

Original Paper

# Cholesterol Triggers Nuclear Co-Association of Androgen Receptor, p160 Steroid Coactivators, and p300/CBP-Associated Factor Leading to Androgenic Axis Transactivation in Castration-Resistant Prostate Cancer

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## Key Words

Prostate cancer • Hypercholesterolemic model • p160

## Abstract

**Background/Aims:** Cholesterol modulates intratumoral androgenic signaling in prostate cancer; however, the molecular mechanisms underlying these changes in castration-resistant prostate cancer (CRPC) are not fully elucidated. Herein, we investigated the effect of cholesterol on androgen receptor (AR) coactivators expression and tumorigenesis *in vitro* and *in vivo*. **Methods:** Herein, we monitored the expression of AR coactivators (SRC-1, 2, 3 and PCAF) genes in PC-3 cells exposed to 2µg/mL of cholesterol for 8 hours by qPCR. We also performed

cell migration at 0, 8, 24, 48 and 72h and flow cytometry assays (viability, apoptosis, and cell cycle) after a 24h exposure. Immunofluorescence assay was performed to evaluate the protein expression of the AR coactivators. Additionally, *in vivo* experiments were conducted using 22 male NOD/SCID mice. Mice were fed a standard (Control) or hypercholesterolemic (HCOL) diet for 21 days and then subcutaneously implanted with PC-3 cells. The tumor volume was calculated every two days, and after four weeks, the tumors were resected, weighed, and the serum lipid profile was measured. We also measured the intratumoral lipid profile and AR coactivators gene and protein expression by qPCR and Western Blot, respectively. Intratumor testosterone and dihydrotestosterone (DHT) concentrations were determined using ELISA. **Results:** Cholesterol up-regulated the gene expression of coactivators SRC-1, SRC-2, SRC-3 and PCAF, increasing AR expression in PC-3 cells. Next, cholesterol-supplemented PC-3 cells exhibited increased cell migration and altered cell cycle phases, leading to changes in proliferation and reduced apoptosis. We found that SRC-1, SRC-2, SRC-3 and PCAF proteins co-localized in the nucleus of cholesterol-supplemented cells and co-associate with AR. In the *in vivo* model, the hypercholesterolemic (HCOL) group displayed higher serum total and intratumoral cholesterol levels, increased testosterone and dihydrotestosterone concentrations, and up-regulated AR coactivator expression. The tumor volume of the HCOL group was significantly higher than the control group. **Conclusion:** Our findings revealed that increased nuclear translocation of the coactivators leads to up-regulated AR gene and protein expression, potentially influencing tumor progression. Studies targeting cholesterol-modulated changes in AR coactivator expression may provide insights into the molecular mechanisms associated with the CRPC phenotype.

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## Introduction

Castration-resistant prostate cancer (CRPC) is the most lethal form of Prostate Cancer (PCa) and is characterized by patients who are resistant to androgen-blocking therapies [1]. In these cases, the resistant cells acquire the potential to synthesize testosterone from cholesterol molecules, stimulating cell proliferation and survival [2]. The escape mechanisms behind CRPC have been attributed to the positive regulation of a complex heterogeneous network of anti-apoptotic and survival genes [3] and growth factors [1, 4]. Indeed, two hypotheses have been proposed to explain these observations. The first involves aberrant Androgen Receptor (AR) activation through common downstream pathways or the positive regulation of AR coactivators in the absence of androgens [5]. The second relies on androgen-regulated pathways in PCa cells by alternative sources of steroidal androgens [6].

Given the fact that AR plays a fundamental role in PCa [7] and that steroid receptor activity is potentiated by a wide range of coactivators [8], it has been proposed that these molecules may also play a role in PCa [9]. The coactivators of the p160 family include SRC-1, SRC-2, and SRC-3 [10], and previous work has shown that these proteins mediate transcriptional functions between nuclear receptors and other transcription factors [11]. Moreover, the overexpression of p160 in PCa has been correlated with increased cell proliferation [12], migration [13], metastatic potential, decreased apoptosis rates and up-regulation of *de novo* lipogenesis [14].

The p300/CBP (PCAF) exhibits histone acetyltransferase activity and is considered an AR coactivator. The expression of this gene has been correlated with an unfavorable PCa prognosis [15-17]. Additionally, PCAF acetylates AR, leading to DHT-induced transcription. Notably, it has been proposed that increasing AR coactivator gene and/or protein expression could result in promiscuous AR activation even by low-affinity AR ligands [18, 19].

Interestingly, compelling evidence has shown that several cancers, including PCa, overexpress lipogenic enzymes [20]. This observation is particularly relevant to cancer progression since prostatic lipogenesis has been observed in both the early neoplastic and invasive stages [21]. AR dysregulation, resulting from mutations and cross-interactions with active signaling pathways in malignant cells, influences CRPC progression. The

biological underpinnings behind the upregulation of lipogenic proteins in PCa and the related mechanisms of how AR coactivators and co-repressors are involved in the onset and progression of this neoplasm are still poorly understood. Therefore, the present study aimed to demonstrate that cholesterol modulates the expression of genes that participate in AR co-activation in cellular and animal CRPC models.

## Materials and Methods

### *Supplementation of PC-3 cells with cholesterol*

The PC-3 cell was seeded in 12-well culture plates at  $2 \times 10^5$  cells/well and grown in MEM medium (Invitrogen, Grand Island, NY, USA) using standard culture methods. The cell line was authenticated by Short Tandem Repeat (STR) (Supplementary Fig. 1 – for all supplementary material see [www.cellphysiolbiochem.com](http://www.cellphysiolbiochem.com)). The cells were supplemented with  $2 \mu\text{g/mL}$  ( $5 \mu\text{M}$ ) cholesterol (C4951-30MG, Sigma Aldrich, St. Louis, MO, USA) for eight hours, according to Moon et al. [22], with modifications. Untreated cells served as the control group.

### *RNA extraction and quantitative real-time polymerase chain reaction*

According to the manufacturer's instructions, cellular RNAs were extracted using the mirVana kit (Ambion, Austin, TX, USA). The total RNA was then synthesized using the High-Capacity cDNA Reverse Transcription Kit for complementary DNA (cDNA) synthesis (Applied Biosystems, CA, USA). The target gene sequences were amplified in a  $10 \mu\text{L}$  reaction mixture containing  $5 \mu\text{L}$  of TaqMan Universal PCR Master Mix and  $0.5 \mu\text{L}$  of TaqMan gene expression (Supplementary Table 1). Data were analyzed using the DataAssist Software (Applied Biosystems, USA). All qPCR reactions were performed in duplicate. GAPDH was used as the endogenous control in the gene expression analysis.

### *Viability, apoptosis, and cell cycle analyses by flow cytometry*

Flow cytometry experiments were performed on a Muse Cell Analyzer (Merck Millipore, Burlington, MA, USA). The assays were performed by supplementing PC-3 cells with cholesterol for 24 hours. After 24 hours, the cells were labeled with the Muse Count & Viability (MCH100102), Muse Annexin V & Cell Death (MCH100105), and Muse Cellular Cycle (MCH100105) kits according to the manufacturer's recommendations.

### *Cell migration assay*

PC-3 cells were transferred to 24-well plates with  $5 \times 10^4$  cells/well; after reaching confluence, a wound was made (risk). The cells were washed, and MEM medium without FBS was added to each well. The plates were photographed at 0, 8, 24, 48 and 72h. Images were analyzed with the NIS Elements D 3.1 software (Nikon). Percent wound closure was calculated based on the risk area at 0, 8, 24, 48 and 72h.

### *Immunofluorescence in vitro*

Cells were cultured on glass coverslips and supplemented with cholesterol ( $2 \mu\text{g}$  for 8 hours) to evaluate co-activating and co-repressor protein expression. The cells were then fixed with 4% paraformaldehyde in 1X PBS for 10 minutes and permeabilized with 0.1% Triton X-100 in 1X PBS for five minutes. Coverslips were incubated with the primary antibodies of the proteins SRC-1, SRC-2, SRC-3, PCAF and AR (Supplementary Table 2) diluted 1:100 in normal horse serum (2.5%) for 12h, followed by the secondary antibody diluted for one hour (VectaFluor™ Duet Immunofluorescence Double Labeling Kit, DyLight® 488 Anti-Rabbit (green), DyLight® 594 Anti-Mouse (red)). Coverslips were washed three times in 1X PBS after each antibody incubation. Where indicated, coverslips were counterstained with DAPI (Cell Signaling), washed three times with 1X PBS, and mounted using ProLong Diamond (Invitrogen). Cells were photographed using a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan) attached to a photographic camera. ImageJ software quantified the fluorescence signal and reported it as fluorescence intensity (arbitrary units).

## *In vivo study*

The *in vivo* portion of this study was approved by our institution's Ethics and Animal Research Committee (Protocol #1119/2018). The Bioterio Central - FMUSP (São Paulo, SP, Brazil) provided 22 NOD/SCID mice. At the age of four to five weeks, male mice were relocated and maintained in laminar flow cabinets under specific pathogen-free conditions, at 20–21°C and 50–60% humidity and a 12h light and 12h dark cycle. The animals' food and water were sterilized before being consumed. Cages were entirely changed once or twice a week. All animal manipulations were performed under sterile conditions. The animals were divided into hypercholesterolemic (HCOL) and control (Ctr). The HCOL group was fed a high-calorie diet (RH19539E) (1.5% cholesterol) (N = 11), and the control group was fed a standard diet (RH19522) (N = 11). Twenty-one days after starting the diet, mice were placed under isoflurane anesthesia, and  $1.5 \times 10^6$  PC-3 cells (in a final volume of 100  $\mu$ L of medium) were injected into the subcutaneous tissue. The mice continued to consume their respective diets until the time of euthanasia, 24 days after injecting the cells. The tumors were palpable between 7 and 14 days after the PC-3 cell inoculation. Tumor volumes and weights were measured every two days until the end of the experimental period. Tumors were palpable, and the length (L) and width (W) were measured with a digital caliper. The tumor volume (V) was calculated every two days using the following formula:  $V = [W \times L \times (W + L) / 2] \times 0.52$  [23].

## *Lipid Profile*

Both groups' serum cholesterol, LDL, HDL, and triglyceride levels were measured using Labtest kits (Labtest Diagnostica, Minas Gerais, BR). The same kit was used to measure intratumoral cholesterol levels in tissue homogenates. The absorbance of the samples was recorded in a SpectraMax 340PC384 microplate reader (Molecular Devices, USA) following the manufacturer's recommendations.

## *Western Blotting*

Tumor tissues were homogenized in ice-cold RIPA buffer enriched with a protease and phosphatase inhibitor cocktail (Merck). The samples were mixed with Laemmli buffer and DTT and heated at 95°C for 5 minutes in a Thermomixer Confort (Thermo Fisher). Aliquots corresponding to 20  $\mu$ g of total protein were resolved by SDS-PAGE using 4%-15% gels (Mini-Protean TGX, BioRad) and then transferred to PVDF membranes (Thermo Fisher). Standard western blotting techniques were used to detect SRC-1, SRC-2, SRC-3, PCAF, AR, and GAPDH. The primary antibodies were diluted at 1:500, and the secondary antibody (Goat Anti-Rabbit IgG, (H+L) HRP conjugate, Merck Millipore AP307P) was applied at 1:5000. The band intensities were quantified by measuring the chemiluminescence in an Alliance Mini 4 16.15 gel documentation system (UVITEC Cambridge, UK).

## *ELISA*

Intratumor testosterone (AVIVA Systems Biology, San Diego, CA, USA [OKCA00142]) and dihydrotestosterone (DHT) (Fine Test. Wuhan. China [EU2551]) concentrations were determined using a competitive Enzyme-Linked Immunosorbent Assay (ELISA) technique with tumor homogenates according to the manufacturers' instructions. Absorbance at 450 nm was recorded. All experiments were performed in duplicate.

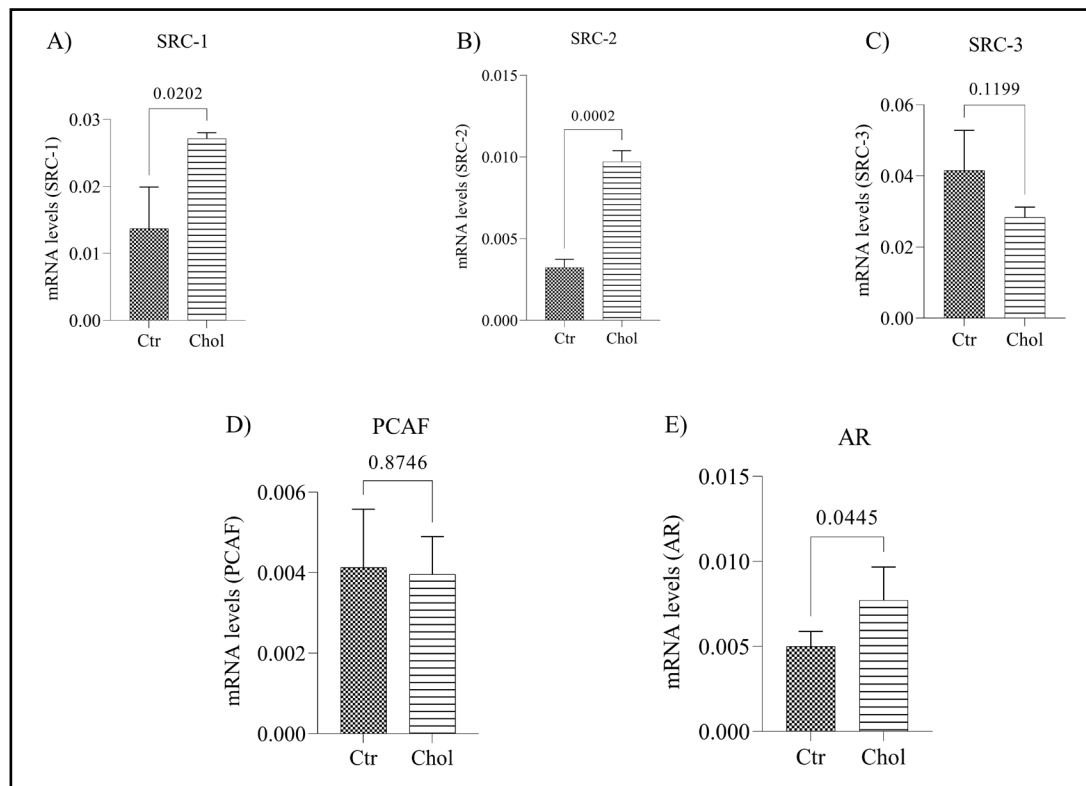
## *Statistical analysis*

Statistical analyses were performed using GraphPad Prism 9.0 software. All *in vitro* experiments were performed in biological triplicates. The Shapiro-Wilk test was used to assess the normality of the data, and the Student's T-test and Mann-Whitney test were used to compare the *in vitro* and *in vivo* data. The level of statistical significance was set to 5% ( $p \leq 0.05$ ).

## **Results**

### *Cholesterol up-regulates coactivator gene and protein, consequently regulating AR in the PC-3 cell line*

The qPCR data showed that cholesterol-supplemented cells displayed upregulated SRC-1 (Fig. 1A;  $p = 0.020$ ), SRC-2 (Fig. 1B;  $p = 0.0002$ ), gene expression compared to the control group, but did not affect SRC-3 (Fig. 1C;  $p = 0.119$ ) and PCAF (Fig. 1D;  $p = 0.874$ )

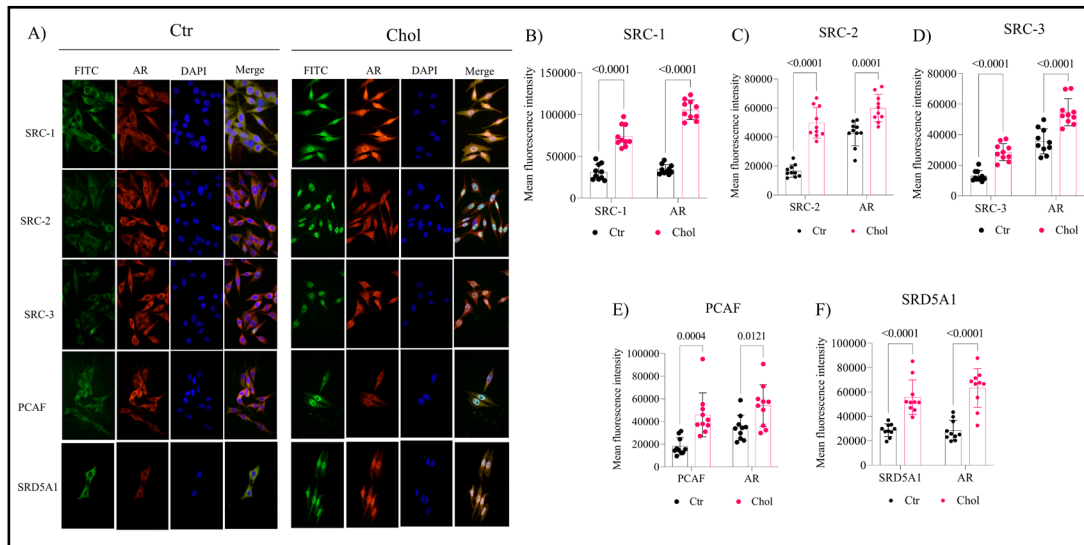


**Fig. 1.** Effect of supplementation of PC-3 cells with cholesterol (Chol) (2 $\mu$ g/mL) for 8h on the expression of AR coactivator genes. A) Expression levels of SRC-1 genes in the absence and presence of Chol. B) Expression levels of SRC-2 gene in the absence and presence of Chol. C) SRC-3 gene expression levels in the absence and presence of Chol. D) PCAF gene expression levels in the absence and presence of Chol. E) Expression levels of the AR gene in the absence and presence of Chol. The p-values obtained from the statistical analyses are shown above the bars in each panel, and the error bar corresponds to the standard deviation of the samples. T-test was used in all analyses.

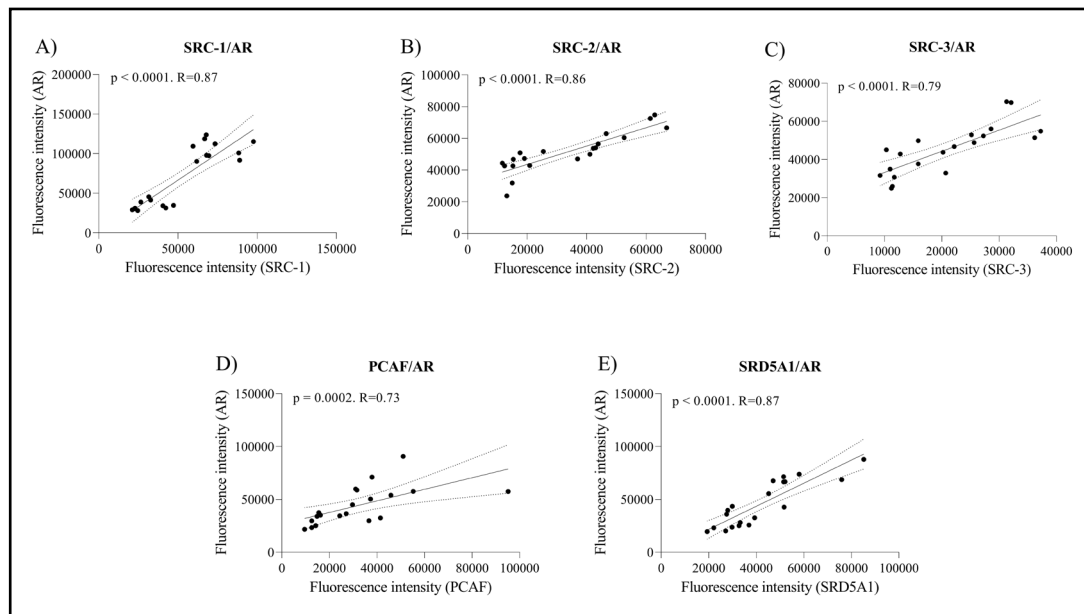
expression levels. The cholesterol-supplemented cells also exhibited significantly higher AR gene expression levels than the control group (Fig. 1E;  $p = 0.044$ ). It is worth mentioning that these gene expression alterations were not observed in LNCaP cells following cholesterol supplementation (2  $\mu$ g for 8 hours) (Supplementary Fig. 2).

As shown in Fig. 2A, cholesterol-supplemented cells display upregulated protein expression of the p160 family coactivators SRC-1 (Fig. 2B;  $p < 0.0001$ ), SRC-2 (Fig. 2C;  $p < 0.0001$ ), SRC-3 (Fig. 2D;  $p < 0.0001$ ), PCAF (Fig. 2E,  $p = 0.0004$ ), and increased levels of AR after increased expression of coactivator proteins and cholesterol supplementation, followed by cytoplasmic-nucleus translocation. Subsequently, we investigated whether there was an increase in the androgen response element genes after increasing AR protein levels. We found that the SRD5A1 (Fig. 2F;  $< 0.0001$ ) protein, which possesses this response element, was increased in the cholesterol-supplemented cells, where the increase in AR is observed.

Subsequently, we investigated whether the increase in the coactivator proteins p160, PCAF and SRD5A1 triggered by cholesterol supplementation showed correlations with AR expression. We found that all proteins showed strong significant positive correlations, being SRC-1 ( $R = 0.87$  and  $p < 0.0001$ , Fig. 3A), SRC-2 ( $R = 0.86$  and  $p < 0.0001$ , Fig. 3B), SRC-3 ( $R = 0.79$  and  $p < 0.0001$ , Fig. 3C), PCAF ( $R = 0.73$  and  $p = 0.0002$ , Fig. 3D) and SRD5A1 ( $R = 0.87$  and  $p < 0.0001$ , Fig. 3E).



**Fig. 2.** Effect of supplementation of PC-3 cells with cholesterol (2µg/mL) for 8h on the expression of AR coactivator proteins by immunofluorescence (IF). A) Representative IF images of the control and Chol groups. We observe a higher concentration of the proteins in the cytoplasm in the control group. After Chol supplementation, a migration of the coactivator proteins to the cell nucleus is observed, consequently increasing AR expression. B) Expression levels of SRC-1 and AR protein in the absence and presence of cholesterol. C) Expression levels of the SRC-2 and AR protein in the absence and presence of Chol. D) Expression levels of the SRC-3 and AR protein in the absence and presence of Chol. E) Levels of PCAF and AR protein expression in the absence and the presence of Chol. F) SRD5A1 and AR protein expression levels in the absence and presence of Chol. The p-values obtained from the statistical analyses are shown above the bars in each panel, and the error bar corresponds to the standard deviation of the samples. The T-test was used in all analyses.

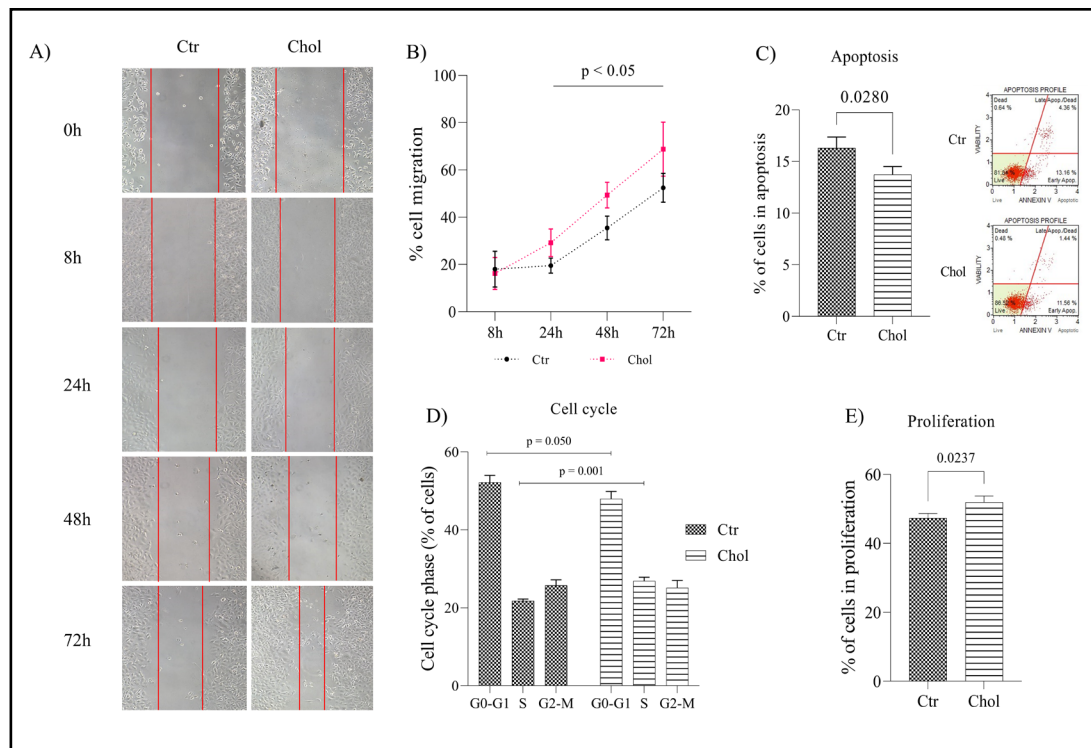


**Fig. 3.** Correlation of the protein expression of the coactivators p160, PCAF and the androgen-responsive element gene, SRD5A1, with the protein levels of AR. We showed strong positive correlations between the protein levels of the cofactors and AR post cholesterol supplementation. A) SRC-1 (R= 0.87 and  $p < 0.0001$ ). B) SRC-2 (R= 0.86 and  $p < 0.0001$ ). C) SRC-3 (R= 0.79 and  $p < 0.0001$ ). D) PCAF (R= 0.73 and  $p = 0.0002$ ). E) SRD5A1 (R= 0.87 and  $p < 0.0001$ ). The p-values obtained from the statistical analyses are shown above in each panel. The Pearson correlation test was used in all analyses.

*Cholesterol supplementation attenuates apoptosis and increases cell proliferation and migration in castration-resistant prostate cancer cells*

Exposing PC-3 cells to 2 $\mu$ g of cholesterol for 24h did not result in any significant difference in cell viability rate compared to control cells (Supplementary Fig. 3). Cell migration assay indicated that cholesterol-supplemented increased PC-3 cell migration after 24, 48 and 72 hours compared to control cells (Fig. 4A-B;  $p = 0.011$ ,  $p = 0.002$  and  $p = 0.022$ , respectively). Cholesterol-supplemented cells also displayed a lower apoptosis rate than the control group (Fig. 4C;  $p = 0.028$ ).

Concerning the cell cycle, we observed a lower percentage of cholesterol-supplemented PC-3 cells in the G0-G1 phase and a higher rate of cells in the S phase than in the control group (Fig. 4D;  $p = 0.050$  and  $p = 0.001$ , respectively). In contrast, there was no difference in the percentage of cells in the G2-M phase when comparing the two groups (Fig. 4D;  $p = 0.685$ ). The percentage of cells in the G2-M and S phases of the cell cycle indicate that the cell proliferation rate of the cholesterol-treated cells was significantly higher than the control group (Fig. 4E;  $p = 0.023$ ).



**Fig. 4.** Influence of cholesterol (Chol) in cell migration and flow cytometry assays with PC-3 cells. A) Representative images of the cell migration assay using unsupplemented control (Ctr) and Chol-supplemented cells at 0, 8, 24, 48 and 72 hours. B) Graphical representation between the increase in cell migration with time progression, T-test. C) Percent of apoptotic Ctr and Chol supplemented cells, T-test. D) Percent of Ctr and Chol supplemented cells in each cell cycle phase, T-test. E) Percent of proliferating Ctr and Chol-supplemented cells. Cell proliferation is based on the percentages of cells in the S and G2-M phases of the cell cycle, T-test. PC-3 cells were supplemented with 2 $\mu$ g/mL of Chol for 24 hours. The p values obtained from the statistical analyses are displayed above the bars in each panel, and the error bar corresponds to the standard deviation of the samples.

## *Diet-induced hypercholesterolemia contributes to castration-resistant prostate cancer progression in NOD/SCID mice*

We employed a subcutaneous model of CRPC in male NOD/SCID mice to evaluate the effect of hypercholesterolemia on PCa (Fig. 5A). The animals were divided into two groups: the experimental group receiving a hypercholesterolemic diet (HCOL, N = 11) and the control group receiving a standard chow diet (Ctr, N = 11). The weights of the animals were measured every two days, and no significant difference was detected between the two groups (Supplementary Table 3;  $p > 0.05$ ). The tumors were palpable 7-14 days after the PC-3 cell inoculation, and the tumor volume was measured every other day until the end of the experimental period. The HCOL group presented significantly larger tumor volumes throughout the experimental period (Supplementary Table 4;  $p < 0.01$ ). At the end of the experimental period, the HCOL group's tumor volume was 231.82% higher than the Ctr animals.

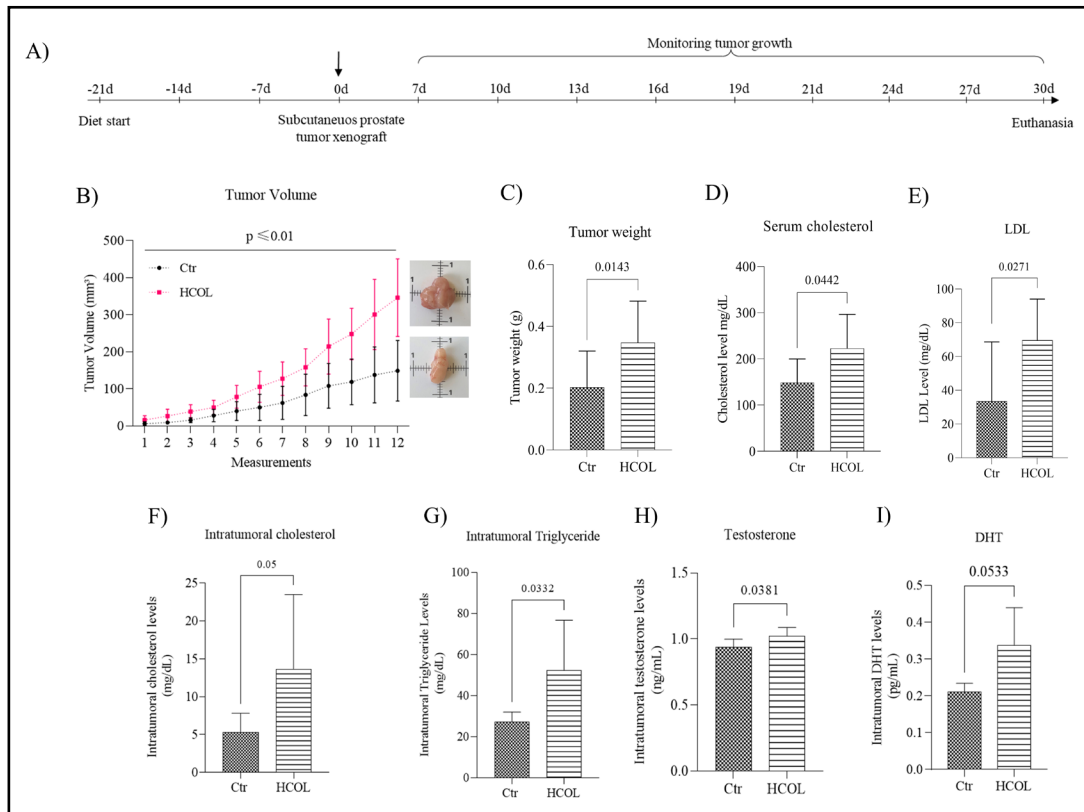
Between 24 and 30 days post-injection, HCOL and Ctr animals were euthanized, lipid profiles were performed on the serum, and the tumors were removed and weighed. Representative images of tumors from each group demonstrate that the tumors from the HCOL animals were more extensive than those from the Ctr group (Fig. 5B). Moreover, the tumors in the HCOL group were 171.78% heavier than Ctr group (Fig. 5C;  $p = 0.0143$ ). Furthermore, while the HCOL group's systemic cholesterol (Fig. 5D;  $p = 0.044$ ) and LDL (Fig. 5E;  $p = 0.027$ ) levels were significantly higher than the Ctr group, the HDL and triglyceride levels were similar (data not shown). The intratumoral cholesterol and triglyceride levels were also measured after tissue homogenization. Equivalent to the serum levels, the intratumoral cholesterol (Fig. 5F;  $p = 0.05$ ) and triglycerides (Fig. 5G;  $p = 0.033$ ) levels were elevated in the HCOL group compared to the Ctr animals. The ELISA assays demonstrated that testosterone levels in the HCOL group were significantly higher than Ctr group (Fig. 5H;  $p = 0.038$ ). There was a tendency for the DHT concentrations to be elevated in the HCOL group compared to the Ctr; however, with a marginal statistical difference (Fig. 5I;  $p = 0.053$ ).

## *Relationship between the high lipid profile and AR cofactors expression in PCa*

We evaluated the gene expression of SRC-1, SRC-2, SRC-3, PCAF, AR, and SRD5A1 in the tumors from HCOL and Ctr animals. As shown in Fig. 6A-C, the expression of p160 family genes, SRC-1, SRC-2 and SRC-3, was upregulated in the HCOL group compared to the Ctr animals ( $p = 0.027$ ,  $p = 0.036$  and  $p = 0.004$ , respectively). Moreover, we observed a 2.45-fold increase in the expression of PCAF in the HCOL group compared to the Ctr group (Fig. 6D;  $p = 0.033$ ). Interestingly, AR gene expression levels were also higher in the HCOL group when compared to the Ctr group (Fig. 6E;  $p = 0.024$ ), a result also observed in the *in vitro* experiments. Furthermore, up-regulated SRD5A1 gene expression, which is responsible for androgen conversion, was higher in the HCOL group than in the Ctr group (Fig. 6F;  $p = 0.009$ ).

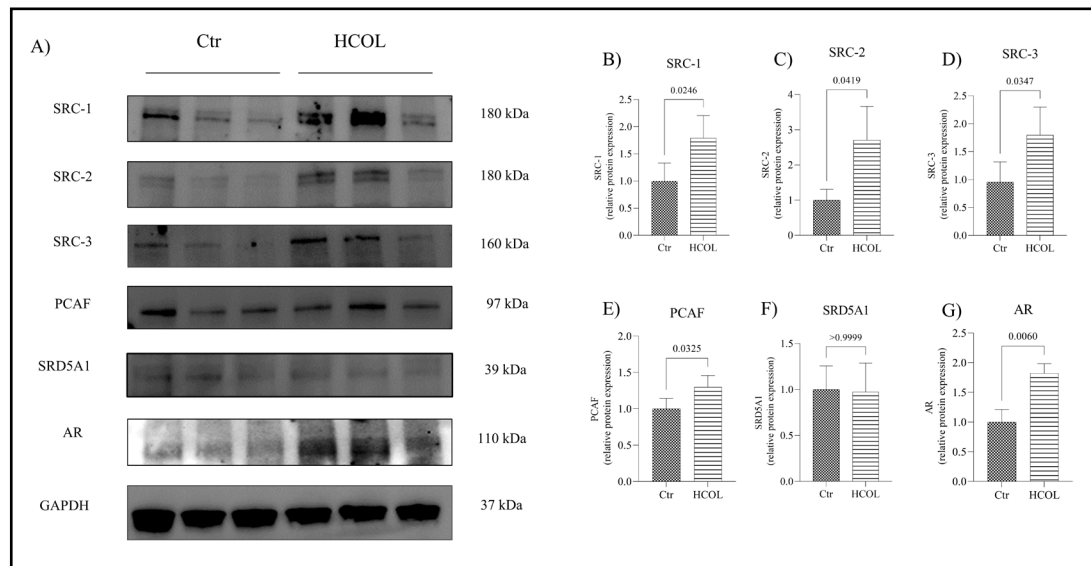
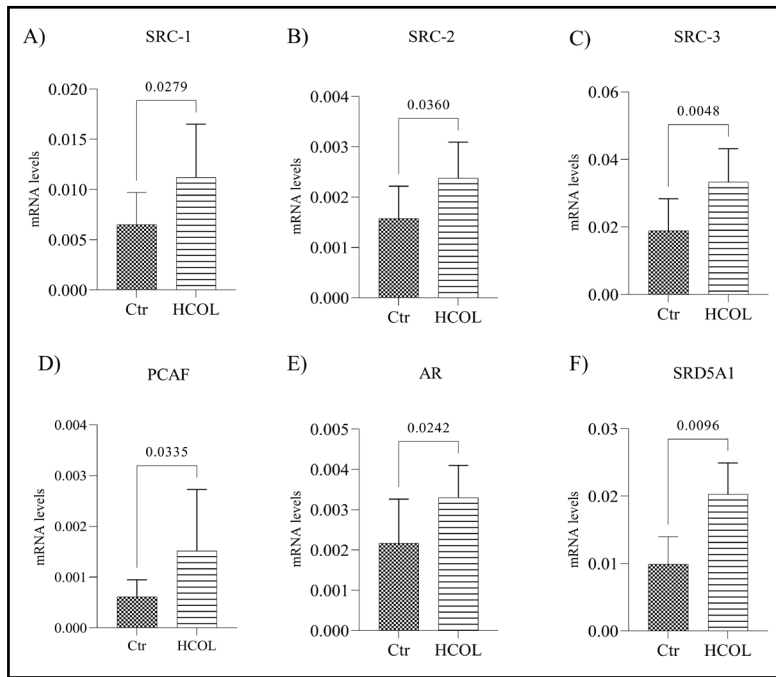
Western blot assay was employed to determine whether cholesterol would also impact the protein expression of the AR coactivators. Representative blots are presented in Fig. 7A. As shown in Fig. 7B-D, SRC-1, SRC-2 and SRC-3 protein expression was upregulated in the HCOL group when compared to the Ctr animals ( $p = 0.024$ ,  $p = 0.041$  and  $p = 0.034$ , respectively). We also detected higher PCAF (Fig. 7E;  $p = 0.032$ ) protein expression in the HCOL group than in the Ctr group. In the *in vivo* model, SRD5A1 protein expression remained unchanged when comparing the two groups (Fig. 7F;  $p > 0.999$ ). However, we did detect significantly increased AR protein levels in the HCOL group compared to Ctr animals (Fig. 7G;  $p = 0.006$ ). The full images are presented in Supplementary Fig. 4.





**Fig. 5.** Effect of cholesterol on tumor volume and serum and intratumoral lipid profile. A) Experimental plan. Twenty-one days before xenograft, male NOD / SCID mice were randomly allocated to either a control diet (Ctr, n = 11) or a hypercholesterolemic diet (HCOL, n = 11). On day 0, cells were injected into the subcutaneous (dorsum) of the animals. B) Graph shows mean tumor volume (mm<sup>3</sup>) over time, Mann-Whitney Test. Representative images of the tumors in the Ctr and HCOL groups at the end of the experimental period. C) Mean tumor weight in the Ctr and HCOL groups at the end of the experimental period, T-test. D) Mean serum cholesterol levels of the Ctr and HCOL groups at the end of the experimental period, Mann-Whitney Test. E) Average serum LDL levels of Ctr and HCOL groups at the end of the experimental period, T-test. F) T-test is the average intratumoral cholesterol levels of the Ctr and HCOL groups at the end of the experimental period. G) Average intratumoral triglyceride levels of the Ctr and HCOL groups at the end of the experimental period, Mann-Whitney Test. H) Average intratumoral testosterone levels after tissue homogenization at the end of the experimental period, Mann-Whitney Test. I) Mean intratumoral DHT levels after tissue homogenization at the end of the experimental period, T-test. The p values obtained from the statistical analyses are displayed above the bars in each panel, and the error bar corresponds to the standard deviation of the samples.

**Fig. 6.** *In vivo* effect of cholesterol on coactivators and AR gene expression. A) Expression levels of SRC-1 gene in Ctr and HCOL groups, Mann-Whitney Test. B) SRC-2 gene expression levels in Ctr and HCOL groups, Mann-Whitney Test. C) SRC-3 gene expression levels in the Ctr and HCOL groups, Mann-Whitney Test. D) PCAF gene expression levels in Ctr and HCOL groups, T-test. E) AR gene expression levels in Ctr and HCOL groups, Mann-Whitney Test. F) SRD5A1 gene expression levels in Ctr and HCOL groups, T-test. All animals were subjected to a PC-3 cell xenograft. The p-values obtained from the statistical analyses are shown above the bars in each panel, and the error bar corresponds to the standard deviation of the samples.



**Fig. 7.** *In vivo* effect of cholesterol on the protein expression of and AR by Western blot (WB). A) Representative WB images of the Ctr and HCOL groups. We observed increased protein in the tumors of the animals receiving the HCOL diet. B) SRC-1 protein expression levels of Ctr and HCOL group, T-test. C) SRC-2 protein expression levels group Ctr and HCOL, T-test. D) expression levels of the SRC-3 protein Ctr group and HCOL, T-test. E) PCAF protein expression levels Ctr and HCOL group, T-test. F) SRD5A1 protein expression levels Ctr and HCOL group, Mann-Whitney test. G) AR protein expression levels Ctr and HCOL group, T-test. The p-values obtained in the statistical analyses are shown above the bars in each panel, and the error bar corresponds to the standard deviation of the samples.

## Discussion

Previous studies have shown that cholesterol plays a crucial role in the development and aggressiveness of PCa [22, 24]. Moreover, epidemiological data suggest that drugs designed to regulate cholesterol levels may prevent PCa progression but not disease incidence [25]. Consistent with our data, a previous study also demonstrated that high cholesterol diets stimulate PCa cells and tumors while low cholesterol diets slow them down [22]. Interestingly, circulating cholesterol levels have been associated with elevated intratumoral androgen levels [26]. Nevertheless, until now, no current evidence has demonstrated that cholesterol can modulate AR coactivators *in vitro* and *in vivo* models of CRPC.

According to our results, in the *in vitro* assays, cells exposed to cholesterol exhibited increased cell proliferation and migration in 24, 48, and 72h and attenuated apoptosis rate. These observations are consistent with other studies [22, 27] and indicate that cholesterol increases the proliferation and survival rate of neoplastic cells leading to PCa progression. Moon et al. observed a positive association between increased systemic cholesterol levels and larger prostate tumors in NOD/SCID mice [22]. These findings follow the results reported herein. Moreover, it has been shown that PCa tumor cells could produce testosterone and DHT from different precursors, including cholesterol [28]. Similarly, our *in vivo* results using a CRPC model showed that the HCOL group displayed increased cholesterol, testosterone, and DHT levels at the intratumoral level.

In the PC-3 cell line, SRC-1, SRC-2, and SRC-3 gene expression were up-regulated in the cholesterol-supplemented cells. Previously, elevated SRC-1 gene expression levels were correlated with increased cell proliferation and migration [29]. Indeed, our results indicate that such events occur *in vitro* and *in vivo* and directly impact PCa progression and tumorigenesis. These results are not entirely surprising, given that increased SRC-2 levels stimulate cell growth in both androgen-sensitive and androgen-resistant environments [30], and SRC-2 knockout mice present attenuated cytoplasmic lipid levels in their LNCaP and PC-3 cells [14]. Herein, supplementing PC-3 cells with cholesterol produces higher cytoplasmic cholesterol concentrations and increases SRC-2 gene and protein expression.

*In vitro*, we observed increased SRC-3 protein expression and reduced apoptosis in cholesterol-supplemented cells, an observation previously reported [31]. Moreover, it has been shown that SRC-3 plays a crucial role in adipogenesis [32], especially in androgen-dependent cells, and is essential for PCa cell proliferation [12]. Thus, it is plausible that the observed increase in SRC-3 protein expression in the *in vivo* model (HCOL) is associated with the increased intratumoral lipid profile, which may contribute to larger tumor volumes.

In cells supplemented with cholesterol, significantly increased levels of PCAF protein were detected. In PCa cells, PCAF has been associated with CRPC cell growth [33], AR acetylation [34], and AR activation [35]. Our results demonstrating increased PCAF expression after cholesterol supplementation are correlated with CRPC progression, culminating in increased cell proliferation *in vitro* and tumor volume in the HCOL group is consistent with previous work [36].

It is known that 5-alpha-reductase is responsible for converting testosterone to DHT. This enzyme has two isoforms, SRD5A1 and SRD5A2, expressed in the prostate [37, 38]. It has been shown that progression to CRPC is accompanied by increased expression of these two isoenzymes [38]. In our *in vivo* model, the isoform SRD5A1 was up-regulated in the HCOL group; however, SRD5A1 protein expression remained unchanged.

There is some debate in the literature about the AR gene and protein expression in the PC-3 cell line [39, 40]. For example, it has been reported that this cell line is AR negative, while our group showed that PC-3 cells express this receptor at low levels [41], which is consistent with Culig et al. [42], Sica et al. [43], and Alimirah et al. [44]. Interestingly, after cholesterol application, AR expression levels in PC-3 cells increased significantly both *in vitro* and *in vivo*. Thus, we demonstrate that high cholesterol levels could increase intratumoral androgen signaling, which is consistent with the literature [23].

At the level of a high cholesterol environment, we infer that the molecular pathway of progression of the LNCaP cell line via AR is partially or wholly different from the progression pathway of the PC-3 cell line. We suggest that the PC-3 cell line uses the cofactor and de novo lipogenesis pathways for its progression. Thus, although at low levels of AR, this is sufficient to trigger tumor progression through an autocrine loop. Furthermore, we believe that cholesterol is an initial effector of a cascade of events leading to tumor progression and that DHT, as the final metabolite, either through canonical or “backdoor” [45] pathways, contributes to the successful advancement of CRPC. These events seem to be linked to mechanisms that we infer positively regulate the cofactors of AR; however, mechanistic studies should be performed to understand this association deeply. The literature has shown that cholesterol can positively impact other proteins, such as CAV1 and IQGAP1 [22]. *In silico* analysis in TCGA database displays that IQGAP1 a positive correlation with the coactivators of AR (NCOA1, NCOA2, NCOA3 and PCAF [KAT2B]) ( $R = 0.52; 0.55; 0.67$  and  $0.60$ , respectively,  $p < 0.05$ ), these data contribute to the knowledge of the pathophysiology of PCa.

Overall, our results show that cholesterol increases AR coactivators SRC-1, SRC-2, SRC-3, PCAF gene and protein expression. In control cells, SRC-1, SRC-2, SRC-3, and PCAF proteins were mainly localized to the cytoplasm. However, higher concentrations of these proteins were detected in the nucleus co-associated with AR protein following cholesterol-supplemented, possibly increasing nuclear activity. Our study does not present functional results directly linking the action of AR coactivators to cholesterol or even AR. Still, they reinforce the role of cholesterol in the progression of the CRPC phenotype. These events directly affect cellular processes such as apoptosis, proliferation, cell cycle progression, and possibly PCa cell metabolism. We postulate that the resistance pathway cross-talk with the coactivators may be modulated under these conditions. Therefore, molecules that attenuate or block AR coactivators could potentially be employed as a molecular-target treatment strategy for the CRPC phenotype.

## Conclusion

We conclude that cholesterol modulates the expression of AR cofactors. Up-regulated SRC-1, SRC-2, SRC-3, PCAF gene and protein expression coincide with increased AR gene and protein expression, which could significantly affect the onset and/or progression of prostate tumorigenesis. Future studies focusing on cholesterol-mediated modulation of AR cofactor expression are required for understanding the molecular mechanisms underlying CRPC.

## Abbreviations

AR (Androgen Receptor); cDNA (complementary DNA); Chol (Cholesterol); CRPC (Castration-Resistant Prostate Cancer); Ctr (Control); DHT (Dihydrotestosterone); ELISA (Enzyme-Linked Immunosorbent Assay); FBS (Fetal Bovine Serum); GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase); HCOL (Hypercholesterolemic); HDL (High-Density Lipoproteins); LDL (Low-Density Lipoproteins); PBS (Phosphate-Buffered Saline); PCa (Prostate Cancer); PCAF (p300/CBP-associated factor); SRC-1 (NCOA1) (Nuclear Receptor Coactivator 1); SRC-2 (NCOA2) (Nuclear Receptor Coactivator 2); SRC-3 (NCOA3) (Nuclear Receptor Coactivator 3); SRD5A1 (Steroid 5-Alpha-Reductase 1); STR (Short Tandem Repeat).

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### *Author Contributions*

Pimenta R: Conceptualization, formal analysis, writing-original draft, writing-review and editing. Camargo J A: Methodology. Candido P: Methodology. Ghazarian V: Methodology. Gonçalves G L: Methodology. Guimarães V R: Methodology. Romão P: Methodology. Chiovatto C: Methodology. Mioshi C M: Methodology. Santos G A: Methodology. Silva I A: Methodology. Leite K R: Supervision, writing-review. Birbair A: Writing-review and editing. Srougi M: Conceptualization, writing-review. Nahas W: Conceptualization, writing-review. Viana N I: Supervision, methodology. Reis S T: Resources, supervision funding acquisition, methodology, writing-review and editing.

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### *Statement of Ethics*

This study was submitted and approved by the Research Ethics Committee of the University of Sao Paulo Medical School under the number #1119/2018.

### **Disclosure Statement**

The authors declare that no conflict of interests exists.

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