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Androgens and low density lipoprotein-cholesterol interplay in modulating prostate cancer cell fate and metabolism

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ABSTRACT

Background: Androgens, the known drivers of prostate cancer (PCa), have been indicated as important metabolic regulators with a relevant role in stimulating lipid metabolism. Also, the relationship between obesity and the aggressiveness of PCa has been established. However, it is unknown if the androgenic hormonal environment may alter the response of PCa cells to lipid availability.

Purpose: The present study evaluated the effect of 5α-dihydrotestosterone (DHT) in regulating lipid metabolism, and the interplay between this hormone and low-density lipoprotein (LDL)-cholesterol in modulating PCa cells fate.

Methods: Non-neoplastic and neoplastic PCa cells were treated with 10 nM DHT, and the expression of fatty acids transporter, fatty acid synthase (FASN), and carnitine palmitoyltransferase 1A (CPT1A) evaluated. PCa cells were also exposed to LDL (100 μg/ml) in the presence or absence of DHT.

Results: Treatment with DHT upregulated the expression of FASN and CPT1A in androgen-sensitive PCa cells. In contrast, LDL supplementation suppressed FASN expression regardless of the presence of DHT, whereas augmenting CPT1A levels. Our results also showed that LDL-cholesterol increased PCa cells viability, proliferation, and migration dependently on the presence of DHT. Moreover, LDL and DHT synergistically enhanced the accumulation of lipid droplets in PCa cells.

Conclusions: The obtained results show that androgens deregulate lipid metabolism and enhance the effects of LDL increasing PCa cells viability, proliferation and migration. The present findings support clinical data linking obesity with PCa and first implicate androgens in this relationship. Also, they sustain the application of pharmacological approaches targeting cholesterol availability and androgens signaling simultaneously.

1. Introduction

Androgens are widely recognized as key stimulators of prostate cancer (PCa) cell survival and growth [1–3], exerting their actions through binding the androgen receptor (AR), a member of the steroid hormone receptor subfamily of ligand-activated nuclear transcription factors [4–6].

In the last years, other actions apart from the control of cell survival and growth have been assigned to androgens. We and others have described that androgens have an important role as metabolic regulators in PCa, modulating glycolysis, nucleotide and amino acid metabolism and lipid handling [5,7–9]. Indeed, lipid metabolism, which is crucial for energy production and membrane synthesis, is one of the main targets of the metabolic actions of androgens in PCa cells [10–13]. Androgens have been shown to stimulate lipid uptake, synthesis, storage and lipolysis from lipid droplets by interacting with the transcriptional machinery and thus regulating the gene expression network [14–16]. Nevertheless, several molecular partners involved in lipid handling remain to be identified as androgens targets.

On the other hand, clinical and epidemiological studies have established the relationship between obesity and PCa. Overall, obese PCa patients develop aggressive forms of the disease displaying accelerated

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progression to treatment resistance, poor prognosis and reduced survival rates [17–21]. Also, studies in animal models showed that diet-induced hypercholesterolemia promotes the development of PCa metastasis [22,23]. However, the direct influence of exogenous low-density lipoprotein (LDL) supplementation in modifying PCa cell survival and proliferative activity remains less clear. Moreover, the presence of an androgenic hormone milieu driving PCa and the role of androgens as lipid metabolism regulators raise the curiosity about the possible interplay between these steroid hormones and LDL-cholesterol actions, namely if androgens may influence PCa cell's response to LDL-cholesterol.

The present study aims to elucidate further the role of androgens in regulating lipid metabolism and determine the effect of 5α-dihydrotestosterone (DHT) shaping the response of PCa cells to LDL-cholesterol. Cell viability, proliferation and migration, and the expression of target regulators of lipid metabolism and lipid content in PCa cells under different LDL-cholesterol availability, with or without DHT, were assessed.

2. Materials and methods

2.1. Chemicals

All chemicals, culture media, and antibodies unless otherwise stated were purchased from Sigma–Aldrich (St Louis, MO, USA).

2.2. Cell lines and treatments

Human prostate cell lines (PNT1A, LNCaP, DU145, and PC3) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PNT1A are non-tumorigenic prostate epithelial cells. LNCaP cells, originated from a PCa lymph node metastasis, express the AR and are an androgen-sensitive model. The 22Rv1 cell line is derived from a xenograft that was serially propagated in mice after castration-induced regression, express the AR and are responsive to androgens [24]. DU145 and PC3 cell lines have origin in brain and bone metastasis of an undifferentiated grade IV prostate adenocarcinomas, and are considered to be non-sensitive to androgens [25,26]. In all experiments were used cells with passage number below.

PNT1A, LNCaP, DU145, and PC3 cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/ streptomycin at 37 ºC in an atmosphere equilibrated with 5 % CO_2. At 60 % confluence, culture medium was replaced by phenol red-free RPMI1640 medium (R8755) containing 1 % lipid-depleted FBS (LD-FBS, Biowest, Riverside, MO, USA) or 5 % charcoal-stripped FBS depending on cells will be treated with LDL or DHT. Cells were maintained for additional 24 h and, then, exposed to 10 nM DHT or vehicle for 12, 24, 48 h [7,27]. Experimental DHT concentrations used in experiments in human PCa cells have been shown to range from 10 to 100 nM, for example [7,27–31]. Therefore, the relatively low 10 nM concentration was selected.

Alternatively, PCa cells were exposed to LDL (100 μg/ml, Merck, Darmstadt, Germany) in the presence or absence of DHT. This LDL concentration was selected as it was previously reported to induce physiological responses in human cancer cells [32,33]. LDL-exposure experiments were repeated in LNCaP cells in the presence of 40 μM etomoxir (inhibitor of fatty acid oxidation) [34–37], or human anti-LDL receptor (LDLR) antibody (5 μg/ml, AF2148, R&D Systems, Minneapolis, MN, USA) [38] for 24 h and 48 h. Antibody was added to culture medium 1 h before LDL-stimulation.

DHT-treated LNCaP cells in LD-FBS conditions were simultaneously cultured with 20 μM fatostatin, an effective concentration inhibiting the activity of the transcription factor sterol regulatory element-binding protein-1 (SREBP-1) [39–43]. At different experimental conditions, cells were used for cell viability and migration assays, Ki-67 immunocytochemistry, and determination of ATP, and fatty acids and lipid content, or, alternatively, harvested for RNA and protein extraction.

2.3. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from LNCaP and 22RV1 cells using the TRI Reagent according to the manufacturer's instructions. The quantity and quality of total RNA was assessed by spectrophotometry at 260 and 280 nm (NanoPhotometer, Implen, München, Germany) and agarose gel electrophoresis, respectively. cDNA was synthetized from 1 μg of total RNA using the First Strand cDNA Synthesis Kit (NZYtech, Lisboa, Portugal). 1 μL of synthesized DNA was used to determine the expression of CD36 (5´–3´, sense: TCTGTCCTATTGGGAAAGTCACTG, antisense: GAACTGCAATACCTGGCTTTTCTC), fatty acid synthase (FASN; 5´–3´ sense: ACCGCTTCCGAGATTCCATC, antisense: ATGGCAGTCAGGCTCACAAA) and carnitine palmitoyltransferase 1A (CPT1A; 5´–3´, sense: TGGATCTGCTGTATATCCTTC, antisense: AATTGGTTTGATTTCCTCCC). The β-2-microglobulin (5´–3´, sense: ATGAGTATGCCTGCCGTGTG; antisense: CAAACCTCCATGATGCTGCTTAC) was used as internal control to normalize gene expression. qPCRs were carried out in the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA (1; 1:3; 1:9). The specificity of the amplicons was determined by melting curve analysis. qPCR reactions were carried out in a 20 μL reaction containing 10 μL iTaq Universal SYBRGreen Supermix (Bio-Rad), 300 nM (CD36, FASN, CPT1A) or 200 nM (β-2-microglobulin) of sense and antisense primers for each gene and 1 μL of cDNA. Samples were run in triplicate in each qPCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ [44].

2.4. Western blot (WB) analysis

Human prostate cells were homogenized in the appropriate volume of radioimmunoprecipitation assay buffer (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail and 10 % PMSF, kept on ice for 20 min with occasional mixing, and then centrifuged at 14,000 rpm for 20 min at 4 °C. Protein concentration was determined using the Pierce™ BCA Protein Assay Ki(Thermo Scientific). Twenty-five microgram of total proteins were resolved by SDS-PAGE on 7.5 or 10 % gels and electrotransferred to a PVDF membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with rabbit anti-CD36 (1:400, ab64014; Abcam, Cambridge, United Kingdom), rabbit anti-FASN (1:1000, no.3180; Cell Signaling Technology, Danvers, MA, USA), mouse anti-CPT1A (1:1000, ab128568; Abcam), rabbit anti-AKT (1:1000, no.9272; Cell Signaling Technology), rabbit anti-phospho-AKT (1:1000, no.9271; Cell Signaling Technology), rabbit anti-p44/42 MAPK (Erk1/2) (1:1000, no.9102; Cell Signaling Technology), rabbit anti-phospho-p44/42 MAPK (Erk1/2) (1:1000, no.9101; Cell Signaling Technology), rabbit anti-phospho-c-Myc (1:1000, no.13748; Cell Signaling Technology), or mouse anti-E-cadherin (1:1000, sc-8426, Santa Cruz Biotecnhnology, Heidelberg, Germany) primary antibodies. Thereafter, membranes were washed and incubated for 1 h at room temperature with anti-rabbit IgG, HRP-linked (1:20,000, no.7074; Cell Signaling Technology) or anti-mouse-IgGκ HRP-linked (1:20,000, sc-516102, Santa Cruz Biotecnhnology) secondary antibodies, according to the species of the primary antibodies. Protein expression was normalized using a mouse anti-β-actin (1:1000, A5441) antibody. Membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and immunoreactive proteins were visualized with the ChemiDoc™ MP System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab software (Bio-Rad) and normalized by division with the respective β-actin band density.

2.5. Cell viability assay

LNCaP (20,000 cells/well), 22RV1 (15,000 cells/well) and PC3 (5000 cells/well) cells were grown in 96-well plates, and cell viability was determined by the colorimetric MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. After LDL, DHT and/or inhibitors stimulation, culture medium was removed and cells incubated with MTT at a final concentration of 0,5 mg/ml, in the dark for 4 h at 37 ºC. After incubation, MTT solution was carefully removed, and the formed formazan crystals were solubilized with 100 μL DMSO. The absorbance of the resultant purple coloured solution was measured at 570 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The value of absorbance is directly proportional to the number of viable cells in each experimental group.

2.6. Ki-67 immunocytochemistry

LNCaP (2.5×10^5 cells/well) and 22RV1 (2.0×10^5 cells/well) cells were fixed with 4 % paraformaldehyde (PFA) and permeabilized with 1 % Triton X-100 for 5 min at room temperature. Nonspecific staining was blocked by incubation with phosphate buffer saline (PBS) containing 0.1 % (w/v) Tween-20 (PBS-T) and 20 % FBS for 1 h. After blocking, cells were washed and then incubated with the rabbit monoclonal anti-Ki-67 antibody (1:50, ab16667, Abcam) for 1 h at room temperature. Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, Darmstadt) was used as secondary antibody. Specificity of staining was assessed by omission of the primary antibody. Cell nuclei were stained with Hoechst 33342 (5 μg/ml, Invitrogen) for 10 min. After washing with PBS-T, slides were mounted in fluorescence mounting medium (Dako, Glostrup, Denmark). Images were acquired using the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany). Proliferation was determined by the percentage of Ki-67-positive cells out of the total number of Hoechst stained nuclei in 10 randomly selected × 40 magnification fields for each section.

2.7. Migration assay

Cell migration assay was performed using 8 μM pore size inserts (35224, SPL, Life Sciences, Naechon-Myeon Pocheon, South Korea). Briefly, LNCaP cells (3.0×10^5 cells/well), in the presence or absence of LDL and DHT, were placed into the upper chambers containing serum-free media. The lower chambers contained 20 % LD-FBS media. After 24 h, cells on the lower surface of the membrane were fixed and stained with haematoxylin. Cells were then counted in 10, randomly selected, × 40 magnification fields in each membrane.

2.8. Oil red O assay

LNCaP cells (5.0×10^5 cells/well) seeded in 12-well plates and exposed to 100 μg/ml LDL and 10 nM DHT for 24 h, were then fixed with 4 % PFA for 30 min. Cells were washed twice with distilled water and rinsed with 60 % isopropanol for 5 min. After washing, cells were stained with Oil Red O for 20 min. Representative microscope images were acquired. Oil Red O (lipid content) quantification was carried out by diluting dye with 100 % isopropanol for 5 min with gentle agitation. Absorbance was measured using xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad) at 492 nm.

2.9. Quantification of free fatty acids

Free fatty acids content in LNCaP cells exposed to 100 μg/ml LDL and 10 nM DHT for 24 h was determined by spectrophotometric analysis using a commercial kit (#MAK044) according to the manufacturer's instructions.

2.10. ATP quantification

ATP measurement in LNCaP cells treated with 100 μg/ml LDL and 10 nM DHT for 24 h was performed using the ATP assay Kit (MAK190) following the manufacturer's instructions.

2.11. Statistical analysis

The statistical significance of differences between experimental groups was evaluated by unpaired t -test with Welch's correction or one-way ANOVA followed by Tukey post-hoc test, as justified and after required assumptions were confirmed. Analyses were performed using the GraphPad Prism v6.00 (GraphPad Software, Inc., La Jolla, CA, USA) software. $P < 0.05$ was considered statistically significant. All experimental data are shown as mean ± standard deviation (S.D).

3. Results

3.1. Fatty acid metabolism regulators are differentially expressed in PCa cells and regulated by androgens

The expression of fatty acid metabolism regulatory proteins (Fig. 1 A) in neoplastic and non-neoplastic prostate cell lines was evaluated by WB analysis (Fig. 1B). The expression of the fatty acid transporter, CD36, was significantly lower in DU145 and PC3 cells compared to the non-neoplastic PNT1A cells (0.66 ± 0.04 and 0.53 ± 0.07 fold-change, respectively, Fig. 1B), as well as in PC3 cells relative to LNCaP cells (Fig. 1b). On the other hand, the expression of FASN (Fig. 1 A), the crucial enzyme in fatty acid synthesis, was significantly higher in all PCa cells relative to PNT1A (2.76 ± 0.10, 1.43 ± 0.13 and 2.15 ± 0.11 fold-change for LNCaP, DU145 and PC3 cells, respectively, Fig. 1B). Amongst the PCa cell lines, DU145 cells displayed significantly lower expression of FASN when compared to LNCaP or PC3 (Fig. 1B); and LNCaP cells showed the highest FASN expression levels (Fig. 1B).

The expression of CPT1A (Fig. 1A), a rate-limiting component in the carnitine-dependent transport of fatty acids across the inner mitochondrial membrane, was significantly higher in LNCaP and PC3 cells (2.39 ± 0.17 and 3.22 ± 0.31 fold-change relative to PNT1A, respectively, Fig. 1B); DU145 cells displayed the lowest CPT1A expression among all PCa cell lines (0.69 ± 0.07 fold-change relative to PNT1A cells, Fig. 1B).

In this work, we evaluated the effect of DHT (10 nM) in regulating the expression of CD36, FASN and CPT1A (Fig. 2A-F) in prostate cells. The mRNA expression of CD36 was significantly decreased in LNCaP cells treated with DHT for 24 h (0.21 ± 0.02 fold-change compared to control, Fig. 2 A), which was followed by the down-regulation of CD36 protein levels (0.72 ± 0.09, 0.63 ± 0.04 and 0.86 ± 0.02 fold-change compared to control for 12 h, 24 h and 48 h, respectively, Fig. 2B). Treatment of PTN1A cells with DHT for 24 h also significantly decreased the expression of CD36 protein (0.2955 ± 0.05 fold-change compared to non-treated cells, Fig. 2B). No effect was observed in 22RV1 (Fig. 2 A), DU145 or PC3 cells (Fig. 2B).

In contrast, DHT treatment significantly increased the FASN mRNA expression in LNCaP and 22RV1 cells (3.96 ± 0.49 and 6.70 ± 1.59 fold-change compared to control, respectively, Fig. 2 C). FASN protein levels were increased at 24 h and 48 h post-treatment in LNCaP cells (1.37 ± 0.09 and 1.27 ± 0.09 fold-change compared to control, respectively, Fig. 2D).

Regarding CPT1A, the mRNA expression was increased in 22RV1 cells in response to DHT (4.00 ± 1.04 fold-change compared to control for 24 h treatment, Fig. 2E). No significant differences were observed in the CPT1A mRNA expression in DHT-treated LNCaP cells (Fig. 2E). However, CPT1A protein expression was increased in LNCaP cells treated with DHT for 24 h (1.63 ± 0.12 fold -change compared to control, Fig. 2 F), as well as in PNT1A cells treated for 24 h or 48 h (2.44 ± 0.55 and 1.59 ± 0.20 fold-change compared to control, respectively, Fig. 2 F). As expected, DHT treatment significantly increased the protein

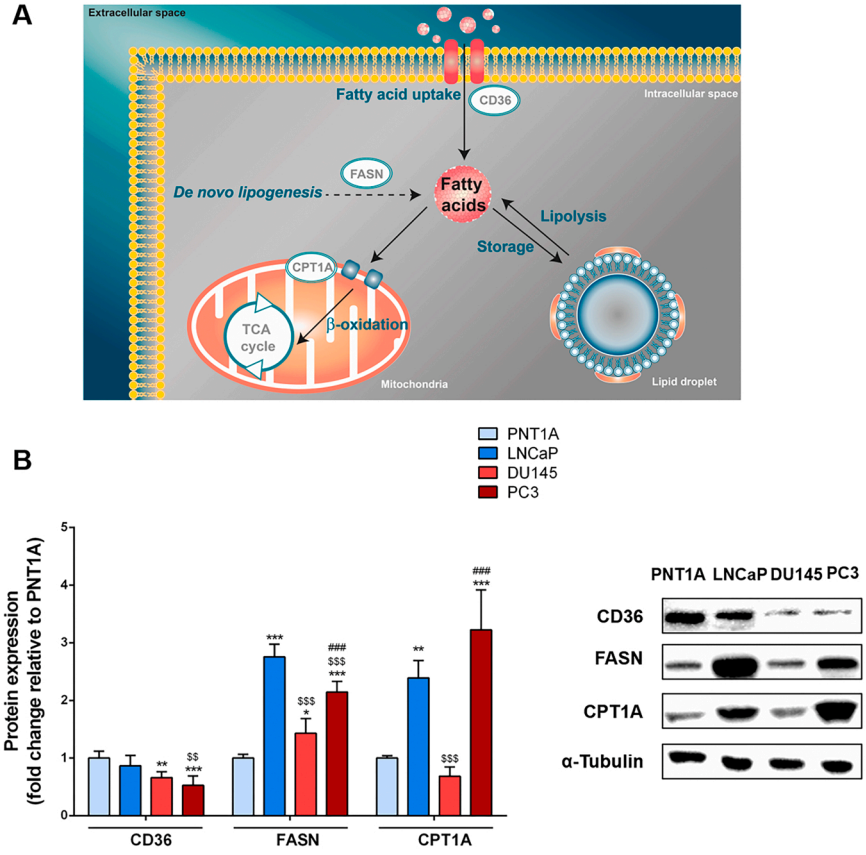

Fig. 1. Expression analysis of the lipid metabolism regulators, CD36, FASN and CPT1A, in PCa cell line models, LNCaP, DU145 and PC3 cells. (A) Fatty acid uptake occurs through the membrane transporter CD36. In the intracellular space, fatty acids can be guided to β-oxidation with the participation of CPT1A, an enzyme at the outer mitochondrial membrane that allows the transport of fatty acid into the mitochondrial matrix, which is a rate-limiting step in fatty acid β-oxidation. The products of lipid oxidation enter the tricarboxylic acid (TCA) cycle generating ATP. Alternatively, incorporated fatty acids can be stored in lipid droplets and recruited by lipolysis when more energy substrates are needed. Cancer cells also have the ability to *de novo* synthesise fatty acids dependently on FASN activity. (B) Protein expression determined by WB analysis after normalization with α-tubulin. All results are expressed as fold-change relative to the non-neoplastic PNT1A prostate cells. Error bars indicate mean ± S.D (n = 5) * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ when compared to PNT1A cells; $^{\$\$}$ $p < 0.01$; $^{\$\$\$}$ $p < 0.001$ when compared to LNCaP; $^{\#\#\#}$ $p < 0.001$ when compared to DU145 cells. Representative immunoblots of three independent experiments are shown in the right panel.

expression levels of a classical androgen target gene, the prostate-specific antigen (PSA), in the androgen-sensitive LNCaP cells (Fig. 2 G).

Overall, no effect was seen on the expression of fatty acid metabolism regulators, CD36 (Fig. 2B), FASN (Fig. 2D), and CPT1A (Fig. 2 F) in DU145 and PC3 cells in response to DHT.

3.2. LDL-cholesterol increased PCa cells viability, proliferation and migration in a DHT dependent manner

The effect of LDL-supplementation, alone or in combination with DHT, on PCa cells viability, proliferation and migration was investigated. LDL-stimulation significantly increased the viability of LNCaP, 22RV1 and PC3 cells both at 24 h and 48 h of treatment (140 ± 1 %, 167 ± 5 %, 114 ± 1, 120 ± 2 and 149 ± 7 %, 186 ± 4 % fold-change compared to LDL-untreated group, respectively, Fig. 3 A). Moreover, the viability of the androgen-sensitive LNCaP and 22RV1 cells was significantly increased by ~ 25 % and ~ 40 %, respectively, when LDL was combined with DHT (175 ± 3 % and 167 ± 3 % fold -change compared to LDL-untreated group for 24 or 48 h of treatment, respectively Fig. 3 A).

Immunofluorescent labelling of the nuclear proliferation marker Ki-67 was used to confirm whether treatment with LDL plus DHT promotes LNCaP and 22RV1 cells proliferation. Indeed, cell proliferation was significantly augmented in the LDL (+)/DHT (+) groups (2.20 ± 0.07 and 1.53 ± 0.06 fold-change compared to LDL-untreated group, respectivelly, Fig. 3B, C). The synergistic effect of LDL in combination with DHT was also observed in a cell migration trans-wells assay. LDL-supplementation significantly increased LNCaP cells migration (1.31 ± 0.06 fold-change compared to LDL-untreated group, Fig. 3D), an effect that was ~ 25% amplified in the presence of DHT (1.64 ± 0.10 fold increased mnigration- compared to LDL-untreated group, Fig. 3D). The enhanced migration of DHT-treated LNCaP cells, was accompained by the decreased expression of the epithelial mesenchymal transition (EMT) E-cadherin, independent of the presence of LDL (0.68 ± 0.07 and 0.65 ± 0.08 fold-change compared to LDL-untreated group, Fig. 4).

We also analysed wheather LDL and DHT treatment affected intracellular signalling pathways by analyzing the expression of total and phosphorylated AKT and ERK proteins (Fig. 4). WB analysis showed that total AKT, a key downstream mediator regulating cell survival and proliferation, was decreased in the LDL (+)/DHT (+)-treated LNCaP cells relative to cells non-supplemented with LDL (0.75 ± 0.04 fold -change, Fig. 4). However, p-AKT levels were only significantly increased in response to DHT (2.26 ± 0.40 fold -change in LDL(-)/DHT (+) relative to LDL-untreated group, Fig. 4). The DHT effect on p-AKT levels was not observed when LDL was present (Fig. 4).

Concerning ERK the presence of LDL significantly decreased p-ERK levels (0.59 ± 0.06 fold-change compared to LDL-untreated group, Fig. 4). Both p-ERK and ERK expression decreased in the LDL(+)/DHT (+) treated group, an effect that was more pronounced for p-ERK (0.32 ± 0.03 and 0.72 ± 0.06 fold -change compared to LDL-untreated group, respectively, Fig. 4).

The oncogene c-Myc is over-expressed in PCa cells and has been indicated as a central player in the regulation of cell metabolism. Therefore, we dediced to analyse its expression in our experimental conditions. The presence of LDL did not affect c-Myc expression, whereas DHT treatment augmented its levels (2.82 ± 0.32 fold-change compared to LDL-untreated group, Fig. 4). Moreover, combination treatment with LDL and DHT, led to a further increase in c-Myc expression (3.89 ± 0.44 fold-change compared to LDL-untreated group, Fig. 4).

3.3. LDL/DHT effects are accompanied by altered fatty acid metabolism with accumulation of lipid droplets

Considering the effect of DHT in modulating the expression of lipid metabolism regulators and the synergistic effect of LDL and DHT, we

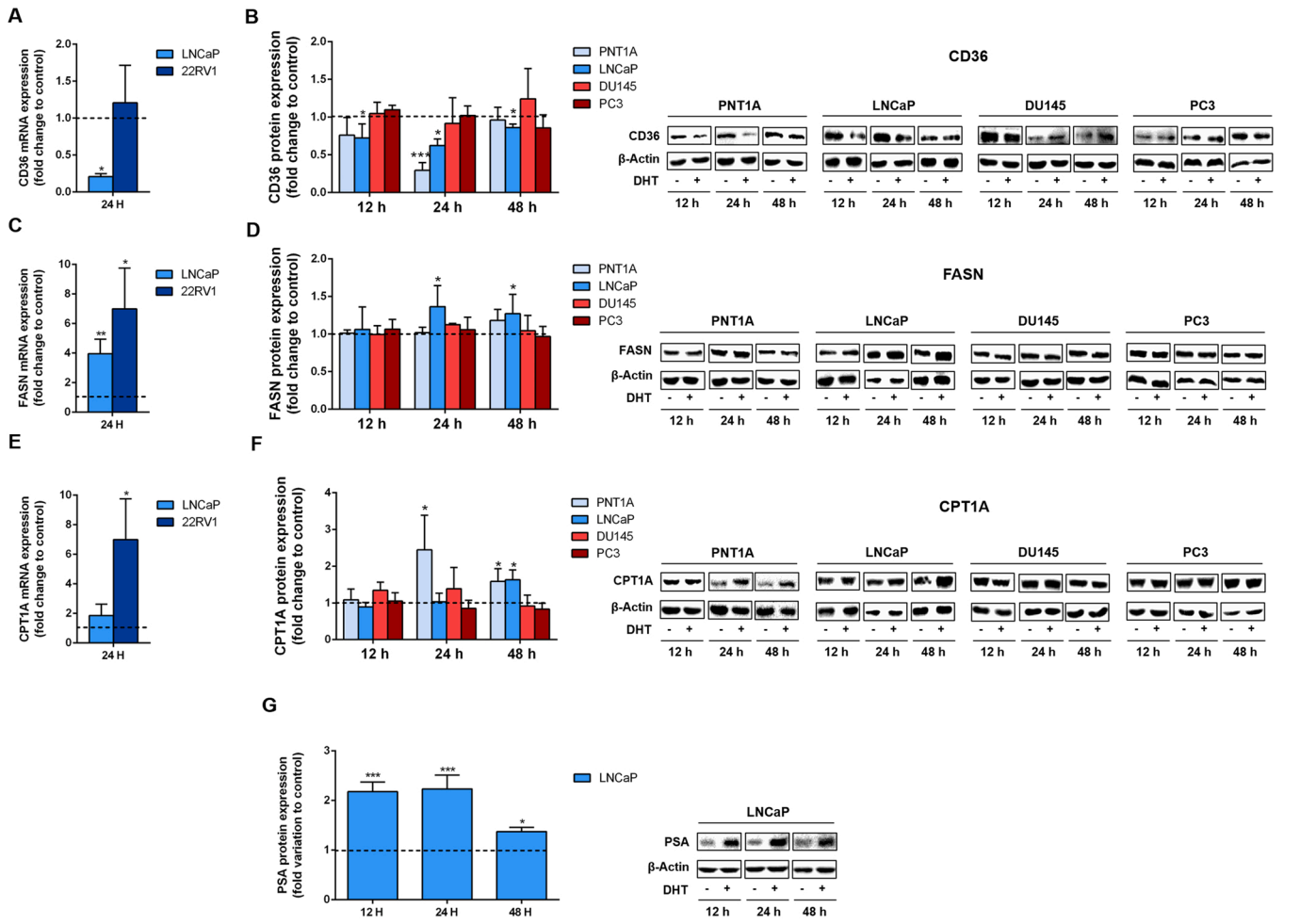

Fig. 2. Effect of DHT on the expression of CD36, FASN and CPT1A in neoplastic (LNCaP, 22RV1, DU145 and PC3) and non-neoplastic (PNT1A) prostate cells. Cells were treated with 10 nM of DHT for 12, 24, or 48 h. (A, B) CD36, (C, D) FASN, and (E, F) CPT1A mRNA and protein expression analysed by qPCR and WB after normalization with the housekeeping gene β-2-microglobulin, and β-actin, respectively. (G) PSA protein expression in LNCaP DHT-treated cells as a positive control of experiments. All results are expressed as fold-change relative to the control untreated group (0 nM DHT, dashed line). Error bars indicate mean ± S.D (n = 5) * $p < 0.05$, * ** $p < 0.001$ when compared to control. Representative immunoblots of three independent experiments are shown in the right panel.

decided to investigate whether LDL availability would affect the response to DHT and lipid handling in prostate cells. The expression of FASN and CPT1A, targeting fatty acid synthesis and oxidation, respectively, was analysed. As expected, 24 h treatment with DHT alone augmented FASN expression (1.38 ± 0.08 fold -change compared to the LDL (-) group, Fig. 5 A). LDL did not affect FASN expression levels, but its presence suppressed the DHT-stimulatory effects over FASN (Fig. 5A).

DHT did not affect CPT1A expression (Fig. 5 A). However, LDL-treated cells alone and in the presence of DHT displayed increased expression of CPT1A (LDL (+), 1.31 ± 0.02 and LDL (+)/DHT (+), 1.40 ± 0.10 fold-change compared to LDL-untreated group, Fig. 5 A).

We also evaluated if LDL availability and DHT-treatment altered the LNCaP cells capability for storing neutral lipids (Fig. 5B). The LDL (+)/DHT(+) combination enhanced the relative number of lipid droplets relative to the LDL-untreated group (1.23 ± 0.03 fold-change, Fig. 5B). The free fatty acids content in LDL- and DHT-treated LNCaP cells also was assessed; however, no significant differences were observed among treatments (Fig. 5 C).

Fatty acid β-oxidation is a source of ATP contributing to generate the cellular energy. LDL or DHT alone did not affect ATP production in LNCaP cells (Fig. 5D). However, total ATP levels were significantly increased in the LDL (+)/DHT(+) group (1.99 ± 0.10 fold-change compared to LDL-untreated group, Fig. 5D).

3.4. The transcription factor SREBP mediates the DHT actions regulating fatty acid metabolism and p-AKT levels

As shown, DHT increased LNCaP cells viability (Fig. 3 A), and the p-AKT (Fig. 4) and FASN (Fig. 3 A) protein expression in lipid-depleted conditions (LDL(-)). It is known that SREBP-1 is involved in the transcriptional activity of AR regulating FASN expression [45–47]. Next, we determined if the chemical inhibition of SREBP through a recognized inhibitor, fatostatin, inhibited the effects of DHT in lipid-depleted conditions. Exposure to fatostatin strongly diminished FASN mRNA and protein expression in LNCaP DHT-treated cells (0.77 ± 0.12 and 0.35 ± 0.07 fold-change compared to the DHT-treated groups, respectively, Fig. 6 A, B). Moreover, fatostatin markedly suppressed AKT and p-AKT expression (0.27 ± 0.10 and 0.06 ± 0.01 fold-change compared to the DHT-treated group, respectively, Fig. 6B).

3.5. Anti-LDLR antibody and etomoxir blocked the LDL-effects increasing viability of PCa cells

Finally, we tested if blocking LDL entry into the cell or fatty acid β-oxidation would reverse LDL effects enhancing PCa cells viability (Fig. 7). LNCaP cells were stimulated with LDL in the presence of an anti-LDLR antibody, which resulted in decreased viability of both LNCaP and PC3 cells (59 ± 18 % and 79 ± 2 % fold-change compared to control

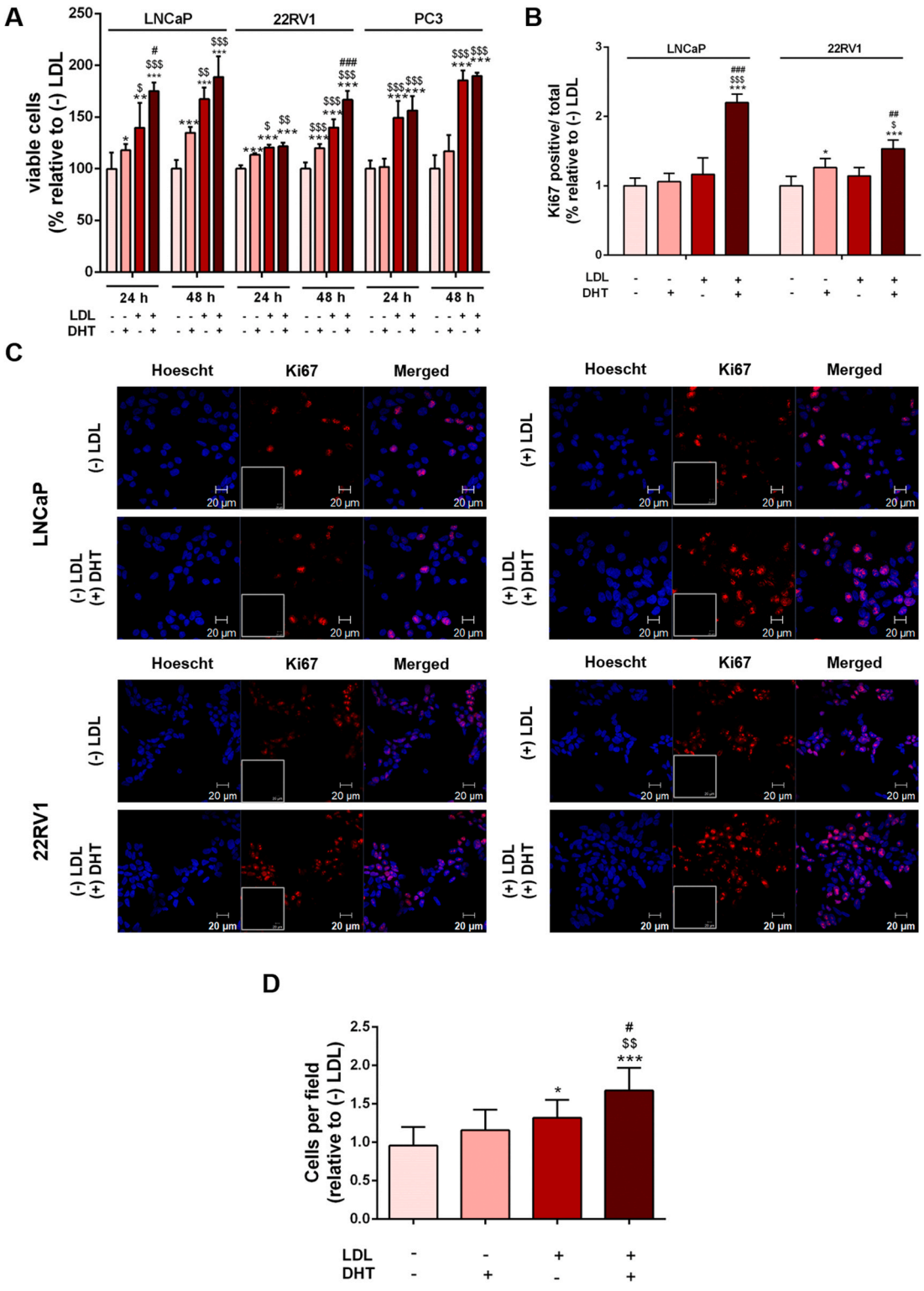

Fig. 3. Effect of LDL and DHT on PCa cells viability, proliferation and migration. LNCaP, 22RV1 and PC3 cells were treated with DHT (10 nM) and/or LDL (100 µg/ml) for 24 or 48 h. (A) Viability of LNCaP, 22RV1 and PC3 cells determined by the MTT assay. (B) Proliferation of LNCaP and 22RV1 cells determined by the immunofluorescence analysis of Ki-67 at 24 h. Data are expressed as the mean of Ki-67-positive cells relative to the total cell number (10 fields were assessed for each experimental condition). (C) Representative confocal microscopy images showing Ki-67 labelling in the different experimental groups. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue) and Ki-67 positive staining is red. Negative controls for Ki-67 obtained by omission of the primary antibody are provided as insert panels (-). (D) Migration of LNCaP cells determined by a trans-wells assay using uncoated chambers. The upper chamber contained serum free medium and LNCaP cells in the presence or absence of LDL and DHT. Lipid depleted medium in the lower chamber was used as chemoattractant. Data are expressed as the mean number of migrating cells per 20x magnification field (10 fields were assessed for each experimental condition). All results are expressed as fold-change relative to the LDL-untreated group (LDL (-)). Error bars indicate mean ± S.D (n = 5) * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ when compared to LDL (-) group; $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ when compared to LDL (-)/DHT (+) group; $^{\#}$ $p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}$ $p < 0.001$ when compared to LDL (+) group.

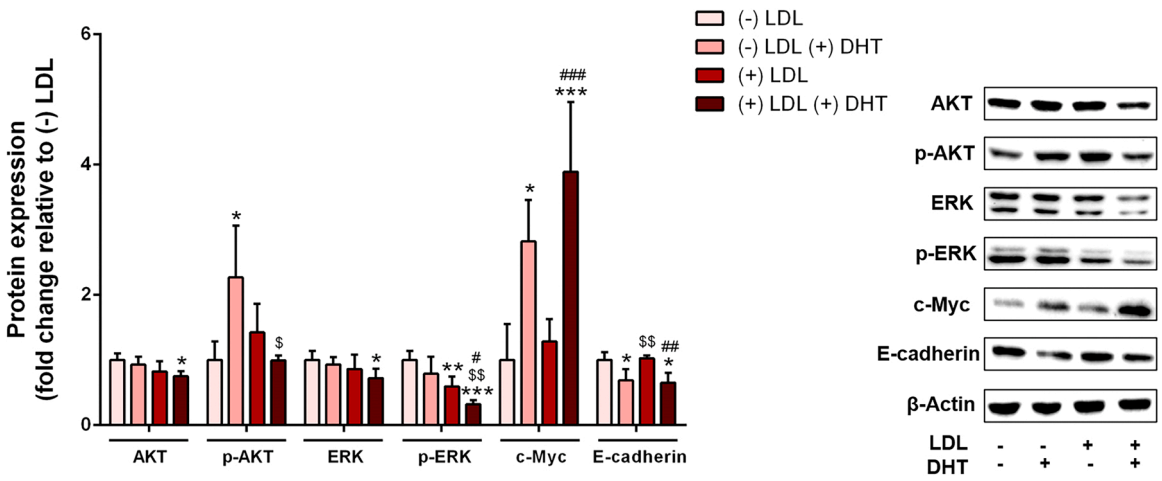

Fig. 4. Effect of LDL and DHT treatment on the expression of AKT, p-AKT, ERK, p-ERK, c-Myc and E-cadherin in LNCaP cells. Cells were treated with DHT (10 nM) and/or LDL (100 μg/ml) for 24 h. Protein expression was analysed by WB after normalization with β-actin. All results are expressed as fold-change relative to the LDL-untreated group (LDL (-)). Error bars indicate mean ± S.D (n = 5) * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ when compared to LDL (-) group; $ $p < 0.05$, $$ $p < 0.01$ when compared to LDL (-)/DHT (+) group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ when compared to LDL (+) group. Representative immunoblots of three independent experiments are shown in the right panel.

group, respectively, Fig. 7 A).

After 48 h of treatment, etomoxir, a well-known inhibitor of CPT1A, completely abolished the LDL effect increasing viability of LNCaP cells (Fig. 7B). A ~ 78 % reduction was observed when etomoxir was present, restoring LNCaP cells viability to that of the control.

4. Discussion

In this study, we investigated the effect of DHT in regulating lipid metabolism, and the influence of this hormone together with LDL-cholesterol in modulating PCa cells fate.

First, we characterized the basal expression of target regulators of lipid metabolism in our prostate cell line models; non-neoplastic (PNT1A) prostate cells, androgen-sensitive (LNCaP) PCa cells, and androgen-insensitive (DU145 and PC3) cells. CD36, FASN and CPT1A proteins were selected as targets of fatty acid uptake, synthesis and β-oxidation (Fig. 1A), respectively. We found that DU145 and PC3 cells displayed a decreased expression of the fatty acid transporter CD36 compared to non-neoplastic PNT1A and the androgens-sensitive LNCaP cells (Fig. 1B). This is the first description of the differential CD36 expression in prostate cell line models dependently on their androgen-responsiveness, and raises the curiosity about the role of androgens in regulating CD36 expression in PCa.

Regarding FASN and CPT1A expression, it was significantly increased in PCa cell models compared to non-neoplastic PNT1A cells (Fig. 1B), which is in agreement with previous reports [48]. FASN overexpression and increased activity have been associated with the onset of PCa, progression and aggressiveness of the disease, and the establishment of bone metastasis [48–51]. Concerning CPT1A, the studies evaluating its expression in human PCa cases or cell lines are almost non-existent, but a report demonstrated that CPT1A is highly abundant in PCa compared with benign tissues, especially in high-grade tumours [52]. This is in accordance with our results showing the highest expression of CPT1A in PC3 cells (Fig. 1B), the most aggressive PCa cell line model under study.

Androgens are widely recognized as the main stimulators of prostate cell proliferation [53,54], and also have been indicated as important regulators of cell metabolism, including glycolysis and lipid metabolism [5,7,9]. Androgen exposure has been shown to modulate lipid handling in PCa cells, with effects on lipogenesis, fatty acid uptake and lipid storage [13]. Herein, we analyzed the effect of 10 nM DHT in regulating the expression levels of FASN, CPT1A and CD36, which was essentially demonstrated in the androgen-sensitive LNCaP and 22RV1 cell lines, both at mRNA and protein level (Fig. 2A-F). The results obtained in response to DHT treatment were underpinned by the increased protein expression of the well-known androgen target gene PSA (Fig. 2 G). This finding validates the experimental setting and was somehow expected, as the supraphysiological 10 nM DHT concentration has been widely used in experimental assays, showing to be effective in regulating the expression of several androgen-target genes and inducing physiological responses in PCa cells [7,27,30,31].

FASN has been indicated as a classical target of the androgens actions regulating lipid *de novo* synthesis in PCa cells [5,55,56]. Accordingly, androgen deprivation was shown to cause a marked reduction in FASN expression that was reverted by the administration of testosterone [57]. Our results corroborate these previous findings, with DHT-treatment enhancing the expression of FASN both in LNCaP and 22RV1 cells (Fig. 2 C, D). The novelty of the obtained results relies on the fact that DHT increased FASN expression even in conditions of lipid depletion, whereas the presence of LDL abrogated its effect (Fig. 5 A). Although no differences were found in fatty acid content (Fig. 5 C), our results suggest that LNCaP cells could maintain the capability of synthesizing *de novo* fatty acids in lipid-depleted conditions if stimulated with DHT. Moreover, the effect of DHT in regulating FASN expression in LNCaP cells was linked to SREBP-1, a central player in the control of lipid metabolism and a mediator of androgens actions regulating lipid handling [58–60]. Fatostatin, a well-known inhibitor of the transcription factor SREBP, suppressed the DHT effects up-regulating the FASN mRNA and protein levels in lipid-depleted conditions (Fig. 6 A, B), which indicates that the observed effect on protein expression is determined at transcriptional level. Similar results were found in breast cancer cells with fatostatin decreasing FASN expression only in the absence of lipids [61].

CPT1A mediates a rate-limiting step in β-oxidation by converting fatty acids to acylcarnitines, which enables their translocation to the intermembrane space of the mitochondria and subsequent energy production. DHT treatment upregulated CPT1A expression in PNT1A, LNCaP and 22RV1 cells (Fig. 2E, F), which may indicate that androgens can stimulate fatty acid β-oxidation both in non-neoplastic and neoplastic prostate cells. However, the effect of DHT upregulating CPT1A in LNCaP cells was absent in lipid depleted conditions (Fig. 5 A). Previous reports indicated that lipid mitochondrial metabolization through β-oxidation is regulated by the AR [62,63]. Our findings suggest that androgens can stimulate fatty acid β-oxidation at the early step of

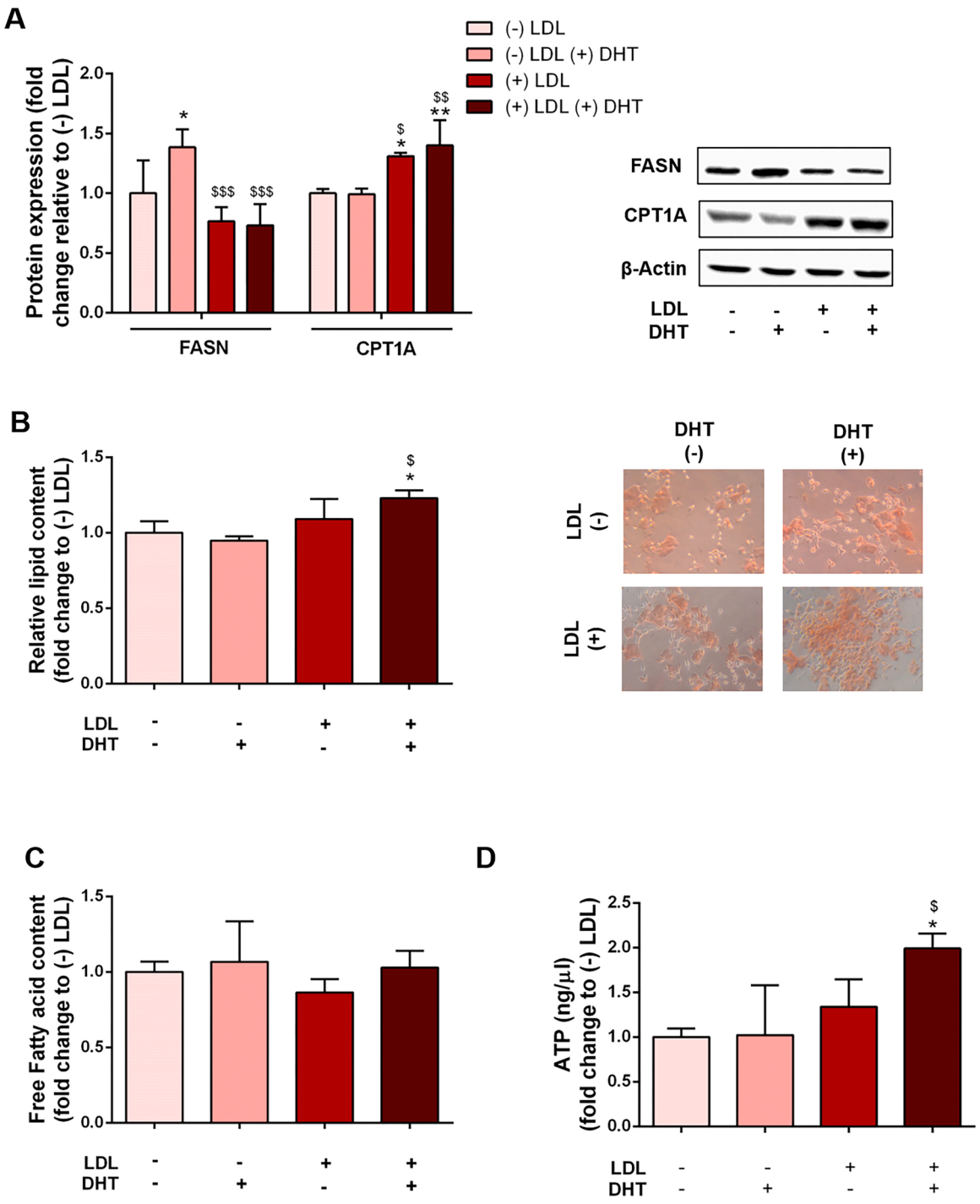

Fig. 5. Effect of LDL and DHT on the expression of FASN and CPT1A, and accumulation of lipid droplets in LNCaP cells. Cells were treated with DHT (10 nM) and/or LDL (100 μg/ml) for 24 h. (A) Protein expression analysed by WB after normalization with β-actin. Representative immunoblots of three independent experiments are shown in the right panel. (B) Lipid droplet staining and quantification determined using the Oil Red-O assay. Representative images of Oil Red staining are shown in the right panel. (C) Free fatty levels determined spectrophotometrically using a commercial kit. (D) ATP measurements using a colorimetric assay. All results are expressed as fold-change relative to the LDL-untreated cells ((-) LDL). Error bars indicate mean ± S.D (n = 5) * $p < 0.05$, * ** $p < 0.001$ when compared to (-) LDL group; $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ when compared to (-) LDL (+) DHT cells.

their translocation to the mitochondria. This is quite relevant since several studies have described that lipid utilization by the mitochondria is crucial for cell survival, activation of proliferation pathways and resistance to treatment [64–67].

As mentioned, the effect of DHT upregulating CPT1A in LNCaP cells was absent in lipid depleted conditions, but LDL supplementation increased the levels of this translocator protein (Fig. 5 A). This suggests the mobilization of fatty acid through β-oxidation under DHT and high LDL availability. However, despite the increased CPT1A expression, free fatty acid content remained unchanged among experimental groups (Fig. 5 C). We cannot exclude that fatty acids could be accumulated in lipid droplets, as these were specifically augmented in the presence of LDL and DHT (Fig. 5B). Nevertheless, ATP production only was increased in the LDL (+)/DHT (+) group (Fig. 6B) that displayed

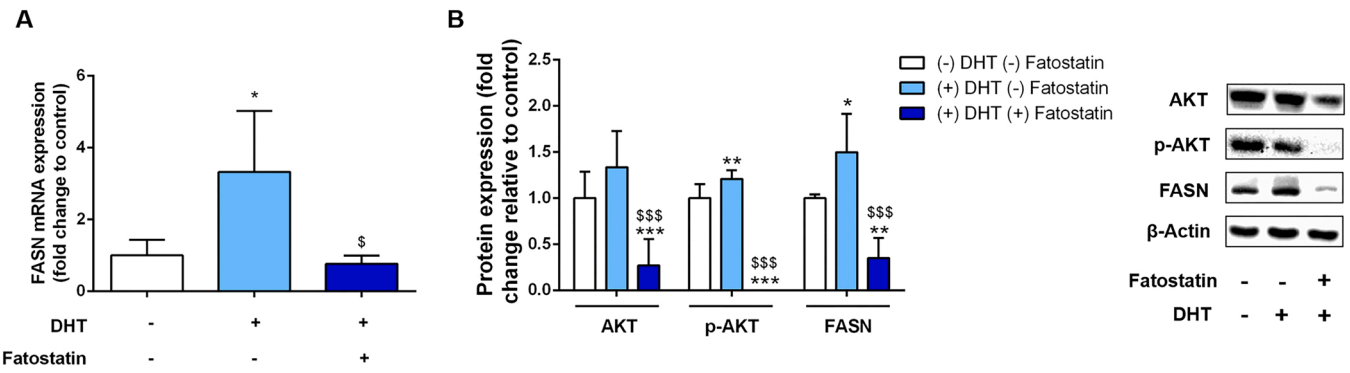

Fig. 6. Effect of fatostatin suppressing DHT actions in regulating the expression of AKT, p-AKT, FASN in LNCaP cells. Cells were treated with 10 nM DHT alone or with 20 μM fatostatin for 24 h, as indicated. (A) FASN mRNA expression determined by qPCR after normalization with the housekeeping gene β-2-microglobulin. (B) AKT, p-AKT, FASN protein expression analysed by WB after normalization with β-actin. Representative immunoblots of three independent experiments are shown in the right panel. All results are expressed as fold-change relative to DHT-treated group. Error bars indicate mean ± S.D (n = 5). * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ when compared to control; $^{\$}$ $p < 0.05$; $^{\$\$\$}$ $p < 0.001$ when compared to (+) DHT.

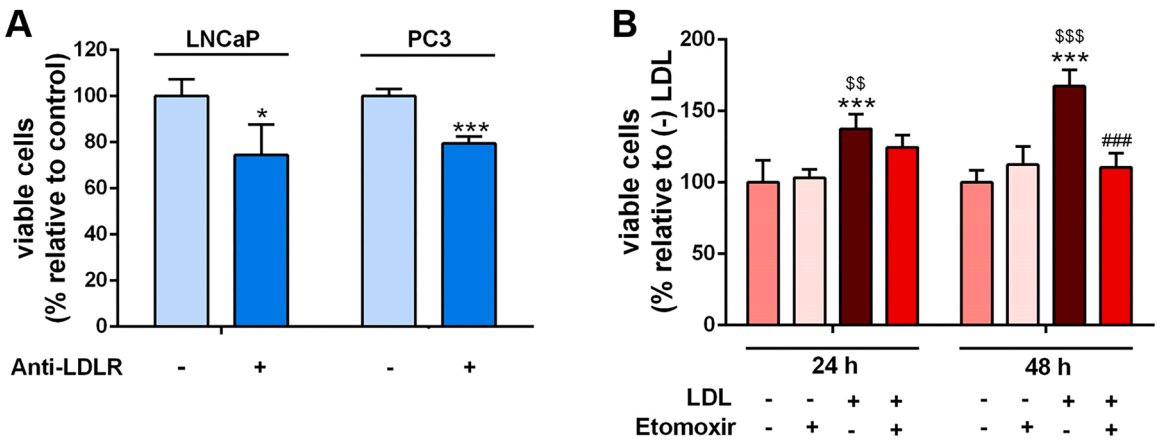

Fig. 7. Effect of anti-LDLR antibody and etomoxir over LDL actions sustaining LNCaP cells viability. (A) Cells were pre-treated with the anti-LDLR antibody (5 μg/ml) for 2 h before exposure to LDL (100 μg/ml). (B) Cells were treated with LDL (100 μM/ml) in the presence or absence of etomoxir (40 μM) for 24 and 48 h. Cell viability was determined by the MTT assay. Error bars indicate mean ± S.D (n = 5) * $p < 0.05$, * ** $p < 0.001$ when compared to the respective control group; $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ when compared to (-) LDL (+) etomoxir group; $^{\#\#\#}$ $p < 0.001$ when compared to (+) LDL (-) etomoxir group.

increased levels of CPT1A, which sustains that is fatty acid β-oxidation that is fueling energy production in the mitochondria. This is further supported by the fact that the CPT1A inhibitor etomoxir has blocked the LDL effects inducing the viability of LNCaP cells (Fig. 7B).

CD36, also known as fatty acid translocase, is a major transporter for fatty acids uptake. Moreover, CD36 was shown to play a relevant role in oncogenic signalling and, consequently, in cancer progression in several types of cancer, including prostate [68–70]. DHT-treatment diminished the expression levels of CD36 in PNT1A and LNCaP cells (Fig. 2 A, B). At least for our knowledge, this is the first report identifying the cell membrane fatty acid transporter CD36 as a target of androgens regulation. Other authors showed that androgens exposure increased medium and long-chain fatty acids, cholesterol, and low-density lipoproteins uptake [71,72]. However, several transporters can mediate lipid uptake in PCa cells [71]. A study indicated that the augmented uptake of lipids in response to androgens was associated with the increased expression of membrane fatty acid-binding protein (FABPm) [72]. In this way, androgens actions stimulating lipid uptake would result from the balance between CD36 and FABPm expression pattern [72]. Overall, the obtained findings broadened the knowledge regarding the role of androgens as central regulators of lipid metabolism.

Next, we analysed the DHT and LDL interplay altering PCa cells fate. LDL exposure augmented LNCaP, 22RV1 and PC3 cells viability, an effect that was amplified in LNCaP cells in the presence of DHT (Fig. 3 A). As a proof of concept, we evaluated LDL actions in the presence of anti-LDLr antibody. Inhibition of LDLr decreased LNCaP cells viability to the control levels (Fig. 7 A), demonstrating the importance of LDL uptake in promoting PCa cell viability. We also found that the combined action of LDL and DHT promoted the proliferation of 22RV1 and LNCaP cells, with approximately a two-fold increase compared to controls in the case of LNCaP (Fig. 3B, C). The presence of androgens potentiating the effects of LDL in AR-positive LNCaP cells is a new important finding that can be quite relevant to the clinics and management of PCa. The existent reports demonstrating the role of androgens increasing the expression of LDLr and LDL uptake support our findings [71].

LDL/DHT effects modulating LNCaP cells behaviour were underpinned by alterations on the protein fingerprint of key targets of survival and oncogenic pathways (Fig. 4), namely, the AKT pathway that plays an important role in the survival of PCa cells, and has been associated with a poor clinical outcome [73–75]. The results obtained herein showed a significant increase of p-AKT in response to DHT in lipid-depleted conditions (Fig. 4), which was linked with increased cell viability (Fig. 3A). Increased p-AKT in DHT-treated LNCaP cells was also concomitant with the increased expression of FASN (Fig. 5 A) and completely blocked by SREBP-1 inhibitor fatostatin (Fig. 6 A, B). These findings are in line with the report of AKT activation, and the involvement of the PI3K/AKT→SREBP-1→ FASN pathway, and fatty acid synthesis, to satisfy the energy demands of cancer cells, their growth and

invasion [76]. Despite the increased viability and proliferation of LDL (+)/DHT(+) treated cells, p-AKT expression levels remain unchanged, which suggests that other pathways are activated when lipid availability is high.

The ERK pathway is another important driver of cell proliferation and survival. The expression of the active p-ERK decreased with LDL supplementation and this effect was significantly more pronounced with the co-administration of LDL and DHT (Fig. 4), whereas LDL or DHT alone had no effect. Interestingly, a study in breast cancer cells showed that LDL-cholesterol enhanced p-ERK expression levels [32]. Another study also showed a decreased expression of p-ERK with DHT treatment alone in LNCaP cells, which we observed only in the LDL(+)/DHT(+) cells, probably because a completely lipid-free medium was used in our study [77]. Nevertheless, the relationship between ERK activity and human PCa cases is also controversial. Some authors have demonstrated the activation of the ERK pathway with malignancy, whereas others observed a decline in ERK activity in advanced tumours [78–81].

Over-expression of the oncogene c-Myc in PCa has been shown to be extremely important for cancer progression and for the establishment of the castration-resistance phenotype [82–84]. Also, the coordinated action of c-Myc and AR has been proposed in PCa development [82,83]. Here we showed an increased expression of c-Myc in LNCaP cells in response to DHT treatment alone and a synergistic effect in LDL(+)/DHT (+)-treated cells (Fig. 4). LDL alone had no effect in c-Myc expression (Fig. 4). c-Myc also has been indicated as a central regulator of metabolic alterations in cancer cells, namely in glucose and glutamine metabolism [85,86]. Furthermore, it was demonstrated that c-Myc is associated with the deregulation of lipid metabolism in PCa cells and triple-negative breast cancer [77,87]. Interestingly, the increased expression of c-Myc in the LDL(+)/DHT(+)-treated cells occurred concomitantly with the accumulation of intracellular lipids. Further studies are needed to confirm the relationship between c-Myc and LDL/DHT actions in lipid metabolism.

Our results also showed that LDL had a stimulatory effect on LNCaP cells migration (Fig. 3D), which was further potentiated by DHT. The EMT marker E-cadherin is a key cell-to-cell adhesion molecule, that when suppressed is associated with cancer cell migration and invasion. Loss of E-cadherin has been shown to facilitate PCa metastasis and enhance PCa chemoresistance [88–90]. Here we observed decreased expression of E-cadherin upon treatment of LNCaP cells with DHT in the presence or absence of LDL (Fig. 4), which is in line with the concept of androgens as enhancers of PCa cell migration and metastasis [91–93]. Our findings are also supported by a study describing the decreased expression of E-cadherin using the same DHT concentration [94]. Concerning other EMT markers, such as vimentin and N-cadherin, their protein expression was undetectable (data not shown). Indeed, LNCaP cells have been shown to present low levels of vimentin [95,96], being negative for N-cadherin [97].

Overall, this study first showed the interplay between androgens and LDL enhancing viability, proliferation and migration abilities of androgen-sensitive PCa cells (Fig. 8).

It has been shown that cancer cells have the capability of metabolic adaptation in response to lipids availability, which has been shown to affect cell fate [98]. When cell lipids are in excess, cholesterol is converted to cholesteryl esters by the activity of acyl coenzyme A: cholesterol acyltransferase (ACAT) and can be stored in lipid droplets [99, 100]. Also, fatty acids have been shown to increase lipid droplet biogenesis, which requires the action of diacylglycerol O-acyltransferase (DGAT) and triglyceride synthesis [101,102]. In accordance with this, we observed that high LDL availability increased the storage of lipids in LNCaP cells, but that required the presence of DHT (Fig. 5B). This suggests that the accumulation of lipid droplets may rely on the stimulatory effect of androgens over expression and/or activity of ACAT and DGAT. Indeed, DHT was shown to increase the expression of these enzymes in rat adipose tissue [103].

The LDL stored in lipid droplets constitute reservoirs to satisfy the cell growth needs, and might support the enhanced proliferative activity and migration of LNCaP cells observed under these conditions (Fig. 3 A, C). Moreover, LDL supplementation, besides suppressing FASN expression, increased CPT1A levels (Fig. 5 A), likely diminishing the lipid *de novo* synthesis and driving fatty acids to β-oxidation, as discussed above. The rate of mitochondrial fatty acid β-oxidation is regulated by CPT1A as it mediates the transport of fatty acids across the inner mitochondrial membrane. Etomoxir is a small-molecule widely used as an inhibitor of fatty acid oxidation by its irreversible inhibition of CPT1A [35]. CPT1A inhibition with etomoxir reverted the LDL-induced LNCaP cells viability (Fig. 7B), which supports that the LDL incorporated in the lipid droplets could being routed for lipolysis, with subsequent fatty acid production and β-oxidation sustaining cell viability (Fig. 8).

The information on the LDL effects and the underpinning mechanistic in the regulation of PCa cell fate is limited, though a study showed that LDL exposure was related to an increased number of cancer cells [33]. However, obesity, fatty diets, and hypercholesterolemia have been associated with more aggressive stages of PCa [17–21, 104]. Also,

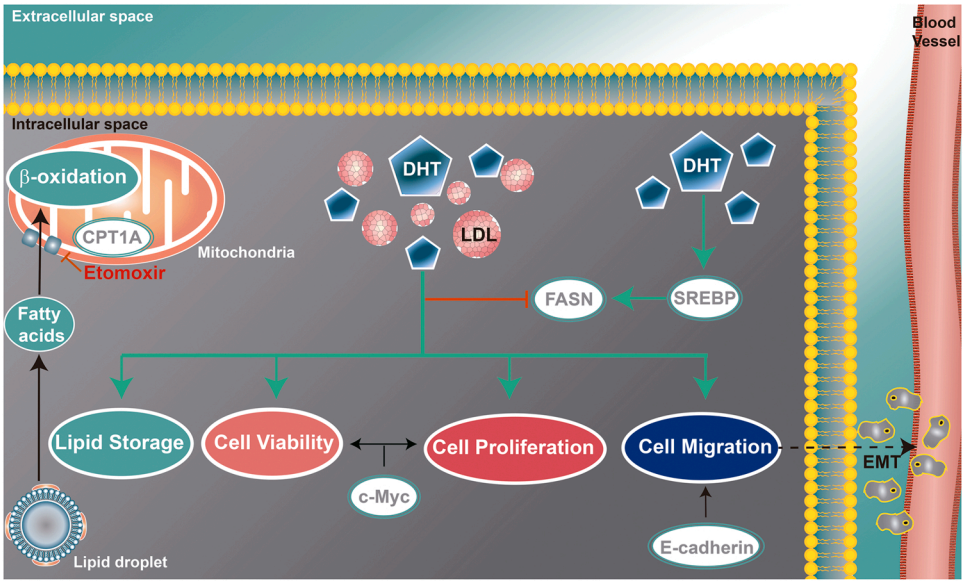

Fig. 8. DHT and LDL-cholesterol actions in modulating PCa cells fate and lipid metabolism. LDL promoted viability, proliferation and migration of PCa cells in a DHT dependent manner. These effects were underpinned by the altered expression of the oncogene c-Myc, and decreased expression of E-cadherin, which is a protein downregulated in the epithelial to mesenchymal transition (EMT). The combination of LDL and DHT augmented the lipid droplets content. Blocking fatty acid β-oxidation with etomoxir, an inhibitor of CPT1A, reverted the effect of LDL promoting cell viability, which indicates that the LDL incorporated in lipid droplets is being routed for fatty acids production and oxidation. DHT increased the expression of FASN, an effect also observed in lipid-depleted conditions and mediated by the transcriptional factor SREBP. The presence of exogenous LDL, regardless of the presence of DHT, turned-off FASN expression.

periprostatic adipocytes were shown to have a significant impact on PCa cells invasiveness [105]. On the other hand, treatment with inhibitors of cholesterol synthesis, such as simvastatin, was shown to reduce LNCaP tumors xenografts growth [106]. These data, together with the cooperative relationship found here between LDL and DHT promoting PCa cell growth, open new perspectives in PCa treatment, namely considering pharmacological approaches targeting cholesterol availability together with the classical anti-androgens therapies.

In conclusion, this study showed that androgens regulate a wide range of lipid metabolism targets, and promote the LDL effects increasing cell viability, proliferation and migration of PCa cells (Fig. 8). These findings support clinical data linking obesity and PCa, and first implicate androgens in this relationship. Furthermore, they sustain the development of future strategies for PCa treatment targeting lipid metabolism/obesity and the AR simultaneously.

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CRediT authorship contribution statement

HJC particiated in conception and design, performed the experiments, analyzed the data and wrote the manuscript. MIF, TMAC and CDMS performed the experiments. CVV and PAM colaborated in supervising experiments and critically revised the article. SS was responsible for conception and design, critical revision and final approval of the version to be published.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and at 10.17632/dbt8mshdk2.1.

Declaration of Competing Interest

The authors report no conflicts of interest.

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