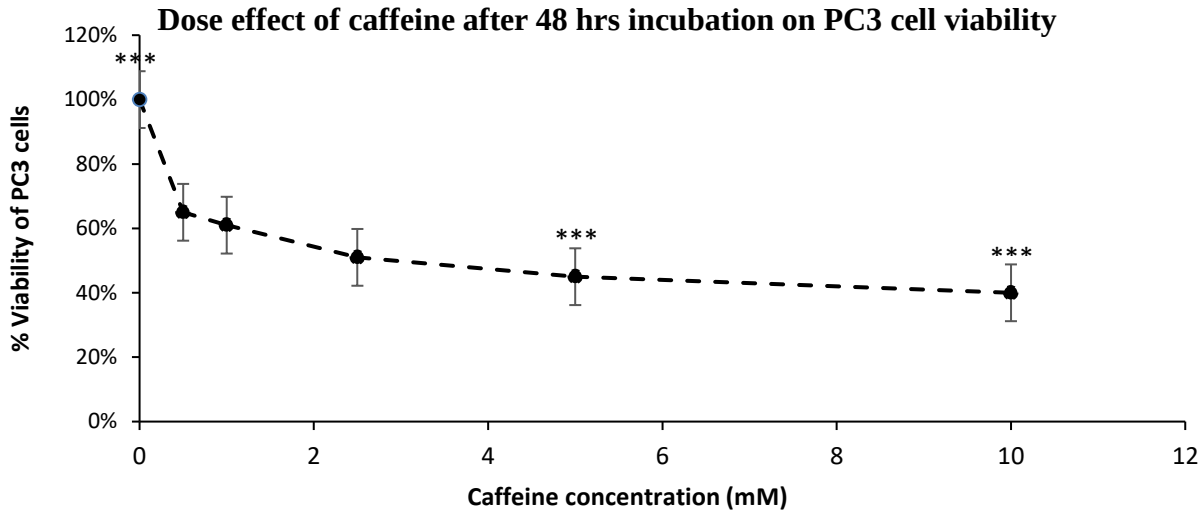
After 48 hrs of incubation, the viability decreased to 65% at 0.5 mM of caffeine and to percentile ranging between 61% and 40% at 1 mM and 10 mM respectively. There is a significant difference in viability between the control and the treated groups at 5 and 10 mM (60.33± 20, p = 0.001) as depicted in figure 5B.

The results showed that the viability significantly decreased, but there is a slight difference between 24 and 48 hrs (figure 5C). Caffeine doses and viability are negatively and linear correlated for both time points (r = -0.76 at 24 hrs, r = -0.73)

A



B



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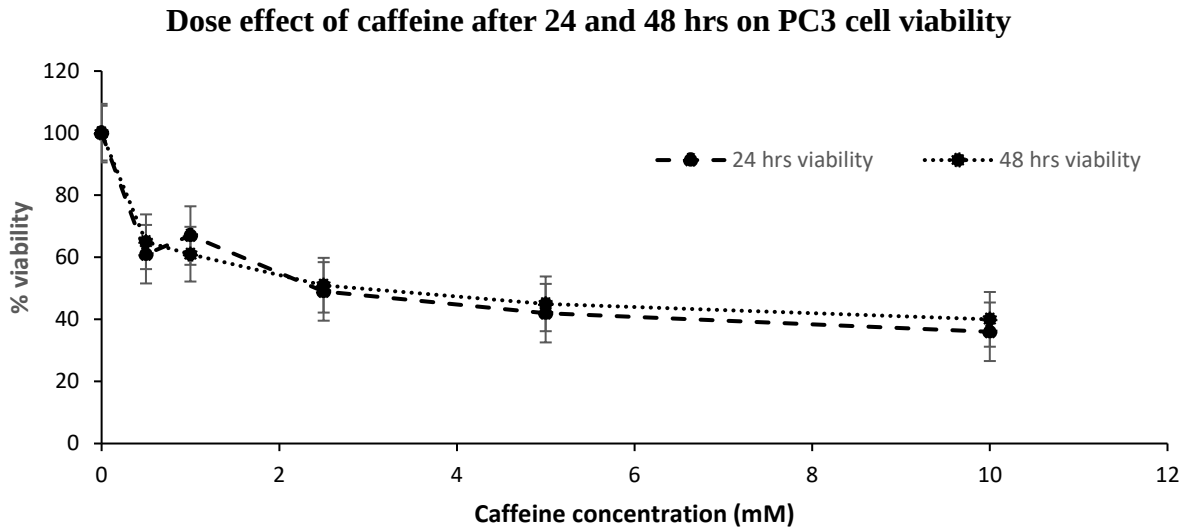


Figure 5: Dose effect of caffeine on viability after 24 and 48 hours on PC3 cells. In panels A and B; cells were plated, treated with different caffeine doses (0, 0.5, 1, 2.5, 5 and 10mM) and incubated for 24 and 48 hrs. In panel C, the two time points show similar patterns. Continuous exposure to high caffeine doses up to two days resulted in two cases of significant decrease in viability. WST1 assay was performed to assess viability after 24 and 48 hours of treatment which was counted using MultiSkan Go plate reader. Values are mean ± SD of three replicas. ** $p \leq 0.01$, *** $p \leq 0.001$

2- Effect of caffeine treatment on IL-6 concentration of PC3 and PC3-PSMA.

IL-6 is a proinflammatory cytokine secreted by androgen-insensitive cells such as PC3 and PC3-PSMA. It has been tested for its fundamental role in the regulation of proliferation, apoptosis and the development and progression of PCa. Paracrine production of IL-6 was tested after seeding the cells into 24 well plates to reach subconfluency. PC3 and PC3- PSMA cells were then treated with caffeine doses (0.5, 1, 2.5, 5, 10 mM) for 24 and 48 hrs. Supernatants were collected and IL-6 concentrations were determined using MultiSkan Go plate reader and expressed in pg/ml.

For PC3-PSMA cells, IL-6 concentration was increased after 24 hours incubation from 30 pg/ml for the control to 88 pg/ml at 10mM caffeine concentration. IL-6 and caffeine doses were positively correlated in a dose dependent manner (r= 0.99). A significant difference in IL-6 concentration as shown in figure 6 was observed between the control and the 0.5, 1 and 10 mM caffeine concentration (46 ± 20, p =0.003). IL-6 was undetectable in cell supernatant after 48 hrs of incubation.

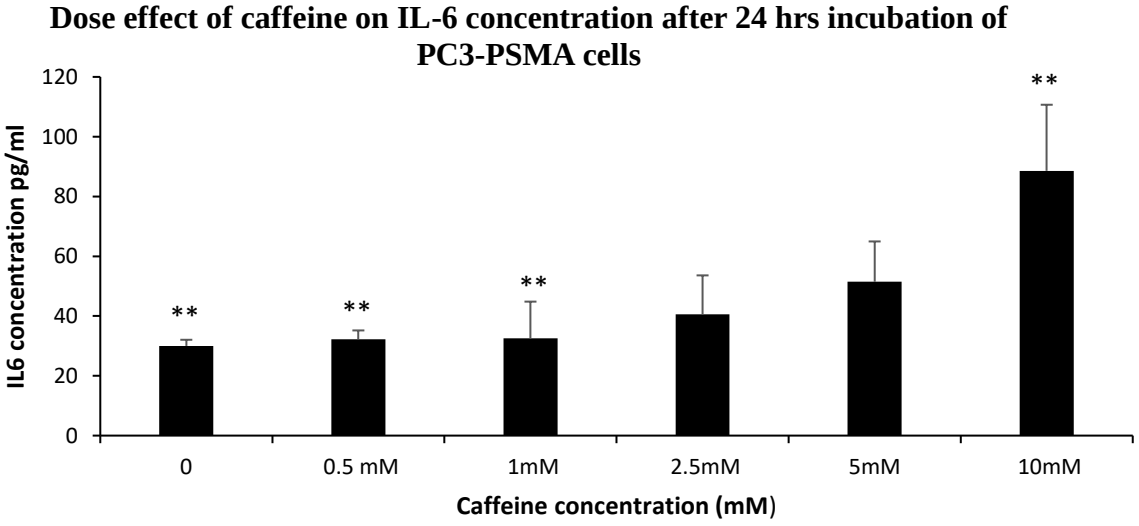


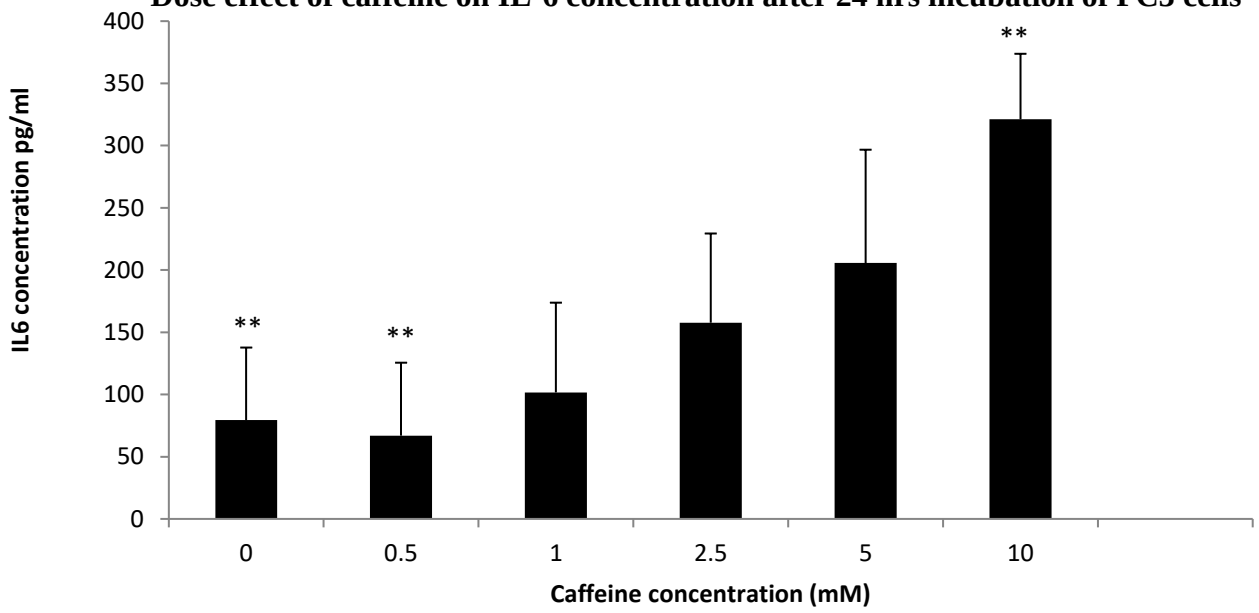
Figure 6: Dose effect of caffeine on IL-6 concentrations after 24 hours on PC3-PSMA cells. An increased up to two fold in secreted IL-6 was observed at 10mM. Cells were plated and treated with different caffeine doses (0, 0.5,1,2.5,5 and 10mM) for 24 hrs. Supernatants were collected and subjected to MultiSkan Go plate reader for IL-6 determination. Values represent mean ±SD for two replicas. ** $p \leq 0.01$.

For PC3 cells, IL-6 concentration increased to 102 pg/ml at 1mM, 205 pg/ml at 5 mM and 321 pg/ml at 10mM after 24 hrs incubation. Whereas at 48 hrs, IL-6 concentration decreased from 68 pg/ml for the control to 34 pg/ml at 10mM caffeine dose. A significant difference in IL-6 concentration between the control and the treated PC3 cells after 24 hrs at 0.5 and 10 mM (155.63±81.4, p= 0.003) as displayed in figure 7A.

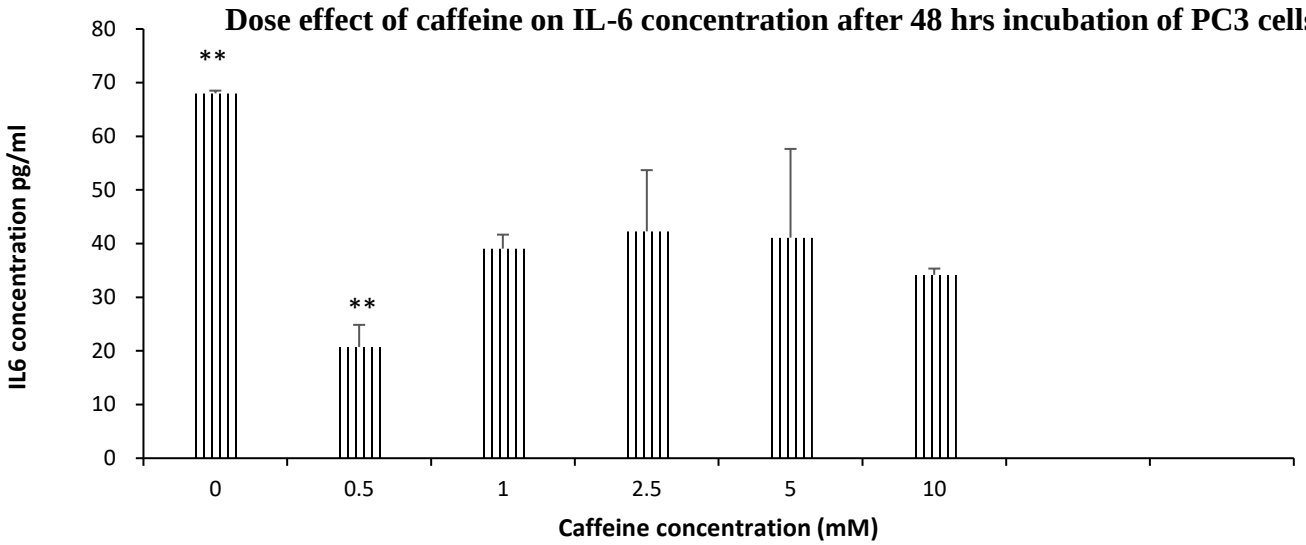
In addition, significance is observed after 48 hrs at 0.5 mM of caffeine (40.9±16.2,p = 0.01) as shown in figure 7B.

Overall as depicted in figure 7C, IL-6 concentration varied depending on the time of incubation. After 24 hrs, IL-6 concentration is positively correlated to increased caffeine doses (r = 0.9). However, after 48 hrs the two parameters are negatively correlated with r = -0.2. IL-6 concentration dropped significantly at 0.5 mM as compared to the control. Slight increase with no significant variations is observed for the remaining caffeine concentrations.

A



B



C

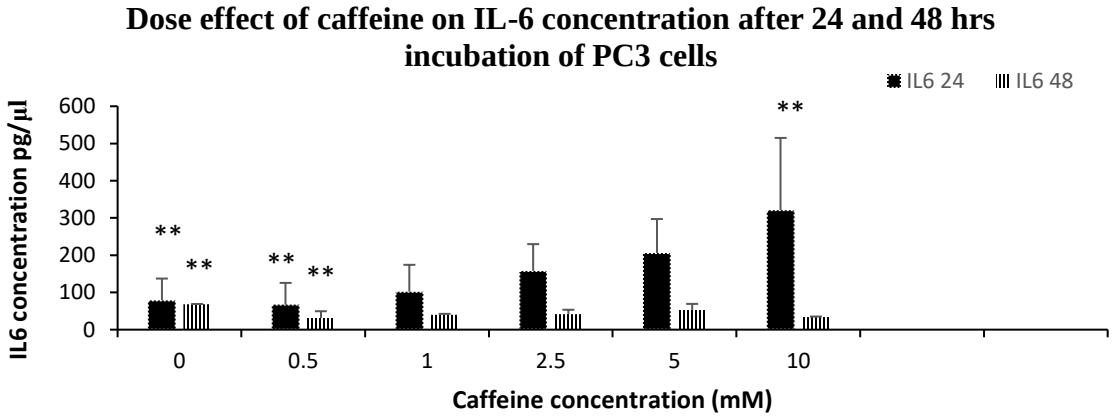


Figure 7: Dose effect of caffeine on IL-6 concentration after 24 and 48 hours on PC3 cells. In panel A, treatment for 24 hrs lead to an increase up to three fold in IL-6. On the other hand, in panel B a decrease in IL-6 concentration was observed after 48 hrs. Panel C, showed the effect of time on IL-6 secretion. IL-6 concentration in cell-free supernatants was measured with ELISA kit. Values were plotted onto a standard curve and expressed as pg/ml. The experiments were performed in duplicates and values represent mean ± SD. ** $p \leq 0.01$.

3- qRT-PCR

In addition to assess IL-6 protein level using MultiSkan Go Plate reader, we opt for qRT-PCR to quantify mRNA gene expression of IL-6 and IκB-α which are highly expressed in PCa.

3.1 Primer optimization

Optimization was done to determine the best primer concentration and annealing temperature for optimum results. Two target genes IL-6 and IκB-α were studied, the first one indicated the inflammation rate and second reflects the amount of NF-κB. GAPDH and 18S were used as internal controls. The melt curve showed that the optimal annealing temperature was 60˚C for both genes and internal controls (figure 8). The amplification curve indicated that the optimum concentrations for each reverse and forward primers were 200ng/µl for IL-6, 100 ng/µl for IκB-α and 100 ng/µl for both internal controls.

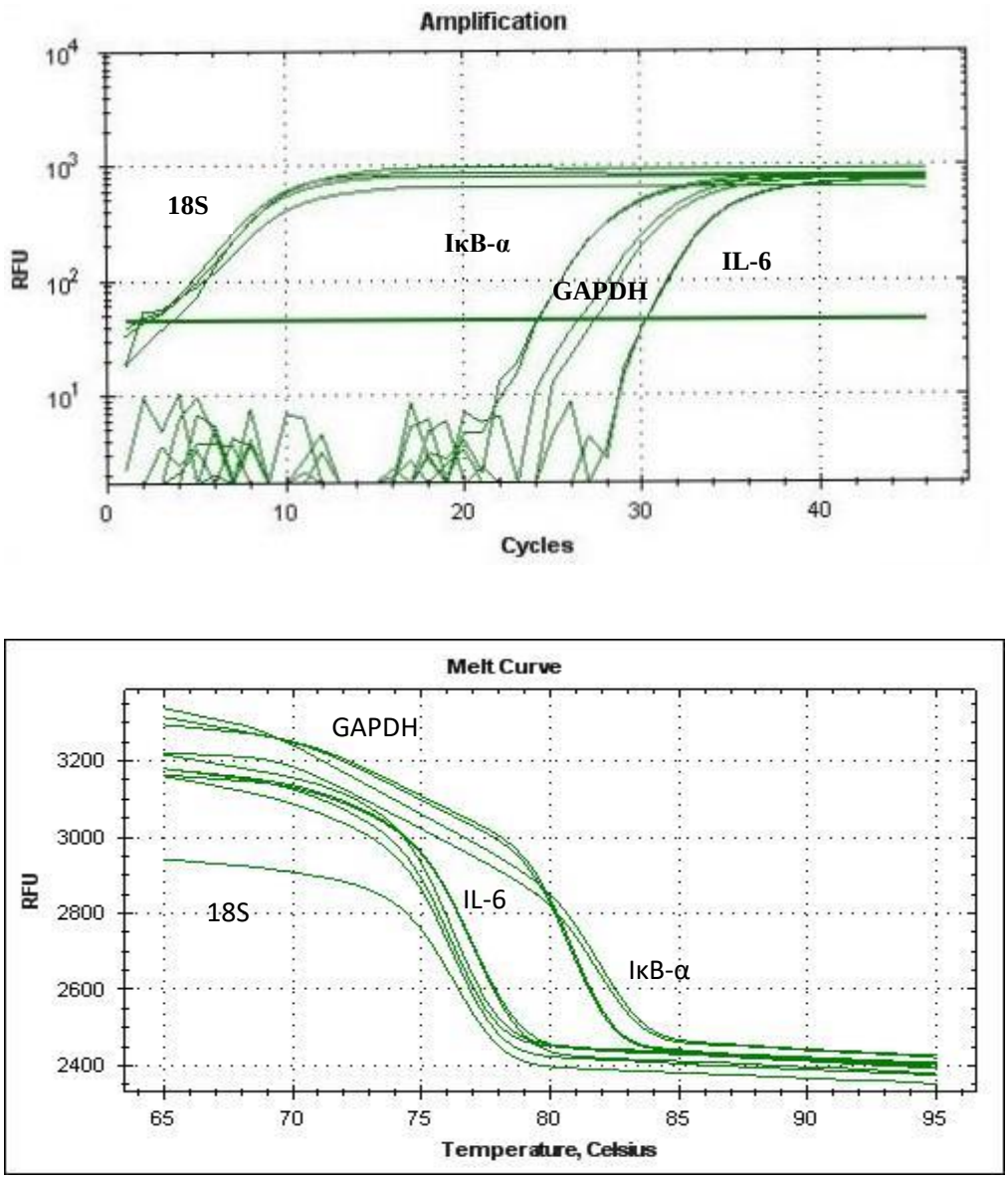


Figure 8: Amplification and melting curves of primer optimization based on RNA mix.

Two different concentrations of 100 and 200 ng/µl were used for each reverse and forward primers and mixed with a fixed RNA concentration mix of 50 ng/µl extracted from PC3 and PC3-PSMA cells at gradient temperature (56℃-60℃). The internal controls 18S and GAPDH were also tested at 100 ng/µl for reverse and forward primers and at the same temperature. Amplification curves of the two target genes and two internal controls shows that 18S appeared after 10 cycles, IκB-α after 20 cycles, GAPDH after 24 cycles and IL6 after 26 cycles. As a result, the primers that gave the earlier and sharper amplification were selected. According to the melting curve, the optimal annealing temperature is 60℃.

3.2 Parallelism

Parallelism is performed to verify that the gene of interest and the internal controls have the ability to detect fold changes in amounts of loaded RNA in a parallel fashion. A parallelism run was prepared with different dilutions of combined RNA mix from PC3 and PC3-PSMA cells. All our tested genes showed fold change detection in a parallel way except GAPDH which had a fluctuation in expression with decreased RNA concentrations (figure 9). Thus, 18 S was chosen as internal control for gene amplification in a dilution of 1/1000.

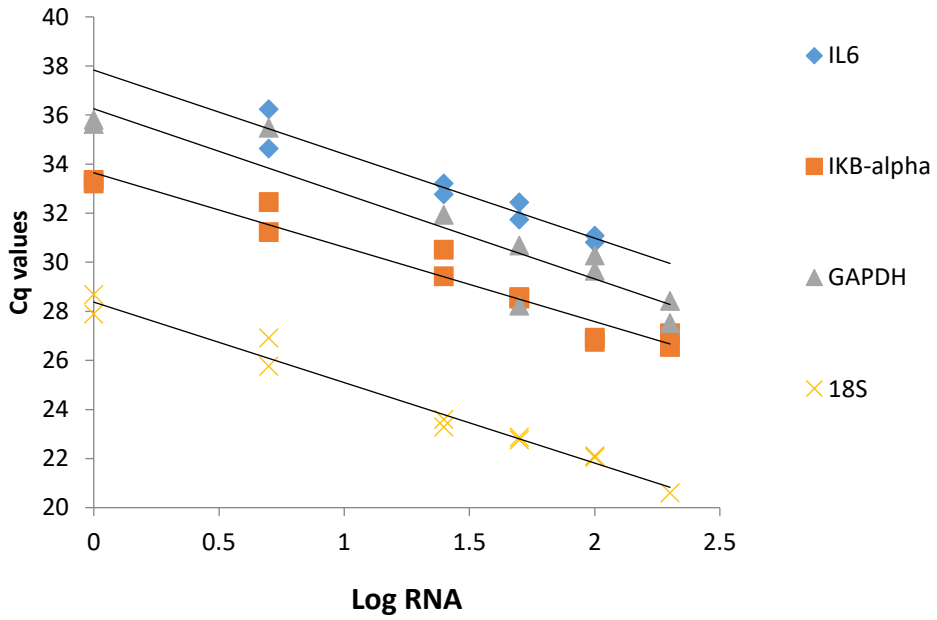


Figure 9: Parallelism graph of 18S, GAPDH and the target genes.

Decreased amounts of total RNA (200, 100, 50, 25, 5, 1 ng/µl) were tested with forward and reverse primers concentrations of 200 ng/µl for IL-6 and 100ng/µl for IκB-α, GAPDH and 18S (for 18S RNA was diluted to 1/1000). RT-PCR Cq values were plotted versus log RNA. Parallelism was validated for IL-6, IκB-α, 18 S, but not for GAPDH.

3.3 Melting curve

For primer validation the melt curve analysis of the parallelism run showed reproducible results. Single peaks for each amplicon at gradient RNA concentrations were observed. Decreased total RNA concentration (200, 100, 50, 25, 5, 1 ng/µl) for IL-6, IκB-α, GAPDH and (200, 100, 50, 25, 5, 1 pg/µl) for 18S followed by RT-PCR quantification of all amplified genes showed the presence of one gene of interest represented by one peak for IL6, IκB-α, 18S and GAPDH. Cycle threshold (Ct) is proportional to the amount of gene amplified by PCR as shown in figure 10.

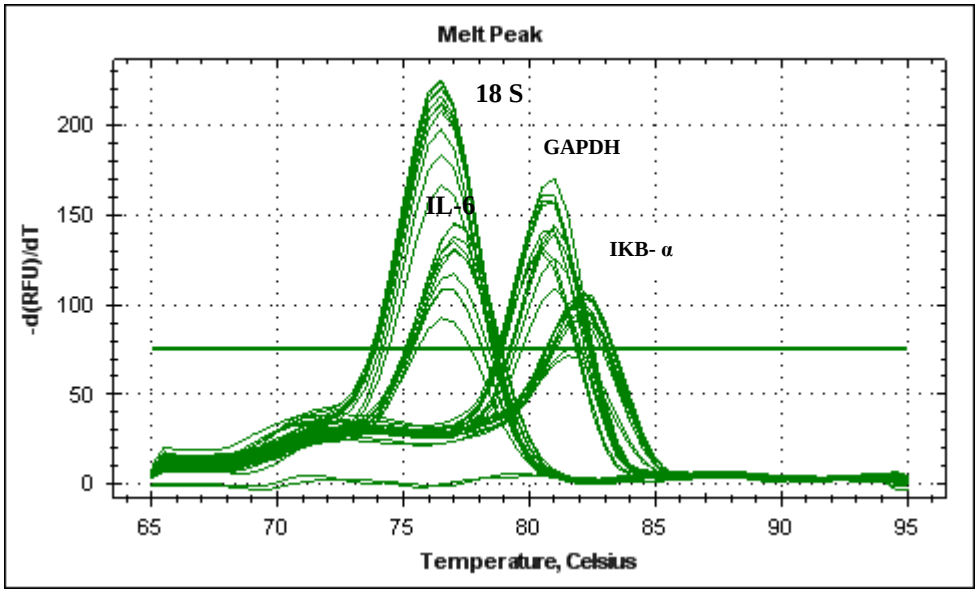


Figure 10: Melting curve analysis of IL6, IκB-α, 18S and GAPDH in qRT-PCR using SYBR Green. Decreased total RNA concentration (200, 100, 50, 25, 5, 1 ng/µl) for IL-6, IκB-α, GAPDH and (200, 100, 50, 25, 5, 1 pg/µl) for 18S followed by RT-PCR quantification with a melt curve cycle set at 65℃ to 95℃, increment 0.5℃ showed unique melting curve for IL-6, IκB-α, 18S and GAPDH.

4- qRT-PCR amplification

IL-6 and IκB-α mRNA levels were analyzed in PC3 and PC3-PSMA cells. After treatment with caffeine concentrations (0, 0.5, 1, 2.5, 5, 10, 20 mM) at different time points (24 and 48 hrs), cells were harvested and total mRNA was isolated and reverse transcribed in one step.

The quantitative RT-PCR was then performed using IL-6 and IκB-α specific primers and 18S as internal control. The amplification products were detected during the course of the PCR reactions using the double-strand intercalating fluorescent dye SYBRGreen. Fluorescence of each reaction was recorded and expressed as a function of progression of the reaction.

The results determined the effect of caffeine doses on the expression of IL-6 and IκB-α genes regardless of the incubation time. The data of the two genes were represented in tables 5 and 7 depending on the dose and cell type.

IL-6 gene expression in function of caffeine doses in PC3 and PC3-PSMA cells is represented as fold and log(fold+1) in figure 11. Compared with the control, dose effect showed a significant increase in gene expression at 10 and 20mM caffeine dose for PC3 cells and at 20mM for PC3-PSMA cells (P=0.0017). Maximum IL-6 expression was recorded at 20mM with 7000 and 10000 fold for PC3 and PC3-PSMA respectively. No significant difference in expression depending on cell type and time was observed as shown in table 5 ($p>0.05$).Gene expression is affected by high caffeine doses irrespective of cell type which is validated in figure 12.

The two 24 and 48 hrs incubation time points did not affect the expression of IL-6 in both cell lines as shown in figure 13. Fold and log(fold+1) of IL-6 data in function of time are displayed in table 6.

Given the aforementioned results, IL-6 expression was only affected by caffeine doses irrespective of cell type and incubation time (table 5).

The quantification of IκB-α mRNA by RT-PCR evaluates the transactivation potential of NF-κB, which has an important role as a survival factor. The mRNA levels of IκB-α correlated with the levels of NF-κB activation. Fold and log(fold+1) of IκB-α gene expression in function of caffeine doses in PC3 and PC3-PSMA cells are represented in figure 14. The expression of IκB-α fluctuated without being affected neither by caffeine

doses nor by cell type as depicted in table 7 ($p>0.05$).Moreover, dose effect is validated irrespective of cell type in figure 15which showed that the gene expression is not affected by caffeine concentrations.
Incubation time did not affect the expression in both cell lines as depicted in figure 16.

For both genes, fold values do not follow a normal distribution and therefore a t-test least squares (LS) mean comparison is not valid. Data transformation for log(fold+1) restores normality of the values which are used for multiple comparisons.

Table 5: Effect of caffeine doses on IL-6 expression in PC3 and PC3-PSMA cells. Values are means ± SEM.

Dose (mM)	TG Cq		18S Cq		DCq =TGCq-18SCq		DDCt =DCq-max DCq		Fold $=2^{-DDCq}$		Log(fold+1)	
	PC3	PC3-PSMA	PC3	PC3-PSMA	PC3	PC3-PSMA	PC3	PC3-PSMA	PC3	PC3-PSMA	PC3	PC3-PSMA
0	29.8785	29.6188	18.3354	18.8703	11.5431	10.7484	-6.62	-7.4098	153.7	364.26	1.9978	2.2391
0.5	29.0889	32.7928	19.0572	18.5181	10.0317	14.2747	-8.126	-3.8835	848.03	56.2875	2.4533	1.2709
1	28.9784	33.5113	18.2678	18.8341	10.7106	14.6772	-7.447	-3.481	228.81	54.6753	2.2456	1.1497
2.5	30.5509	30.6526	18.9647	19.8929	11.5862	10.7597	-6.57	-7.3985	199.87	301.75	1.9878	2.2321
5	29.323	32.2187	18.4348	20.9399	10.8882	11.2788	-7.27	-6.8795	219.16	159.43	2.193	2.0761
10	29.2563	32.1217	20.3263	21.6598	8.93	10.462	-9.228	-7.6963	715.17	315.35	2.7788	2.32
20	34.0105	32.0473	27.1978	24.7974	6.8127	7.25	-11.34	-10.908	10944	7084.74	3.4162	3.2844
SEM	±1.0289		±0.9274		±0.8329		±1.2425		±2337		±0.3626	
Cell type	0.0041		0.4046		0.07		0.0626		0.5734		0.0741	
Dose	0.1089		<.0001		<.0001		0.0017		0.004		0.0017*	
Time	0.4499		0.2169		0.6514		0.7618		0.5563		0.7739	
Cell type * dose	0.0287		0.2467		0.0143		0.2357		0.9786		0.2797	
Cell type* time	0.4673		0.6626		0.1708		0.3549		0.6212		0.356	
Dose*time	0.0107		0.0053		0.1545		0.6079		0.8746		0.6734	

Table 6: Effect of time on IL-6 expression irrespective of cell type and caffeine dose. Values are means ± SEM.

Time	Fold	Log(fold+1)
24 hrs	30.79	2.232
48 hrs	31.214	2.2884
SEM	±0.389	±0.137

Table 7: Effect of caffeine doses on IκB-α expression in PC3 and PC3-PSMA cells. Values are means ± SEM.

Dose (mM)	TG Cq		18S Cq		DCq =TGCq-18SCq		DDCt =DCq-max DCq		Fold $=2^{-DDCq}$		Log(fold+1)	
	PC3	PC3-PSMA	PC3	PC3-PSMA	PC3	PC3-PSMA	PC3	PC3-PSMA	PC3	PC3-PSMA	PC3	PC3-PSMA
0	26.5248	27.6353	18.3354	18.8703	8.1895	8.765	-4.028	-3.452	19.2662	18.3052	1.2418	1.116
0.5	26.0938	28.1061	19.0572	18.5181	7.0366	9.5881	-5.181	-2.629	38.7866	8.2166	1.5724	0.871
1	26.632	28.8804	18.2678	18.8341	8.3642	0.0463	-3.853	-2.171	15.3425	7.1935	1.1909	0.7763
2.5	27.7495	27.9681	18.9647	19.8929	8.7848	8.0752	-3.433	-4.142	21.4663	20.564	1.1187	1.2752
5	28.0436	30.3146	18.4348	20.9399	9.6088	9.3747	-2.609	-2.842	7.3013	11.3562	0.8591	0.9551
10	27.6275	29.692	20.3263	21.6598	7.3011	8.0322	-4.917	-4.185	39.004	19.0055	1.4981	1.2842
20	35.4204	33.2503	27.1978	24.7974	8.2226	8.453	-3.995	-3.764	19.285	21.5374	1.2346	1.1972
SEM	±0.99		±0.9274		±0.6756		±0.7424		±7.2083		±0.191	
P- values												
Cell type	0.0438		0.4046		0.0647		0.0913		0.0522		0.0917	
Dose	<.0001		<.0001		0.172		0.2664		0.1297		0.2303	
Time	0.6764		0.2169		0.2747		0.3195		0.3863		0.3275	
Cell type * dose	0.2611		0.2467		0.2558		0.3649		0.1748		0.3133	
Cell type* time	0.9007		0.6626		0.4358		0.4779		0.6395		0.5889	
Dose*time	0.0008		0.0053		0.5288		0.6377		0.7736		0.6744	

Table 8: Effect of time on IκB-α expression irrespective of cell type and caffeine dose.Values are means ± SEM.

Time	Fold	Log(fold+1)
24 hrs	17.35	1.106
48 hrs	20.736	1.2072
SEM	±2.725	±0.072

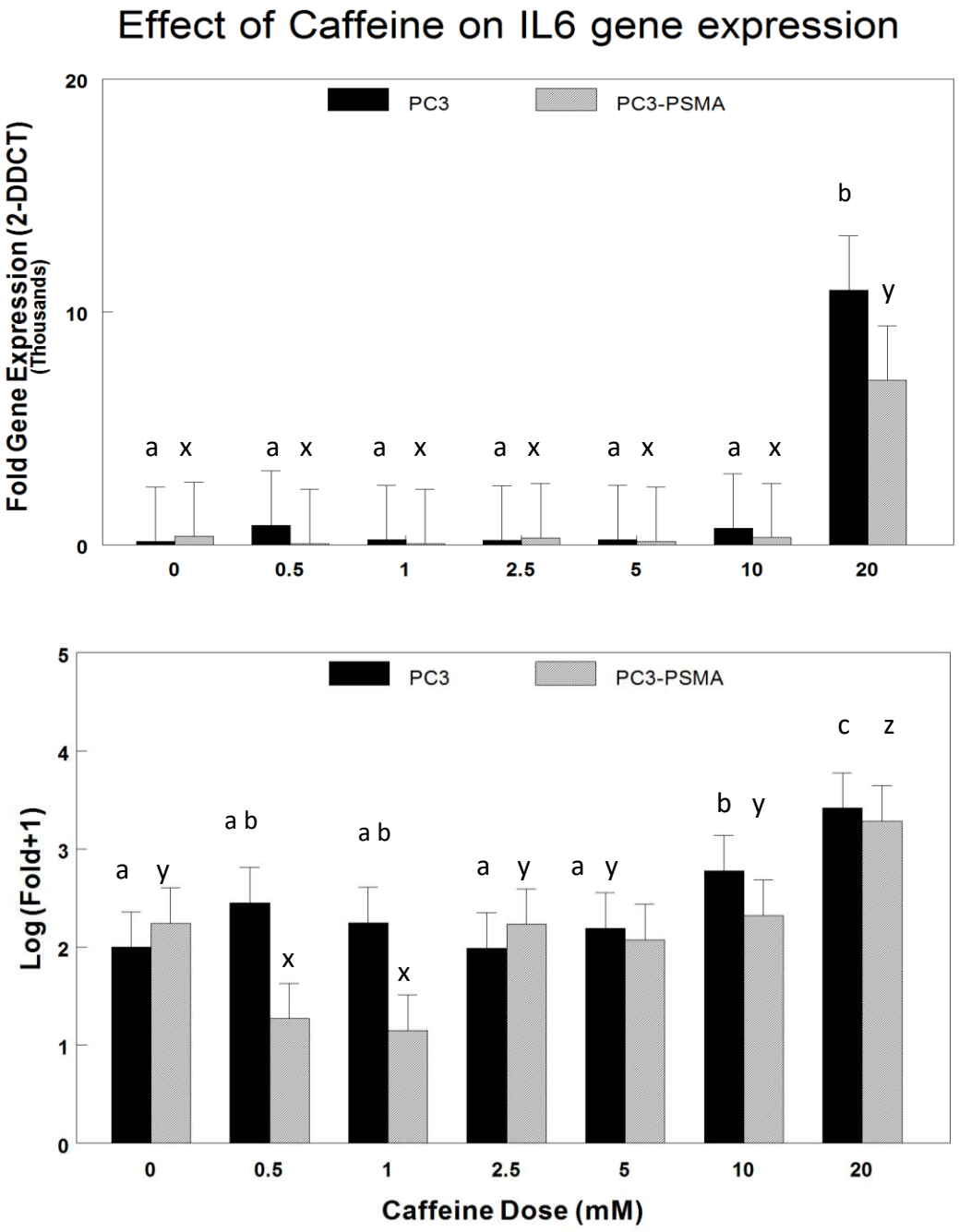


Figure 11: Quantification of IL6 mRNA levels in PC3 and PC3-PSMA cells. Cells were cultured and incubated with different caffeine concentrations (0, 0.5, 1, 2.5, 5, 10, 20 mM).Total mRNA was isolated and reverse transcribed. Fold and log (fold+1) panels for both cell lines show a significant increase in IL6 expression at 20mM for both cell lines ($p<0.05$). Values are presented as mean ± SEM. Means without a common superscript differ (ab,xy within cell type).

IL-6 expression in function of caffeine dose irrespective of cell type

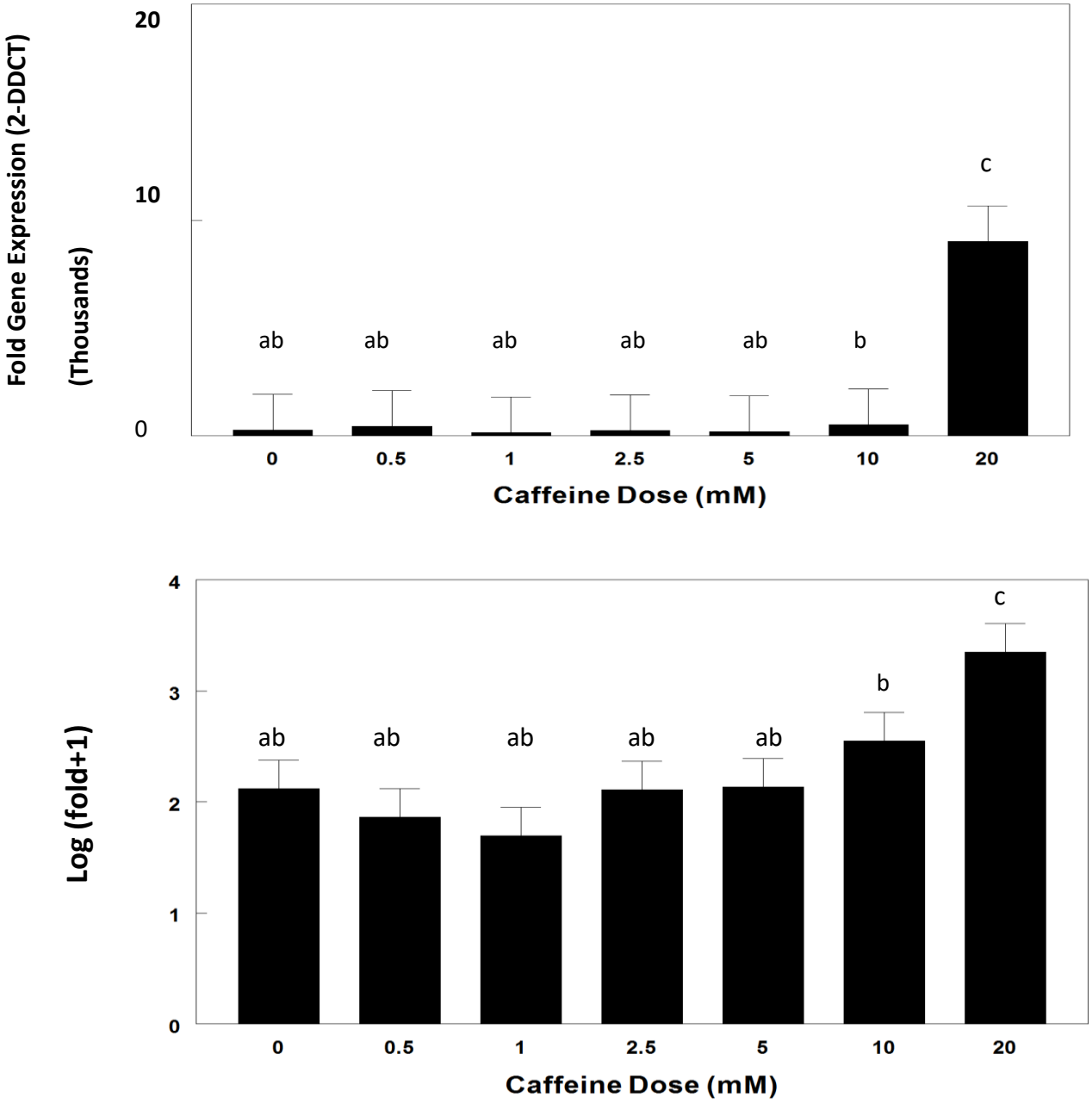


Figure 12: Effect of caffeine doses on IL-6 expression. After culture and incubation of PC3 and PC3-PSMA cells with the appropriate caffeine concentrations, dose effect is clearly validated irrespective of cell type ($p<0.05$). High expression is observed at 10 and 20mM, this is indicated by different letter codes (b,c). Values are presented as mean ± SE

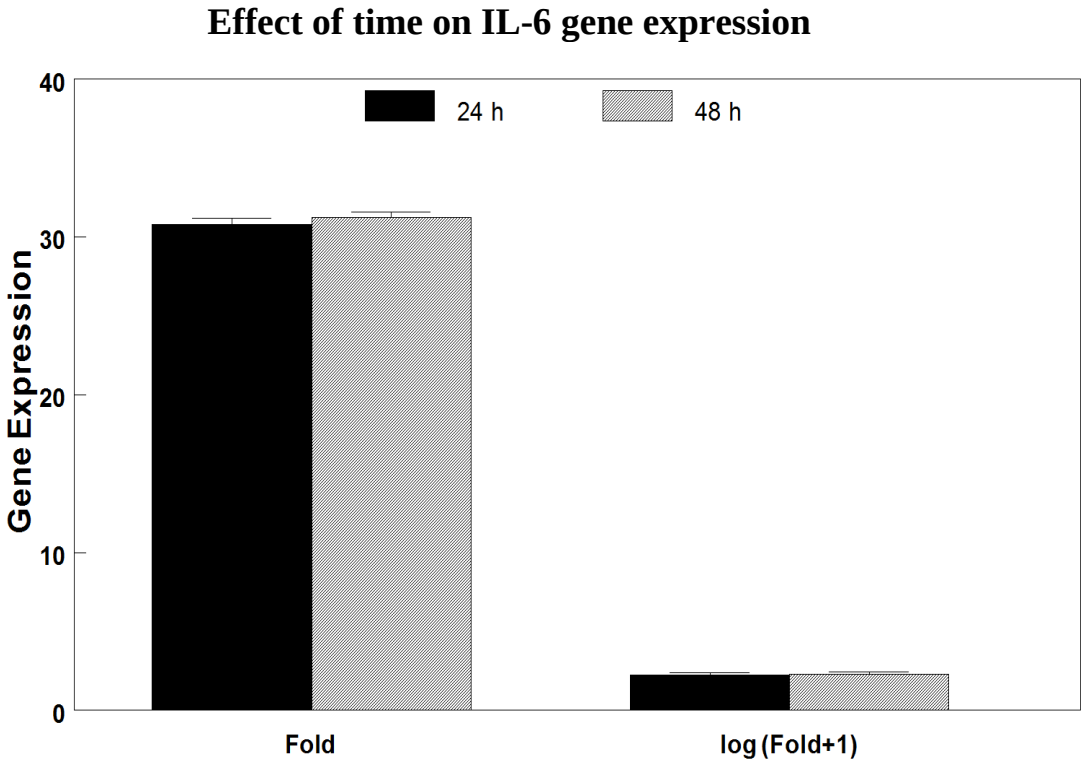


Figure 13: Effect of time on IL-6 gene expression in both PC3 and PC3-PSMA cells. Fold and log (fold+1) of the two time points (24 and 48 hrs) are expressed in function of the IL-6 gene expression. Both panels are equal at 24 and 48 hrs which confirm that time did not alter the expression levels of IL-6 irrespective of cell type (p=0.7, table 1).Values are presented as mean ± SEM.

Effect of caffeine doses on IκB-α gene expression in PC3 and PC3-PSMA cells

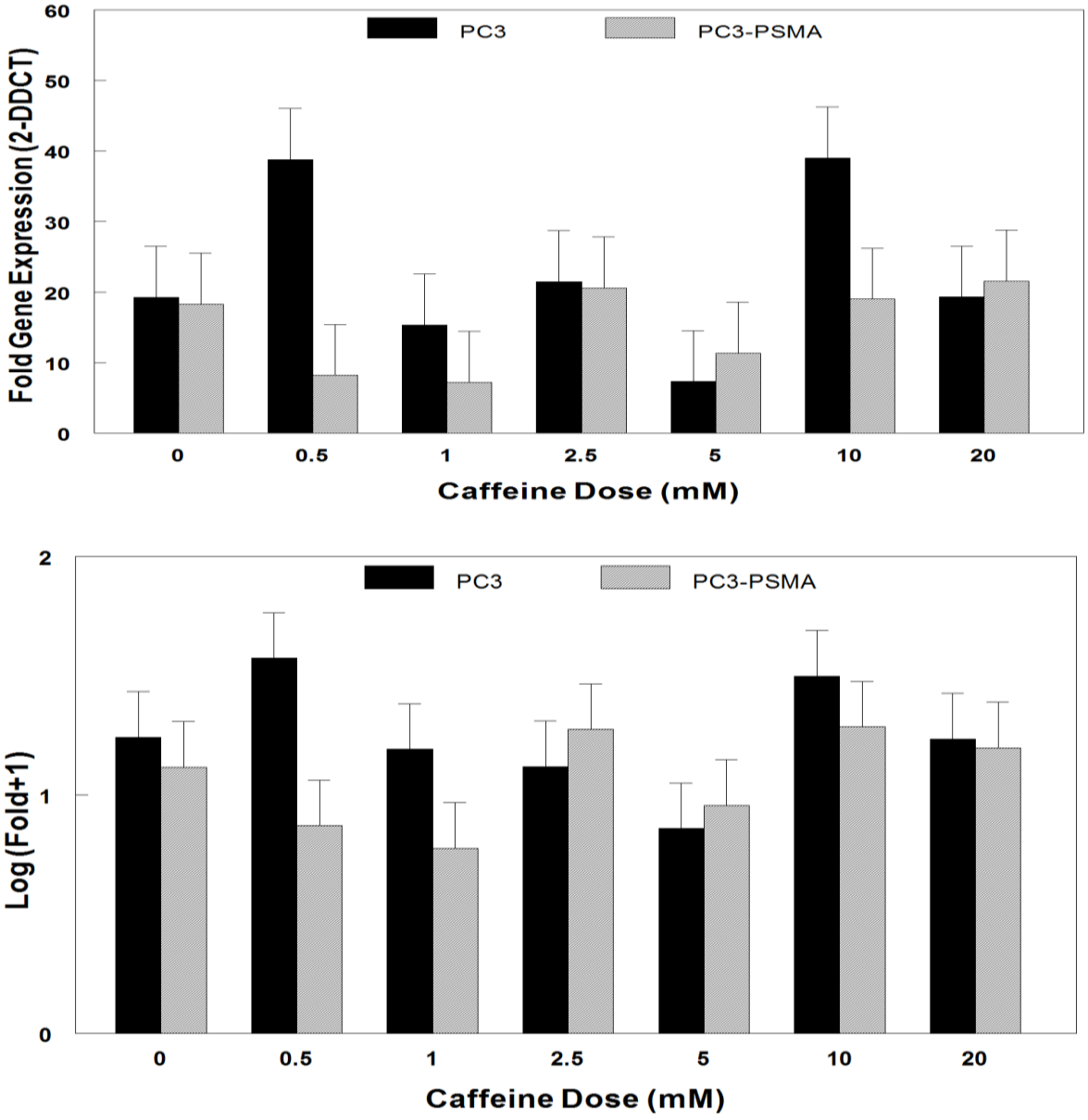


Figure 14: Quantification of IκB-α mRNA levels in PC3 and PC3-PSMA cells. Cells were cultured and incubated with different caffeine concentrations (0, 0.5, 1, 2.5, 5, 10, 20 mM) at two time points (24 and 48 hrs). Total mRNA was isolated and reverse transcribed. The two panels show fluctuation in the gene expression for both cell lines with no significant difference depending on caffeine dose ($p>0.05$). Values are presented as mean ± SEM.

Effect of caffeine doses on IκB-α gene expression irrespective of cell type

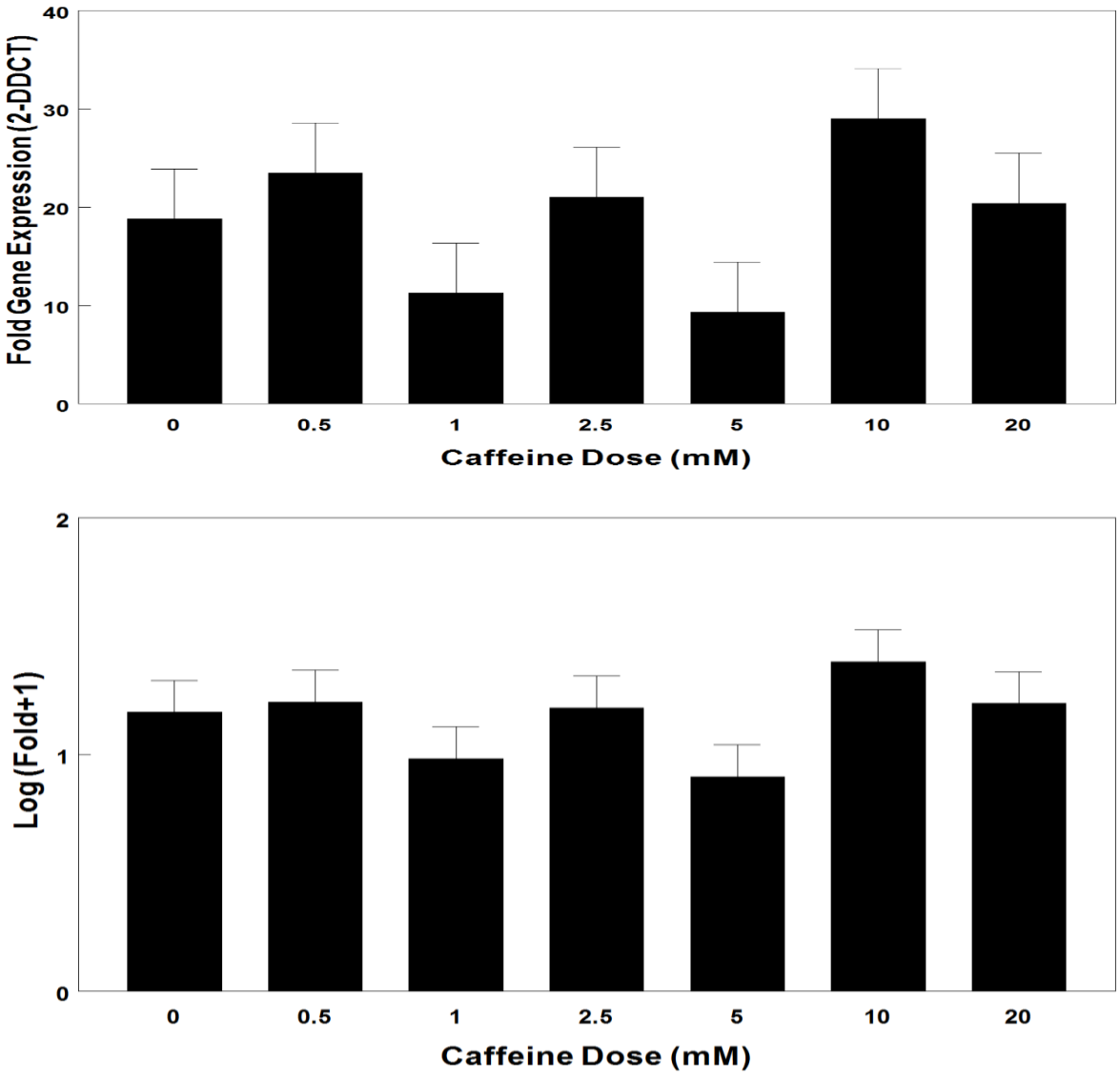


Figure 15: Effect of caffeine dose on IκB-αexpression. PC3 and PC3-PSMA cells were cultured and incubated with the appropriate caffeine concentrations. Fold and log(fold+1) are expressed in function of caffeine dose irrespective of cell type and show that caffeine doses do not affect gene expression. Values are presented as mean ± SEM.

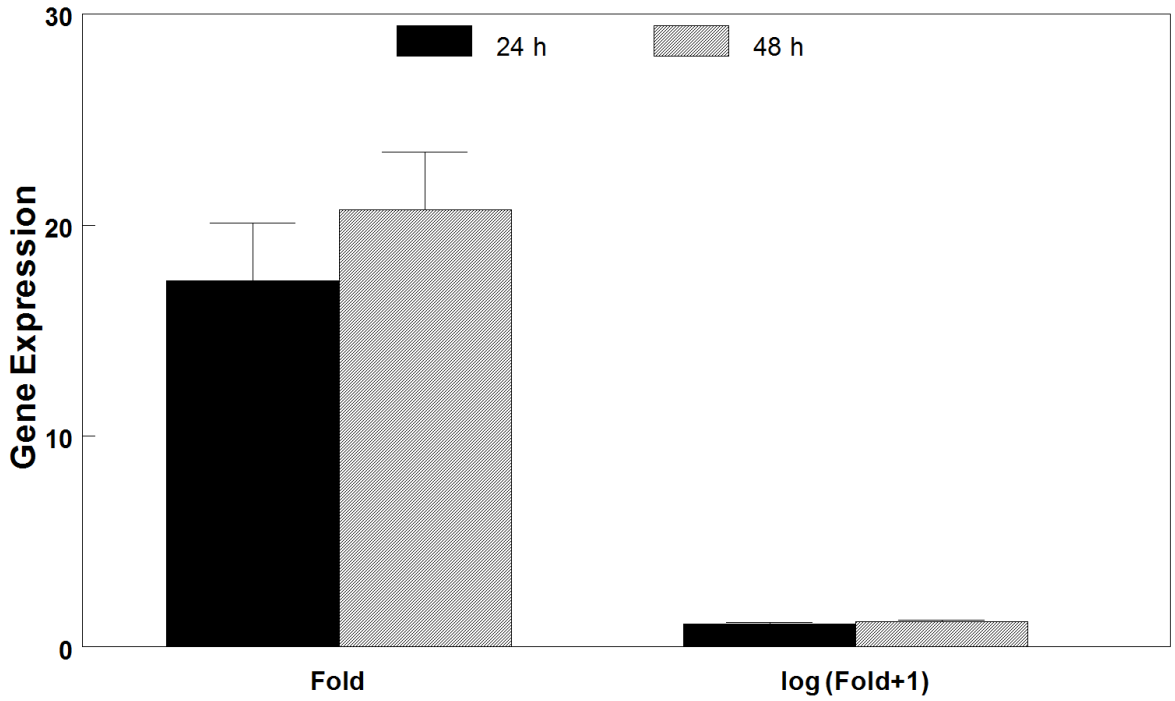


Figure 16: Effect of time on IκB-α gene expression. Fold and log (fold+1) of the two time points (24 and 48 hrs) are expressed in function of IκB-αgene expression. Time do not have any effect on the expression and there is no significant difference between the two bars of the time points. Values are presented as mean ± SEM.

V.DISCUSSION AND CONCLUSION

Understanding the effect of coffee on the incidence of PCa development requires collective evidence from cohort, meta-analysis studies, and *in vitro* applications. The incidence rate of PCa is inconsistent and influenced by several determinants, especially if generated in human subjects. Diet, environmental and epidemiological factors could act as variables. Most *in vivo* studies on PCa prognosis in light of coffee consumption support a lower risk of PCa aggression in patients administering daily up to three cups[14], [15], [16], [17], [18]. However, coffee does not have a protective effect against PCa [19].Despite the well-identified active compounds of coffee, little is known about its *in vitro* effect on PCa growth and inflammatory responses. Thus, more *in vitro* studies are recommended to provide conclusive effects of coffee dose and exact mechanism of action that lies behind its anti-cancerous properties.

Caffeine one of coffee constituents was chosen in our study due to its pharmacological effects toward diseases. Cumulatively, articles published over the past 6 years, support the pharmacological and physiological effects of caffeine mainly linked to its anti-oxidant, anti-inflammatory and anti-carcinogenic properties. The *in vitro* effect of caffeine on PCa cells was not previously studied. PC3 and PC3-PSMA were chosen as model PCa cell lines with the latter overexpressing PSMA with increase aggressiveness most likely leading to metastasis.

To evaluate caffeine anti-cancerous effects, we assessed the viability of various PCa cell lines at the cellular level; and molecular markers at both the protein and RNA levels, namely, IL-6 concentration and IL-6 and IκB-α gene expression. It was previously *in vitro* demonstrated that caffeine had a negative effect on PCa viability depending on the dose, cell type and incubation time[7], [8], [9], [10], [20]. Hashimoto *et al.* (2004) reported a cell proliferation suppression in a dose dependent manner of caffeine by inducing cell cycle arrest at G0/G1 phase. A recent study for Li *et al.* (2016) proved that even at low concentrations ranging from 50 to 600 μM, caffeine inhibits viability and induces apoptosis of melanoma cells at 48 and 72 hrs.

Similarly, our data showed that after 24 and 48 hrs viability decreased when the cells were subjected to six concentrations starting from the lowest to the highest. Interestingly, both cells had a viability strike in a dose and time dependent manner.

Based on data from 24hrs exposure, PC3-PSMA are more resistant to caffeine with 45% survival at high dose (10mM). On the other hand, PC3 are more sensitive to caffeine even at low doses (2mM) reaching 45% at such doses. However, after 48hrs PC3-PSMA cells become more sensitive with only 19% survival at 10mM compared to 40% for PC3 cells. This difference might be due to the fact that PC3 cells androgen insensitive expressing less PSMA which is lost from androgen dependent to androgen independent state. Transfection of PC3 cells with PSMA gene is able to restore PSMA expression in PC3 cells which is the case of PC3-PSMA cells [32]. This makes a difference in protein profiles between PSMA-transfected and PC3-mock model cell lines [32].

In addition, PC3-PSMA cells are three fold higher confluent than PC3 cells thereby rendering them more vulnerable to environmental stress (less nutrient, high acidity and toxicity) leading to apoptosis.

Besides WST1 viability test, IL-6 concentration in supernatants was determined to study the effect of caffeine on inflammation. A number of studies have recently demonstrated that a prominent role in tumor survival and progression can be attributed to soluble mediators present in the tumor microenvironment. Among these, IL-6 has a fundamental role in the regulation of proliferation, apoptosis, angiogenesis and differentiation in many cell types and it is also implicated in the development and progression of several forms of tumors including that of the prostate[33, 34]. The levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer [54].

Moreover, many studies were controversial regarding the effect of caffeine doses on IL-6 concentrations. Caffeine can decrease or increase inflammatory markers such as IL-6. Studies on male rats , human blood cells, human adipose tissue cells demonstrated that caffeine administration decreased IL-6[22], [23], [24], [25].However, increased serum levels of IL-6 were observed *in vitro* on male mice, male and female human subjects[26][27],[28]. Amer *et al* (2017) and Li et al (2011) proved that caffeine reduces inflammation by decreasing IL-6 serum levels. Moreover in 2019, Fang *et al* and Xu *et al* demonstrated that IL-6 was increased in blood after the administration of caffeine in non-alcoholic liver disease and OVX female rats.

Data from our study showed that after 24 hrs, IL-6 concentration increased to 321pg/ml for PC3 cells and to 88 pg/ml for PC3-PSMA at 10mM. However, at low caffeine concentrations the increase is less significant for both cell lines. We deduce that the inflammation process is linked to caffeine-dependent doses which is confirmed by Barcelos *et al* (2020). At high concentrations, caffeine becomes antagonistic to A3 adenosine receptors which are overexpressed in prostatic cancerous cells causing an increase in cAMP production and consequently increases IL-6 in PC3 cells[55].PC3 cells were able to secrete more IL-6 as being more sensitive to caffeine than PC3-PSMA.

On the other hand, linking viability with IL-6 levels in supernatants we deduce that when cells approach apoptosis the levels of IL-6 decrease. This hypothesis was valid at 48 hrs where the IL-6 concentrations decreased to 34 pg/ml at 10mM in PC3 cells and was undetectable in PC3-PSMA because of its fast cell growth leading to a significant viability decrease. This is also explained by the fact that IL-6 peaks at 12 hrs after release and has a half-life of 15 hrs[56].

Besides the study of the caffeine effect on inflammation, IL-6 gene expression was studied using qRT-PCR technique. Caffeine has been shown to affect the expression of genes involved in inflammation such as IL-6 by acting on the signaling pathway that modulates the transcription of NF-κB [56]. NF-κB gene expression was studied for its role in mediating chronic inflammation via the expression of inflammatory cytokines such as IL-6 and TNF. It is an important transcription factor involved in the regulation of immune responses as well as in cell proliferation and survival [40].Since the termination of NF-κB transcription is mediated through the NF-κB-dependent synthesis of the IκB-α inhibitory subunit, IκB-α mRNA gene expression levels directly correlate with the levels of NF-κB activation [40]. We ought at quantifying their expression as a means to check the proliferation and survival effects of caffeine.

Results of the real time-PCR showed that the expression of IL-6 was upregulated in PC3 and PC3-PSMA at high doses of 10mM and 20mM in both cell lines in a dose dependent manner regardless of time. This is reflected by an increase in IL-6 concentrations in supernatants. Because of the few studies on the effect of caffeine on gene expression,

only one study done by Dray *et al* (2007) has been found to confirm our results stating that caffeine upregulates IL-6 gene expression in adipose tissue.

The expression of IκB-α fluctuated without being affected neither by caffeine doses nor by the incubation time period.NF-κB expression was not affected by caffeine resulting in a decrease in viability in both cell lines. Since caffeine did not affect NF-κB gene expression, we speculate that another signaling pathway induces inflammation via adenosine receptors antagonism specifically A3.

Based on the PCa cell lines that we used *in vitro*: PC3 and PC3-PSMA serve as model of progressive PCa stages with low and high aggression, respectively. This was noticeable while culturing the cells; PC3-PSMA grow much faster than PC3 cells.

From my results caffeine showed more cytotoxicity effect on PC3 than PC3-PSMA. Thus caffeine might act as a promising therapeutic agent to treat early developmental stages of PCa. However, at high doses, caffeine induces inflammation as tested by IL-6 release. The immunomodulatory role of caffeine was not correlated to NF-κB expression. Indicating another signaling pathway to modulate inflammation, most likely as we speculate through adenosine receptors and calcium channels.

Our data support other studies from the literature. It is worth noting that the doses used *in vitro* are quite high and cannot be applied *in vivo*. At the end, further studies are recommended to unravel the exact molecular mechanism of caffeine action.

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