Conjugated Linoleate Reduces Prostate Cancer Viability Whereas the Effects of Oleate and Stearate Are Cell Line-dependent

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Abstract. *Background: In this study, responses to fatty acid treatments in commonly used prostate cancer cell culture models and variability of gene expression between them were determined. Materials and Methods: PC3, DU145, LNCaP, VCaP and PNT2 cells were treated with 100 μM of either oleate, stearate or conjugated linoleate. Cell proliferation and viability were assessed using trypan blue and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay respectively. Gene expression was measured using real-time polymerase chain reaction (PCR). Results: Conjugated linoleic acid reduced cell proliferation and viability in all prostate cancer cell lines, whilst the effects of oleic and stearic acid on proliferation were found to be cell line-dependent. A reduction in gene expression of fatty acid desaturases was observed in prostate cancer cell lines compared to normal prostate cells. Conclusion: Differential responses of the cell lines investigated here to fatty acid treatment suggest that multiple prostate cancer cell line models should be used when designing experiments aimed at examining lipid metabolism in prostate cancer.*

One increasingly relevant area in prostate cancer research is the investigation of whether dietary lipids can alter prostate cancer biology and clinical outcome in patients. Increased glycolysis is often defined as a metabolic characteristic of tumour cells. However, recent studies have characterized prostate tumour cells as having low glycolysis and glucose uptake rates, resulting in the dominant uptake of fatty acids over glucose (1). These studies suggest that catabolism of fatty acids, not glucose, may be the dominant bioenergetic source in prostate cancer and thus be an important fuel source for cell proliferation.

Epidemiological studies suggest an association between dietary fat intake, prostate cancer risk and unfavourable prognosis; however, results have often been difficult to interpret and controversial (2, 3). A common finding has been that the essential polyunsaturated fatty acids (PUFAs), omega-6 and omega-3, are associated with increased and reduced prostate cancer risk, respectively (4). However, less is known about the effects of specific unsaturated and saturated fatty acids on prostate cancer and normal prostate cells. Cell lines are commonly used *in vitro* to examine prostate cancer cell pathophysiology as they are easy to maintain and manipulate genetically. There are approximately 63 established prostate cancer cell lines and 46 immortalised prostate cancer cell lines available, isolated from a variety of sites and exhibiting different characteristics. The most commonly used cell lines are PC3, DU145, LNCaP and VCaP, termed classical cell lines (5-8).

It is important to note that different cell lines are often cultured in different media. It is also the case that the same cell line can be cultured in different media by different laboratories and thus it can often be hard to interpret study results. We utilised four human prostate cancer cell lines (DU145, PC3, LNCaP, VCaP) and one normal human prostate cell line (PNT2) (9), all cultured in the same medium and conditions, to investigate for potential similarities and differences with respect to gene expression, cell proliferation and viability in response to fatty acid treatment. We specifically concentrated on fatty acids that are 18 carbons in length but differ in their degree of saturation: a monounsaturated fatty acid, oleic acid (OA), a saturated fatty acid, stearic acid (SA) and a polyunsaturated fatty acid, conjugated linoleic acid (CLA). Their use excludes the added variability that fatty acid chain length can have on cell physiology.

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Table I. *PCR Oligonucleotide sequences used.*

Gene	Accession No.	Forward (5'-3')	Reverse (5'-3')
HK	NM_000188.2	GGCGTTTCCACAAGACTCTA	CTTGGTGAGGTGGAAATGAG
G6PDH	NM_000402.3	TGGAACCGGGACAACATC	CAACACCTTGACCTTCTCATCAC
FAS	NM_004104.4	TCGACTGCATCAGGCAG	ACAGGAAGAGGCTGT
ACC1	NM_198834.1	ACTACCGGAACTTCTCTTGAAAAAT	CAATCTTATCCCCTAAAGCCCACAT
SCD1	NM_005063.4	CTCCACTGCTGGACATGAGA	AATGAGTGAAGGGGCACAAC
FADS1	NM_013402.4	CAGGCCACATGCAATGTC	ATCTAGCCAGAGCTGCCCT
GPAT	NM_001244949.1	AAGTCCTGTGCCATTATGTCCA	CAATTCCCTGCCTGTGTCTG
PPARG	NM_138712.3	GTCGGATCCACAAAAAAAGTAGAA	AGCGGGAAGGACTTTATGTATGA
SREBP1	NM_004176.4	TCAGCGAGGCGGCTTTGGAG	CATGTCTTCGATGTCGGTC
CPT1B	NM_004377.3	CTTTGGCCCTGTAGCAGATGA	TCGTCTCTGAGCTTGAGAACTT
CPT1C	NM_001199752.1	TGGAACTCAGTGCCCCTGTG	GCAGGAAACACACCGGTGA
18S	NR_003286.2	ACCCGTTGAACCCCATTCGTGA	GCCTCACTAAACCATCCAATCGG

Materials and Methods

Materials. All materials, unless otherwise stated, were obtained from Sigma (St. Louis, MO, USA).

Cell culture. The benign prostate cell line PNT2, and prostate cancer cell lines, PC3 and VCaP (all obtained from the European Collection of Cell Cultures (ECACC), DU145 and LNCaP (both kind gifts from Professor Jeff Holly, University of Bristol) were all cultured in Dulbecco's Modified Eagle's Medium (DMEM - 5 mM glucose) containing 10% fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin for two weeks after defrosting. For fatty acid supplementation OA, SA and CLA were prepared as described elsewhere (10). Briefly all fatty acids were dissolved in ethanol to a stock solution of 100 mM. Stock solution was then added to complete DMEM medium to the final concentration of 100 μM. Control cells were incubated with complete DMEM medium containing comparable amounts of ethanol only. Cells were incubated with 100 μM of the specific fatty acid for 48 h and then RNA was isolated.

Quantitative real-time PCR. RNA was extracted using TRI Reagent as per the manufacturer's instructions. RNA was quantified using Nanodrop Spectrophotometer Thermo Scientific (Wilmington, DE, USA). RNA (1 μg) was converted to cDNA using MMLV Reverse Transcriptase Promega (Madison, WI, USA) as per the manufacturer's instructions. Quantitative PCR was performed using 2x SensiFAST SYBR Hi-ROX master mix from Bioline (London, UK) using the StepOne Plus Real-Time PCR System (Applied Biosystems, Life Technologies, CA, USA). Primer sequences are provided in Table I. PCR reaction conditions were 20 s at 95˚C then 40 cycles of; 3 s at 95˚C and 30 s at 60˚C. Fold changes in expression were calculated by using a standard curve according to established methods (11). Gene expression was normalised to the corresponding 18S rRNA value for each sample.

Cell proliferation analysis. Cell proliferation was measured using trypan blue (0.4%). Cells were seeded in 12-well plates (70,000 per well) and incubated overnight in DMEM media to stabilize (5mM glucose). Cells were then serum-starved overnight before treatment with 100 μM fatty acids for 48 h. Only live cells (unstained cells) were counted using a haemocytometer.

Cell viability analysis. Prostate cells were seeded in 96-well plates and incubated overnight to stabilize in DMEM medium (5 mM glucose). Cells were serum-starved overnight before treatment with medium containing fatty acids (100 μM) and cells cultured for 48 h after which the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. MTT was added to cells at a final concentration of 0.4 μg/ml. Cells were incubated at 37˚C for 1 h. Dimethyl sulfoxide (200 μl) was then used to solubilize the formazan crystals. The optical density of the contents of each plate was measured at 570 nm. Readings were taken from three independent experiments, each carried out in triplicate, and the average of these three experiments is presented. Each reading was normalized to that of corresponding control cells incubated in normal culture DMEM (5mM glucose with no additional fatty acids) to eliminate variation in cell proliferation observed between the cell lines.

Statistical analysis. Data from experiments are presented as the mean±standard error of the mean (SEM), with numbers of replicates stated in figure legends. Statistical significance was tested using a two-way analysis of variance test (ANOVA).

Results

Effects of fatty acids on cell viability. Prostate cancer cell lines were incubated with fatty acid-supplemented media for 48 h in order to assess effects on cell viability (Figure 1). OA resulted in a significant reduction in viability of DU145 cells, with a trend for reduced viability of PNT2 cells. In contrast, there was a trend for OA to increase cellular viability of VCaP cells. However, in LNCaP cells there is an indication that SA may increase cellular viability. CLA significantly reduced the cell viability of all cancer cell lines tested, the greatest reduction being observed in DU145 cells, with the VCaP cell line exhibiting the least reduction.

CLA reduces cell proliferation of all prostate cancer cell lines whilst the effects of OA and SA are cell line-dependent. As

the MTT assay measures the metabolic capacity of mitochondria rather than cell number *per se*, we next assessed whether alterations in cell viability observed when cell lines were incubated with specific fatty acids were attributed to alterations in cell number or whether it was an indication of alterations in mitochondrial function. Cells were incubated in fatty acid-supplemented media and cell numbers evaluated using a haemocytometer after 48 h (Figure 2).

OA significantly reduced the proliferation of PNT2 cells. In contrast, OA increased proliferation of PC3 cells. SA treatment significantly reduced the proliferation of PC3 cells while it increased that of LNCaP cells. CLA reduced cell proliferation of all cancer cell lines tested but did not significantly alter cell proliferation of PNT2 cells.

In summary, whilst prostate cancer cell lines appear to have reduced proliferation and viability in response to CLA, they have differential proliferation responses to unsaturated and saturated fatty acids.

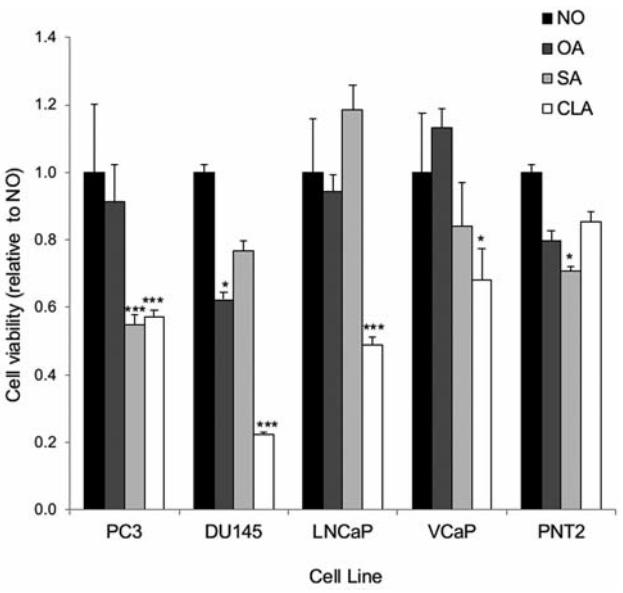

Figure 1. *Cell viability after 48 h fatty acid treatment (100 μM) in all cell lines was determined and expressed relative to values obtained with no fatty acid (NO) (n=3). Differences in NO vs. OA, SA or CLA significant at: *p<0.05, **p<0.01 and ***p<0.001.*

Basal expression of genes involved in fatty acid metabolism in prostate cell lines. We next measured basal expression of a select number of genes involved in glycolytic, lipid metabolic and oxidative pathways to determine whether this could explain the cell line-specific differences recorded. We assessed whether there were intrinsic differences between the cancer cell lines themselves and also between the cancer cell lines PC3, DU145, LNCaP, VCaP and a normal prostate epithelial cell line (PNT2) (Figure 3). No significant difference in *18S* rRNA expression was detected between the cell lines and so this was used for normalisation for gene expression analysis using PCR.

Hexokinase (*HK*) mRNA was expressed at higher levels in the cancer cell lines compared with the benign PNT2 prostate cell line, suggesting that the cancer cell lines had an increased capacity to utilise glucose for energy production. The PC3 cell line had higher *HK* expression compared with the other cancer cell lines. DU145 cells had significantly greater expression of glucose 6 phosphate dehydrogenase (*G6PDH*) compared with PNT2, whilst VCaP cells had a very low level of G6PDH mRNA expression.

Fatty acid synthase (*FAS*) and acetyl coA carboxylase (*ACC*) mRNA expression was reduced in PC3 cells compared with PNT2 cells, whilst both LNCaP and VCaP cell lines had significantly greater *FAS* mRNA expression. The DU145, LNCaP and VCaP cell lines had higher glycerol-3-phosphate acyltransferase (*GPAT*) expression, whilst PC3 cells had significantly reduced *GPAT* expression when compared with PNT2 cells; the opposite was observed for peroxisome proliferator-activated receptor gamma (*PPARG*) mRNA expression. Of note LNCaP and VCaP had very low levels of *PPARG* expression when compared with PNT2 cells. Expression of sterol-regulatory element-binding protein (*SREBP1*) was significantly reduced in the PC3 cell line compared with PNT2 cells. Stearoyl-CoA desaturase (*SCD1*) and fatty acid desaturase-1 (*FADS1*) mRNA expression was significantly reduced in PC3, LNCaP and VCaP cell lines compared with PNT2 cells. Carnitine-palmitoyl transferase 1-B (*CPT1B*) mRNA expression was significantly higher in all prostate cancer cell lines (>4-fold) compared with PNT2 cells. In contrast, all cancer cell lines had reduced *CPT1C* mRNA expression compared with PNT2 cells.

Discussion

A number of prostate cancer cell lines are available to help aid further understanding of prostate cancer progression. In this study we highlighted differential responses in gene expression and cellular proliferation which can be seen when using different cell lines. In addition, we identified a number of genes that are consistently de-regulated in prostate cancer cell lines *in vitro* compared with the normal PNT2 cell line such as *FADS1*, *SCD1* and *CPT1C*.

Fatty acids are known to affect the growth response of cancer cells (12, 13). In particular, CLA has been demonstrated to inhibit the growth of human cancer *in vitro* and reduce tumour size and metastatic spread of PC3 cells in mice, confirming its anticancer activities (14-16). Our study reinforces the potential use of conjugated linoleic acid as a therapeutic agent in reducing prostate cancer cell viability, whilst having a minimal effect on normal prostate epithelial cells. However, we also note there to be a variability in the sensitivity to CLA treatment in the prostate cancer cell lines we tested.

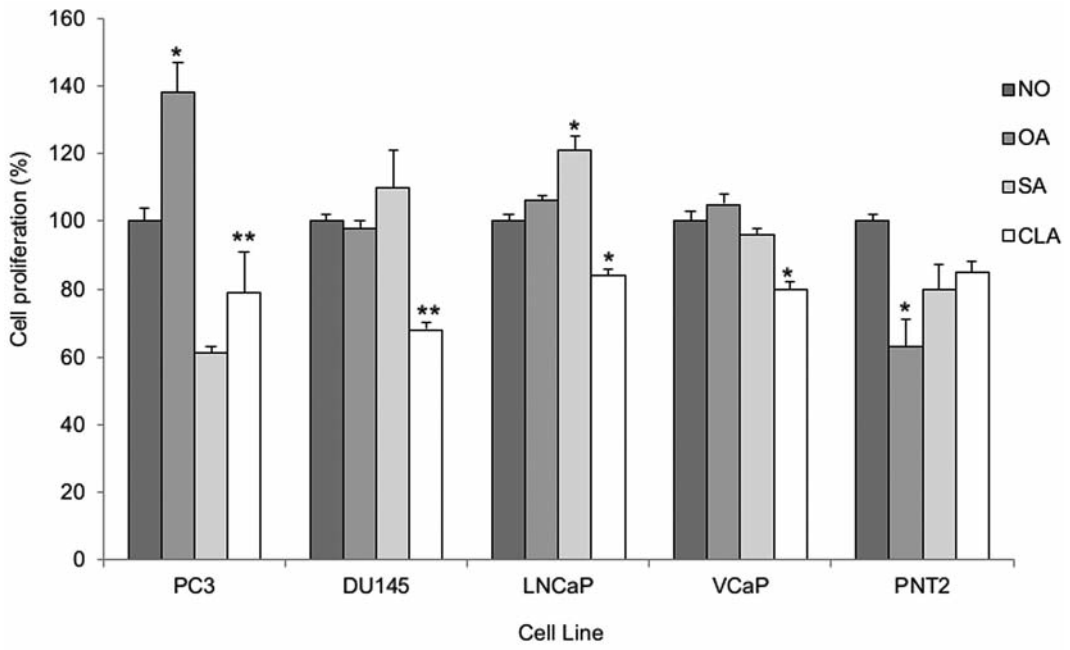

Figure 2. *Cellular proliferation for all cell lines after 48 h of fatty acid supplementation (100 μM) was measured using trypan blue and only live cells were counted. All values are expressed as percentages relative to the control cells that received no fatty acid supplementation (NO) (n=3). NO vs. fatty acid: *p<0.05, **p<0.01, ***p<0.001.*

In addition, we found that OA had a stimulatory effect on PC3 and LNCaP cancer cells but reduced proliferation of the benign PNT2 cells. This is in contrast to another published report that found that OA reduced proliferation of PC3 and LNCaP cells (13). These discrepancies may be due to different culture conditions and fatty acid concentrations used. SA was found to reduce proliferation of PC3 cells here. As PC3 cells had reduced expression of *SCD1* compared with the other cell lines, it may be that the inability to convert saturated fatty acids to unsaturated fatty acids may reduce proliferation or increase apoptosis in this cell line. In hepatocytes, it has been found that saturated fatty acids increase apoptosis through modulation of endoplasmic reticulum homeostasis and increased ceramide synthesis (17). In contrast, SA increased LNCaP proliferation. Of note was the difference observed in results between the MTT assay and direct cell counting, in particular when analyzing the effects of OA treatment. Whilst different seeding approaches may be a contributing factor, it may also be that OA has a direct effect on mitochondrial function independently of the effect on cell numbers. Further investigation to understand the differential responses to OA and SA and the effect on mitochondrial activity between the prostate cancer cell lines is needed.

Each prostate cell line has a distinct genetic background and is derived from a specific origin which will undoubtedly affect the cell biology and gene expression signature. PC3 and LNCaP cells lack phosphatase and tensin homolog (PTEN) expression and have constitutive protein kinase B activity which could result in increased glycolysis, explaining why PC3 cells had increased glycolytic gene expression compared to the other cell lines (18). The androgen-sensitive LNCaP and VCaP cell lines had the highest mRNA expression of *FAS* and *ACC1*, which would be expected as androgens (present in the FBS and media) are known to up-regulate *FAS* and *ACC1* gene expression (19). In addition, PC3 cells had significantly reduced *SCD1* mRNA expression compared to the other cell lines, which may explain why PC3 cells were particularly sensitive to SA treatment, resulting in reduced cell viability and numbers.

Interestingly a reduction in *FADS1* gene expression in prostate cancer cells compared with the benign PNT2 cell line was observed. FADS1 is a delta-5 desaturase that converts di-homo-γ-linoleic acid to arachidonic acid, which is a precursor for eicosanoid production. Studies that have analyzed fatty acid composition of prostate tumours have shown a marked reduction in arachidonic acid in prostate carcinomas (20, 21). As our studies showed reduced mRNA expression of *FADS1* in prostate cancer cells, it may be that the reduction in arachidonic acid in prostate tumours is due to a direct reduction of *FADS1* expression. The functional consequences of loss of FADS1 in prostate cancer is unknown, but it could be hypothesised that de-regulation of desaturase activity would impact production of eicosanoid precursors and several signalling pathways. We also observed a significant reduction in the expression of fatty acid oxidation gene, *CPT1C*, in the cancer cell lines compared with the normal cell line. The up-regulation of *CPT1B* may lead to it being the dominant

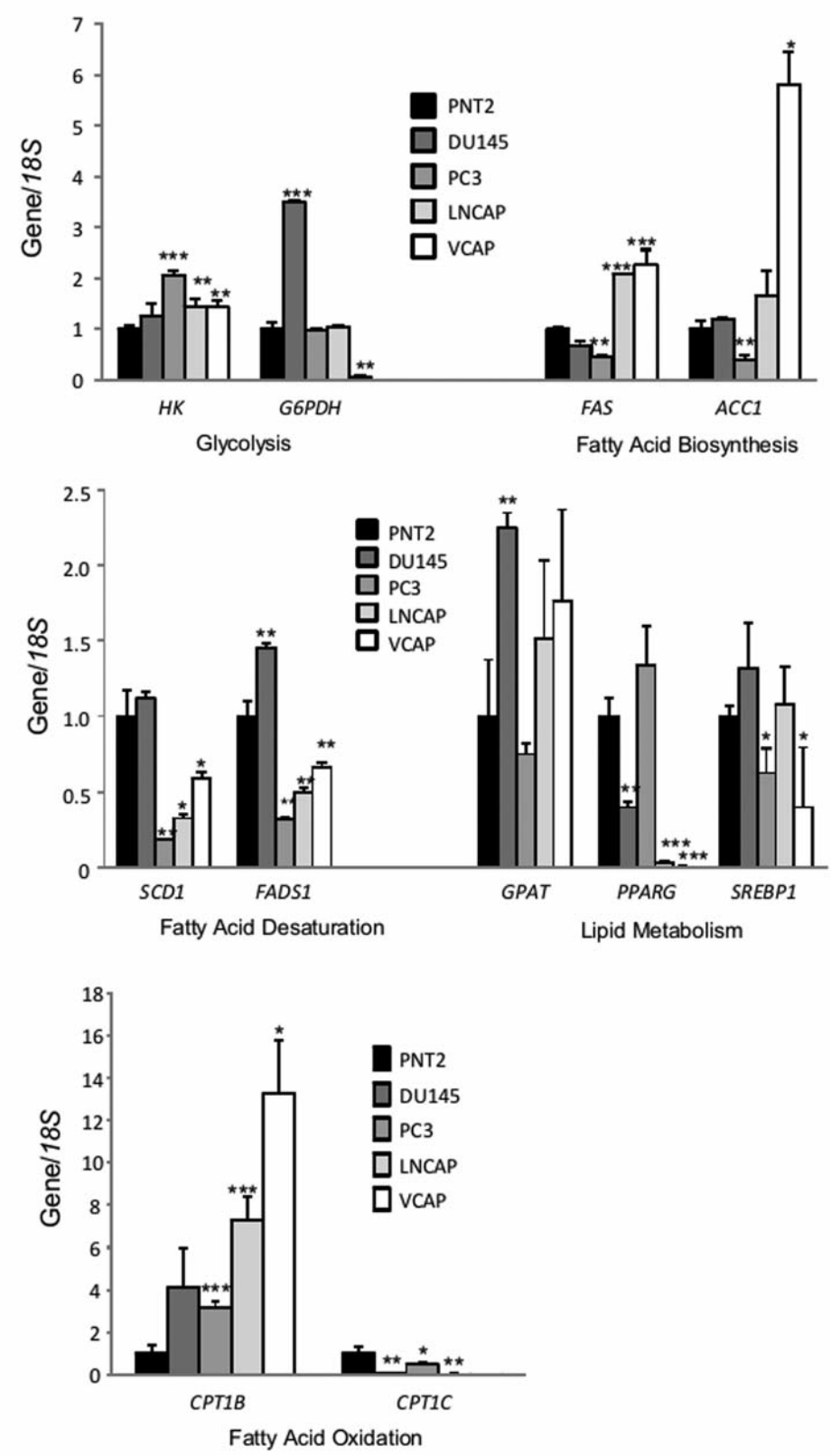

Figure 3. *Real-time PCR analysis of basal mRNA expression of HK, G6PDH, FAS, ACC1, SCD1, FADS1, GPAT, PPARG, SREBP1, CPT1B and CPT1C in prostate cell lines. All cell lines were cultured in fresh (5 mM glucose) medium for 48 h with no fatty acid supplementation (n=3). Normal PNT2 cell line vs. PC3/DU145/LNCaP/VCaP cancer cell lines: *p<0.05, **p<0.01, ***p<0.001.*

isoform in prostate cancer cells and thus a reduction in *CPT1C* expression may be inconsequential, or *CPT1B* expression may negatively regulate *CPT1C* mRNA expression in this system; however, further research is warranted to determine whether reduction of *CPT1C* expression contributes to the metabolic phenotype of prostate cancer cells.

In conclusion, this is the first study that we are aware of that has directly compared and analyzed the effects of fatty acids on different cell lines cultured in the same media to assess variability that can be observed when using different prostate cancer cell lines. Our study showed that fatty acid treatments can have a pro- or anti-proliferative effect. However, the extent and effects are dependent on the prostate cancer cell line used and as such, care should be taken when extrapolating findings to an *in vivo* situation. CLA gave the most consistent reduction in prostate cancer cell proliferation and thus may be an effective non-invasive strategy to reduce the severity of prostate cancer. In addition, we found that *SCD1*, *FADS1* and *CPT1C* mRNA expressions were significantly reduced in prostate cancer cell lines. This area of research is important as revealing the metabolic changes and the study of particular metabolic pathways, such as the desaturase pathways, may broaden therapeutic options for prostate cancer treatment.

Conflicts on Interest

None.

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