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Transcriptional regulation of ASPA in prostate cancer cells

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1. INTRODUCTION

Prostate cancer (PCa) is a remarkably widespread disease. According to the International Agency for Research on Cancer, it is the second type of cancer with the highest incidence worldwide, only after breast cancer[1]. This incidence is far from fading since it has increased in the last decades as a result of the amount of screenings done yearly[2]. The most prominent type of screening for early diagnose is the measurement of a biomarker in serum called prostate-specific antigen (PSA)[3]. Even if PSA quantification is routinely adopted as a gold standard parameter for PCa diagnosis, it is not completely reliable due to its low specificity and high sensitivity and it can give false positive as well as false negative results[4]. In fact, recent studies have shown that although approximately 15% of men will test negative in PCa detection relying on PSA concentrations, they will contract PCa[5]. For this reason, other techniques, such as, digital rectal exam, biopsies and histological studies are also used for the diagnosis[4]. Nevertheless, the finding of new biomarkers will lead to successful early diagnostic techniques.

There are different therapeutic approaches to treating PCa based on tumour stage, such as active surveillance, radiation therapy, chemotherapy or radical prostectomy[4]. However, if the patient does not respond to those therapies given the aggressive development of the disease or metastatic stages, androgen deprivation therapy can be used, though it does not always succeed and resistance can be developed[6]. In that aspect, some biological pathways may play an essential role in metastasis and therefore, they may be targets of interest for future therapies.

The role of modulator peroxisome proliferator-activated receptor gamma co-activator 1α (PGC1α) could be of distinguished relevance for prognosis and treatment of PCa. Recent studies from our lab and others have proved the functions of PGC1α as modulator of cell metabolism, regulator of mitochondrial biogenesis and oxidative metabolism and tumour suppressor in metastasis[7,8,9]. Nonetheless, the role of PGC1α is tumour-dependent; its overexpression reduces metastatic stages in some types of cancer, such as prostate and melanoma[8,10], whilst in other types of cancers, breast cancers or renal carcinoma, for instance, it enhances aggressive features of the disease[11,12].

PGC1α functions as a major transcriptional regulator of metabolic gene networks that help normal cells maintain energy homeostasis[13]. On account of that, it interacts with different nuclear receptors and transcription factors acting as an anchor platform, due to its lack of DNA-binding domain (DBD). Furthermore, for modulating its targets LXXLL leucine-rich motifs or NR boxes are used[7]. PGC1α interacts with several targets; among them, the orphan nuclear receptors named estrogen-related receptor α (ERRα)[14]. The ERRα, like other nuclear receptors, have functional domains for binding ligands (LBD) and DBDs, as well as a N-terminal region[15]. The ERRα's LBD is used in the interaction with PGC1α's NR box or with other of its targets[14].

Current studies have demonstrated the significance of the PGC1-ERR signalling axis in cancer. For instance, the ERRγ is believed to be a favourable biomarker in breast tumours, while ERRα expression is linked to reduced survival rates in ovarian tumours[13] and unfavourable progress of breast cancer[16]. The expression of ERRα is also altered in PCa, associating its overexpression to reduced survival rates[13]. Nonetheless, further studies have shown the opposite effect of ERRα when is in complex with PGC1α; ERRα activation

downstream of PGC1α induces a metabolic rewiring that reduces proliferation, shrinks aggressiveness of PCa, and hence, eases metastatic stages[8]. PGC1α-regulated ERRα-dependent transcriptional programme was investigated to a greater extent and it was shown that not only is PGC1α-ERRα axis important for the aggressive features of PCa, but also for its invasiveness and migration[17]. As mentioned, the activity of PGC1α in PCa is linked to an ERRα-regulated metabolic pathway. This interaction is preserved in PCa patient samples, defining a gene signature with predictive significance[8]. Anyhow, the role of PGC1α is conditional on its microenvironment; hence, the targeting of PGC1α-associated pathways might result a profitable therapy for various types of cancer[18].

Even though PGC1α's tumour suppressor effect has been proved[8,9,10] molecular mechanisms remain unclear. Accordingly, the lab performed an RNA sequencing (RNAseq) starting from PCa cells overexpressing PGC1α and obtained a list of genes that were significantly altered upon the co-transcriptional factor was expressed. Next, the co-expression of these genes with PGC1α was evaluated in PCa patients' dataset in order to identify those genes that could have a relevancy in tumour development and progression. As mentioned above, PGC1α is a metabolic transcriptional co-regulator, and so are many of the genes that arose from the analysis in PCa patients. Amongst PGC1α-inversely correlated genes, such as CDK16, MDH2 or PPCB can be found. In the case of directly correlated to PGC1α, there are genes like PDK4, PPP3CC or ASPA[19].

The gene ASPA codes for an enzyme called aspartoacylase which catalyses the conversion of N-aceryl_L-aspartic acid (NAA) into L-aspartate and acetate via deacetylation. This enzyme is found to be bounteous in the brain owing to its important role in the maintenance of white matter; whereas, in other tissues it operates in body fluids, specifically acting as a NAA scavenger[20]. Together with those functions, it is involved in many biological processes, such as aspartate's catabolic process, aspartate family amino acid's metabolic process, central nervous system myelination and positive regulation of oligodendrocyte differentiation[20,21].

Considering the location of ASPA, it is logical to ascertain neurological diseases related to it. The rare neurodegenerative disorder, Canavan disease, is directly associated with the mutations that occur in ASPA; in consequence, the spongy white matter of the brain is degenerated[22]. Nevertheless, ASPA has not been implicated in other diseases until the day; therefore, this project may lead to an interesting discovery of a new mediator of biological event of PGC1α and it could be employed for future therapies.

2. HYPOTHESIS AND AIMS

As mentioned earlier, ASPA has shown direct correlation with PGC1α in preliminary data analysis. This is, while expression of PGC1α-ERRα decreases in advanced stages of PCa, the expression of ASPA is downregulated. Thus, together with PGC1α-ERRα anti-tumour activity, ASPA may result in an effective tumour suppressor in PCa. Hence, the main aims of this project are the following: firstly, analyse ASPA's expression in PCa cell lines and its relationship in regard to PGC1α-ERRα dependant pathway; secondly, determine changes of ASPA's expression in PCa mouse xenografts; and thirdly, correlate patient and cell data, studying the effect of ERRα on ASPA.

3. MATERIALS AND METHODS

3.1 - Cell culture

3.1.1) **PC3 cell line**

Human prostate cancer cell line PC3 provided from Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH was used to carry out the experiments. The origin of this cell line goes back to a 62-year-old Caucasian man with grade IV prostate cancer after androgen suppression therapy. The cells are epithelial-like, growing adherently in monolayers or in multilayer foci[23].

However, once the cell line was purchased, modifications were done to achieve a stable cell line with the characteristics of interest. On the one hand, cells were transduced with a modified TRIPZ doxycycline (Dox)-inducible lentiviral vector containing the sequence of mouse PPARGC1A gene, so that PGC1α expression was inducible via Dox. On the other hand, ERRα knock outs were done using CRIPSR-Cas9 technology, specifically, two single guides (sg) RNA against ERRα were used: sg69 ERRα and sg90 ERRα.

3.1.2) **General procedures**

PC3 cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium) medium, supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. All procedures in cell-culture room were executed in sterile conditions, in laminar flow cabinet and plated cells were stored in incubators with normoxia conditions (21% O_2), 37ºC and 5% CO_2 saturation.

Cell passes were undertaken starting with the absorption of the medium, followed by phosphate-buffered saline (PBS) tampon's wash and detachment from plate with 1 mL trypsin (diluted 1:4 from original stock). Cells were resuspended in certain volume of medium depending on the final dilution.

For freezing, cells were centrifuged (1200 rpm, room temperature, 4 minutes); the supernatant was absorbed and the pellet resuspended with 1 mL of freezing media (FBS+ 10% dimethyl sulfoxide (DMSO)). The volume was transferred to sterile labelled criovials and they were frozen progressively (using a freezing container) until the storage at -80ºC.

Cells' thawing was achieved adding fresh medium into criovials and transferring them into P100 plates (final volume 10 mL), assuring medium was changed the following day to prevent cell's death due to the toxicity of DMSO used for freezing.

Seeding for molecular and cellular assays was performed in presence and absence of Dox (0.5 μg/mL) in technical triplicate for each condition. To count cells, 10 μL of the mixture made of cells and trypan blue dye (1:1) was added to Neubauer's chamber. Once the number of cells was known, an accurate number was seeded according to the type of experiment.

Either cellular or molecular assays were done following a 3+3 treatment; meaning, cells were preinduced with Dox during three days (72 hours) before plating for the experiment and then induced for other 72 hours adding Dox again.

3.2 - Cellular assays – Cell growth curve

5,000 cells per well were seeded in 12 well-plates. Three cell lines and two conditions per each (+Dox/-Dox) were used for experiments, all plated in technical triplicates in a final volume of 1 mL. Growth of cells was

measured at day 0, day 3 and day 6. In order to fix cells, medium was absorbed, cells were washed with PBS 1x, fixed in the plate with 10% formalin (1 mL/well) and stored at 4ºC.

When the whole experiment was collected, formalin was discarded, PBS washings were done twice and 0.5 mL of 0.1% crystal violet (in 20% methanol) was added to each well. After shaking the plates at room temperature (RT) for 30-60 minutes, plates were cleaned with distilled water (until water came out transparent) and left to dry overnight.

Crystal violet was resuspended in 0.5 mL of 10% acetic acid per well, shaking plates for 30-60 minutes at RT. Once a homogeneous mix was obtained, 70 µL of each sample was displaced to 96 well-plate and optical density was read at 570 nm wavelength using the PowerWave XS Microplate Spectrophotometer provided by BioTek.

3.3 - Molecular assays

3.3.1) **Gene expression analysis**

RNA extraction

75,000 cells were seeded per well in 6 well-plates in a final volume of 2 mL, in which 3+3 treatment was done.

For RNA extraction, firstly medium was absorbed, plates were washed with PBS and directly used for RNA extraction with NucleoSpin® RNA isolation kit provided by Macherey-Nagel (ref: 740955.240C) and following user manual supplied. The method is based in filtered columns and elutions, attaining RNA concentrated in RNAse free water by the end, which was quantified with BioDrop microvolume measurement platform supplied from Biochrom.

RNA retrotranscription

For further analysis, 1 µg of RNA was retrotranscribed to complementary DNA (cDNA). The final volume of the reaction was 10 µl, containing RNA, the enzyme Thermo Scientific Maxima H Minus cDNA synthesis Master mix (5x) and mqWater. The retrotranscription programme used was the following: 10 minutes at 25ºC, 15 minutes at 50ºC and 5 minutes at 85ºC.

Quantitative real time Polymerase Chain Reaction (qPCR)

For qPCR, cDNA was diluted in 1:10 ratio with mqWater and 3 µL of each sample were added in triplicates to a 384 well-plate, together with 3 µL mix for qPCR which contained a qPCR master mix 2x Fast Start Universal SYBR® Green Master (ROX) – it included all the reagents (except primers and template) needed for running a qPCR (buffer, dNTPs, thermostable DNA polymerase and SYBR® green dye) – and primers (supplementary T1) for the genes of interest: human ASPA, mouse PGC1α and human GAPDH.

The programme used by QuantStudio 5 Real-Time PCR Instrument (384-Well Block) supplied from Applied Biosystems was the following: hold stage consisted of 2 minutes at 50ºC, followed by 10 minutes at 95ºC; PCR stage consisted of 15 seconds cycles (40 cycles) at 95ºC and 1 minute at 60ºC. Lastly, melt curve stage contained 15 seconds at 95ºC, 1 minute at 60ºC and 15 minutes at 95ºC. After that, a picture was taken and another cycle began with denaturalisation at 95ºC.

To quantify gene amplification SYBR® green molecule was used. This method is based on the fact that the molecule binds to dsDNA and it emits fluorescence. The molecule binds inespecifically all the double strands of DNA, thus the primers assure the specificity of the amplifications of the genes. Hence, fluorescence raise is proportionally related to the amount of amplified cDNA and in consequence, it is possible to quantify it[24].

3.3.2) **Protein expression analysis**

Protein extraction and quantification

For protein experiments, the same setup of the RNA plating was done - 75,000 cells per well in 6 well-plates in a final volume of 2 mL, with 3+3 treatment.

To lyse the cells, 75 µL of RIPA lysis buffer (supplementary T1) were added per well. Lysates were collected in Eppendorf tubes, incubated on ice for up to 20 min vortexing every 5min and then centrifuged at 15000 rpm at 4ºC for 15 min. Supernatant was carefully transferred to a new tube and protein concentration was determined using Pierce™ BCA protein Assay Kit (ThermoFisher, reference: 23225). Due to the reaction that occurred when combining the solutions of the kit, colorimetric detection is employed using the PowerWave XS Microplate Spectrophotometer provided by BioTek.

A standard curve was built per protein quantification with known concentrations of bovine serum albumin (BSA, 2mg/mL) in duplicates and the information attained could be used to deduce protein concentrations in triplicates of samples.

Electrophoresis and Western Blot

For the analysis of protein expression, SDS-PAGE technique was performed, in order to separate proteins based on their size. This was achieved thanks to the addition of the Laemmli Loading buffer 5x (supplementary T1) in the samples, which, besides, enabled to get the same initial protein concentration together with water. Adding SDS assured that all proteins were negatively charged and denatured, meaning that the only factor determining the proteins' migration in the gel was their size. For that, Criterion™ XT precast gels provided from BioRad were used (reference catalogue #3450123 and #3450125) and as protein marker the Nippon MWP02, DDBiolab protein weight marker was used. The precast gel consisted on an increasing acrylamide concentration (gradient Bis-Tris 4-12%). Proteins were added in the gels after denaturalisation (5 minutes at 95ºC) and they were run in MOPS 1x buffer at 150-180V.

After electrophoresis, transfer of the protein from the gel to nitrocellulose membrane was done in cold transfer buffer (supplementary T1) at 100V for 1h.

After the transfer, the membrane was stained with Ponseau to make sure proteins were transferred correctly to the nitrocellulose membrane.

Membrane blocking was done prior to primary antibody incubation with 5% milk in TBST (supplementary T1) through an hour. After TBST wash, membranes were incubated overnight at 4ºC with the following primary antibodies: PGC1α (Santa Cruz PGC h300 #13067), ERRα (Cell Signaling Technology #13826), ASPA (Abcam #223269) and GAPDH (Cell Signaling Technology #2118L). ERRα and ASPA were used at 1:1000 dilutions in TBST+0,002% Sodium Azide, PGC1α at 1:1000 in TBST-5% milk and GAPDH at

1:2000 in TBST+0,002% Sodium Azide. Prior to secondary antibody incubations, three TBST washings of 10 minutes were done. Secondary anti rabbit antibody (Vitro s.a. S30111-035-144) was diluted at 1:4000 in 5% milk in TBST and incubation of it was executed within an hour in the shaker, doing three consecutive TBST washings after it.

In order to re-use the membrane, stripping of the membrane was done using a NaCl 5% solution during 5 minutes, obtaining a membrane without antibodies and therefore, it was possible to block and incubate it again with another antibody.

For the detection of the proteins, membranes were incubated 5 min with commercial Clarity Max ECL purchased from BioRad (Solution A+Solution B 1:1 ratio). This technique lied in the employment of secondary antibodies conjugated with the horseradish peroxidase. When the substrate of the enzyme (luminol) was added, chemioluminiscence could be measured at 428 nm by virtue of the light emitted in the reaction. The chemiluminescence signal was detected using ChemiDoc Imaging system[25].

3.3.3) **Xenograft Samples**

cDNA and protein extracts from xenograft samples previously generated in the lab were used[17]. The conditions analysed in the xenografts were four: control –Dox, control +Dox, sg69 ERRα –Dox and sg69 ERRα +Dox.

3.4 - Statistical analysis

GraphPad Prism 8.0.1 software was employed for statistical analysis. The confidence level applied for all test was %95 (significant values below $\alpha = 0'05$).

A minimum of three biological replicates were performed per experiment. Data was analysed assuming Gaussian distribution, either in *in vitro* samples and in *in vivo* western blot samples, in exception of qPCR data of xenografts, in which non-normality was used, because normality test was negative.

One sample t-test was applied in growth curves and *in vitro* data for comparing normalised conditions. This test compared the mean of the samples with a hypothetical mean (1 for every case).

For the comparison of two components, student T test was employed. Unlike xenograft samples, in the case of cell lines, parametric paired T test was used in which differences between paired values were consistent; for xenograft samples, non-parametric unpaired was utilised.

For correlations, Cancertool[26] was used in which gene expression correlation analyses graphs are obtained based on gene expression data from different clinical PCa datasets. In the graphs Spearman correlation coefficient and p-values are shown.

4. RESULTS

4.1 - Validation of the cellular model

To validate the anti-proliferative capabilities of PGC1α in PC3 TRIPZ-HA-PGC1α cells with or without ERRα, growth curves were performed. As it is shown in Figure1, in the presence of ERRα PGC1α reexpression in PC3 cells induced a reduction in cell proliferation at Day3 that was more pronounce at Day

6. Validating previous studies from the lab that the anti-proliferative effect of PGC1α was driven by ERRα, as the knocking out of ERRα results in a rescue of PC3 TRIPZ-HA-PGC1α cell growth.

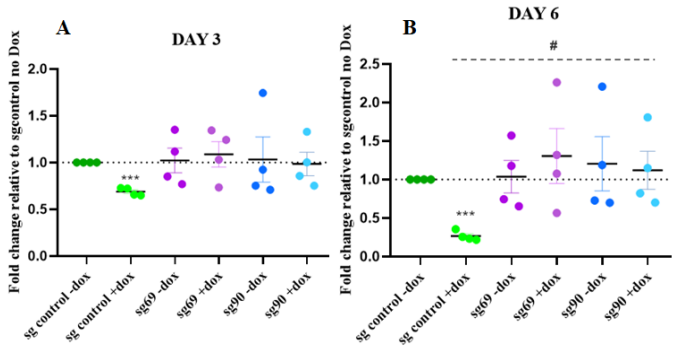

Figure 1. Growth analysis of PC3 TRIPZ-HA-PGC1α sgcontrol ERRα and ERRα deletion, sg69 ERRα and sg90 ERRα. A) Fold change relative to non-treated sgcontrol ERRα in day 3. B) Fold change relative to non-treated sgcontrol ERRα in day 6. Asterisks (*) represent statistical difference between 1.0 and each condition; pounds (#) represent statistical difference between the two compared conditions. # indicate p-value < 0'05 and *** indicate p-value < 0'001. Error bars denote standard error of the mean (SEM).

We performed PGC1α gene expression analysis by qPCR in cell lines. PC3 TRIPZ-HA-PGC1α sgERRα knock-out cell lines' (sg69 and sg90) and PC3 TRIPZ-HA-PGC1α sgERRα control (from now on referred as sg69 ERRα, sg90 ERRα and sgcontrol ERRα, respectively) in conditions of Dox and no Dox were studied with that purpose. The results attained assured the induction system of the cell lines is functioning properly. Hence, when Dox is added, cells overexpress PGC1α with a considerably significant difference towards no treated cell lines. The expression of PGC1α is not influenced by the deletion of ERRα, just by the treatment of Dox (fig. 2).

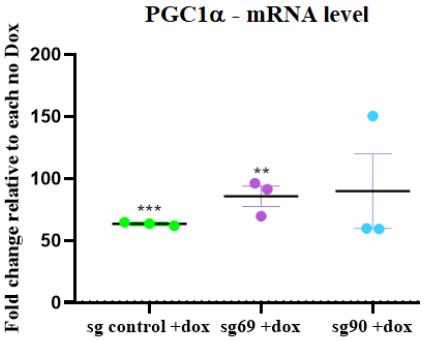

Figure 2. Effect of Doxycycline's induction on the expression of PGC1α in cell lines PC3 TRIPZ-HA-PGC1α sgcontrol ERRα and ERRα knock-outs analysed by qPCR. Fold change of PGC1α relative to non-treated cell lines. Asterisks (*) represent statistical difference between 1.0 and each condition. ** indicate p-value < 0'01 and ***, p < 0'001. Error bars denote standard error of the mean (SEM).

4.1.2) **ASPA is regulated by PGC1α through ERRα in cell lines**

As already mentioned, a previous study proved PGC1α's tumour suppressor activity is subject to ERRα interaction[8]. Taking into consideration that ASPA is one of the genes associated with PGC1α expression both in cell lines (RNA Seq from the lab) and in prostate cancer patients (fig 3.), we aimed at first, monitoring and confirming the regulation of ASPA expression *in vitro* by PGC1α-ERRα axis.

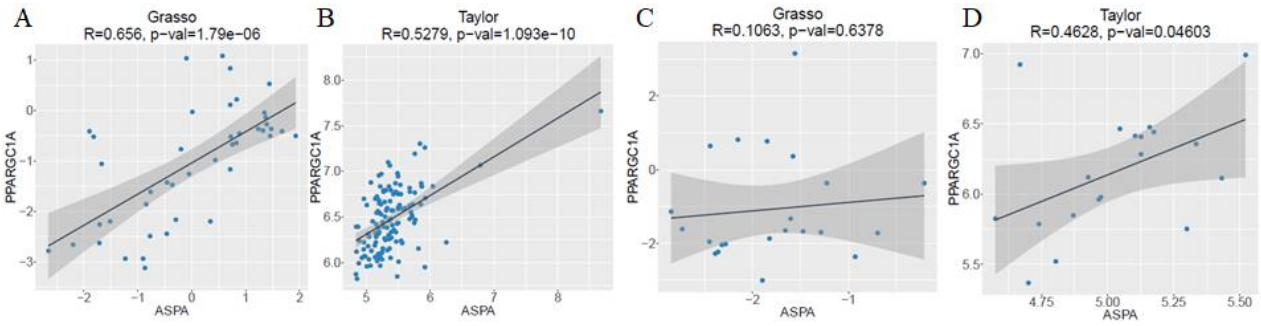

Figure 3. Correlation analysis between ASPA and PGC1α expression in primary tumours (A and B) and metastasis (C and D) in prostate cancer datasets Grasso and Taylor.

To determine whether ERRα is required for PGC1α-driven ASPA regulation, sgERRα knock-out cell lines' ASPA expression was analysed at mRNA and protein level, together with sgcontrol ERRα in conditions of Dox (PGC1α's overexpression) and no Dox.

In regard to ASPA expression, results obtained from qPCR revealed that it is expressed differently in the various conditions. In the case of sgERRα control cell line, it is shown that PGC1α re-expression (Dox treatment) increased ASPA's mRNA expression significantly; whereas, in the case of the ERRα knock-outs, ASPA's mRNA reduces significantly, with or without the induction of PGC1α. In sg90 ERRα Dox treated cells, even the decrease in the fold change is not significant, the tendency for the downregulation can be suspected (fig. 4A, asterisks *). Furthermore, when comparing the effect of ERRα in PGC1α overexpressing conditions (fig. 4A, pound #), we concluded that ASPA is reduced significantly in the ERRα knock-outs, compared to control Dox-treated cells. Overall, ASPA mRNA expression is regulated by PGC1α through ERRα.

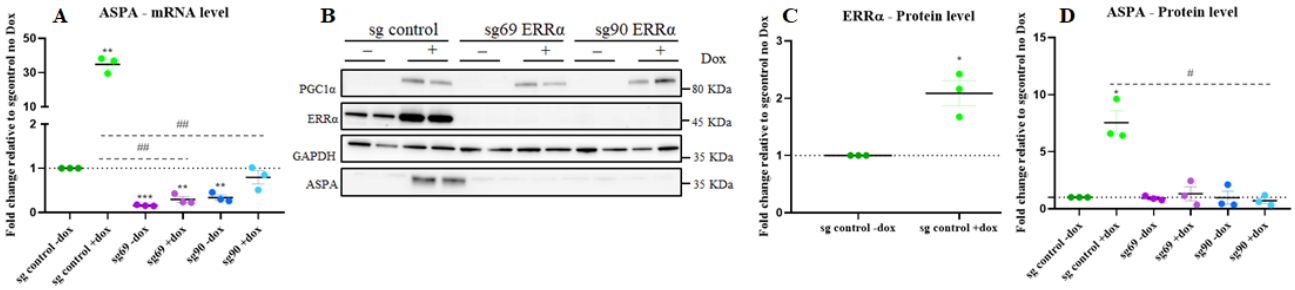

Figure 4. Effect of PGC1α induction (via Doxycycline treatment) and ERRα deletion in ASPA's expression in cell lines PC3 TRIPZ-HA-PGC1α sgcontrol ERRα and ERRα knock-outs analysed by qPCR (A) and western blot (B-D). A) Fold change of mRNA data is calculated relative to non-treated sgcontrols. B) PGC1α, ERRα, ASPA and the housekeeping gene GAPDH (used for normalisation) are shown in nitrocellulose membrane. C) Fold change of ERRα in controls. D) Fold change of ASPA relative to non-treated sgcontrols. Asterisks (*) represent statistical difference between 1.0 and each condition; pounds (#) represent statistical difference between the two compared conditions. * or # indicate p-value < 0'05; ** or ##, p < 0'01 and ***, p < 0'001. Error bars denote standard error of the mean (SEM).

Verification of induction system at protein level proved that cell lines treated with Dox do overexpress PGC1α, while in the case of non-treated cell lines the expression of the protein is undetectable (fig. 4B). Furthermore, PGC1α induces ERRα expression as it can be seen in Figure4C. Nevertheless, this induction is not observable at the protein level in the knock-outs. Therefore, this proves CRISPR-Cas9 system has worked properly and it has removed ERRα's gene from cells' genomes.

The results obtained in the qPCR analysis were confirmed at the protein level. As it is represented in figure 4D, ASPA expression is increased when PGC1α is re-expressed in PC3 cells. However, in the ERRα knock-outs, owing to the lack of ERRα, ASPA is barely expressed and the difference in contrast to non-treated control cells is not big enough to detect it (fig. 4D).

4.1.3) **Validation of ASPA regulation in PGC1α-ERRα Xenograft samples**

In order to validate our *in vitro* results in a *in vivo* scenario, we took advantage of RNA and protein samples from xenograft tumours that the lab previously obtained from PC3 TRIPZ PGC1α sgControls and sgERRα cells[17].

In regard to the xenograft samples, results achieved were in line with cell lines', meaning that Dox treatment induces PGC1α's expression. Notwithstanding, to the contrary of cell lines, ERRα knock-out does affect PGC1α's expression, either in non-treated and treated conditions (fig. 5A). Moreover, the expression of PGC1α is influenced by the deletion of ERRα, and not only by the treatment of Dox. If non-treated xenograft samples were compared, in the knock-out PGC1α's expression increased significantly relative to the control (fig. 5A).

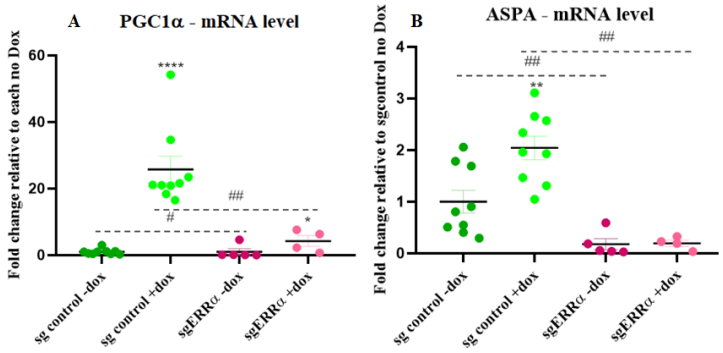

Figure 5. Effect of PGC1α induction (via Doxycycline treatment) and ERRα deletion in ASPA's expression in ERRα knocked out and non-knocked out xenograft samples analysed by qPCR. A) Fold change of PGC1α relative to non-treated xenografts. B) Fold change of ASPA relative to non-treated sgcontrol xenografts. Asterisks (*) represent statistical difference between non-treated or treated conditions; pounds (#) represent statistical difference between the two compared conditions. # indicate p-value < 0'05; ** or ##, p < 0'01 and ****, p < 0'0001. Error bars denote standard error of the mean (SEM).

<u>ASPA downregulation at mRNA level in Xenograft samples</u>

Given the direct correlation between PGC1α and ASPA *in vitro* (fig. 4A and 4D) and in patient data (fig. 3), xenografts were employed for the study of ASPA's regulation in PCa tumours. As we predicted and in line with *in vitro* data, at mRNA level ASPA is upregulated in tumours expressing PGC1α and ERRα when compared to the control ones (sgControl – Dox). Nevertheless, in comparison, in Dox treated ERRα knock-outs tumours, due to lack of ERRα, PGC1α is no longer able to induce ASPA expression, as well as in non-treated xenografts samples, in line with cell lines' results (fig. 5B). Therefore, qPCR results prove ERRα's absence hampers ASPA's transcription. These results purport to emerge that ASPA is regulated by PGC1α through ERRα expression.

PGC1α does not regulate ASPA protein levels *in vivo*

At protein level, cell lines and xenografts led to the same conclusion concerning the induction system, as xenografts samples overexpress PGC1α, associated to an upregulation of endogenous ERRα (fig. 6A); nonetheless, in this case, there are significant differences in PGC1α expression due to ERRα, PGC1α being more expressed in the knock-outs as it can be seen in Figure6A, on the contrary to what resulted at mRNA level. This could suggest the knocked out xenografts are more sensitive to PGC1α expression. In addition, ERRα's expression increases significantly with Dox induction in control xenografts, alike in cell lines (fig. 6A). As well as in cell lines, CRISPR-Cas9 procedure has functioned and deletion of ERRα has been achieved.

ASPA's transcription was analysed, alongside translation to protein levels. Even though xenografts' results at mRNA concur with results obtained in cell lines, expression of ASPA at protein level in xenografts does not harmonise with cell lines. Unlike hypothesised, results acquired in the Western Blot demonstrate that ASPA is not modulated by PGC1α. In fact, ASPA is slightly reduced in Dox samples, even if the difference is non-significant (fig. 6B). On account of that, it could be gathered that *in vivo* ASPA is not regulated by PGC1α, unlike results show till the point.

Furthermore, in regard to the effect of ERRα's deletion, it seems ASPA's expression is stabilised or increased, rather than being downregulated, since the results of the Western Blot show no significant changes of ASPA in comparison to control mouse. Nonetheless, this increase is only significant in Dox condition, as when mouse were non-treated, ASPA's expression did increase but not significantly (fig. 6B).

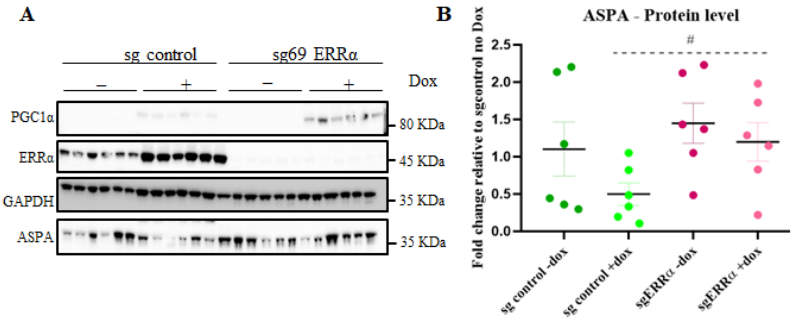

Figure 6. Effect of PGC1α induction (via Doxycycline treatment) and ERRα deletion in ASPA's expression in ERRα knocked out and non-knocked out xenograft samples analysed by Western Blot. A) PGC1α, ERRα, ASPA and the housekeeping gene GAPDH (used for normalisation) are shown in nitrocellulose membrane. B) Fold change of ASPA calculated relative to non-treated sgcontrols. Pounds (#) represent statistical difference between the treated conditions. # indicate p-value < 0'05. Error bars denote standard error of the mean (SEM).

4.2 - Data mining

As mentioned, ASPA's protein expression in mouse xenografts is different from hypothesised. Hence, further analyses were done to discuss that fact. Following that aim, mRNA correlation analyses were done in prostate cancer patients: on the one hand, between ASPA and ERRα and on the other hand, between ASPA and ERRα target genes. As shown in Figure7A, ERRα mRNA expression is inversely correlated to ASPA in PCa's primary tumours. Therefore, this would not pursue our initial hypothesis; this is, in PCa patients, while aggressiveness of the disease expands towards metastasis, ERRα decreases and ASPA increases.

Notwithstanding, it is of relevance to mention that direct correlation in patients between PGC1α and ERRα does not exist in primary tumours and if it exists, it is inverse (fig. 7B). In consequence, as in the case of the correlation between ASPA and ERRα, this would not support *in vitro* results; however, correlation between PGC1α and ERRα targets is observable (fig. 7C). Thus, this would suggest that the initial hypothesis may not be wrong and there are other unknown factors that are involved in the correlation between ASPA and ERRα or PGC1α and ERRα that makes them be opposite to hypothesised.

Apart from that, ERRα's targets correlation with ASPA was analysed. Three targets were chosen (ANGPT1, MAP1A and RNF128) and as it can be observed in Figure7D, they are directly correlated in some of the datasets. This would suggest the initial hypothesis could be correct, meaning that when ERRα is upregulated, and therefore, its targets, ASPA is also upregulated, as seen in cell lines and mouse xenograft at mRNA level, as happens in the correlation between PGC1α and ERRα targets (fig 7D).

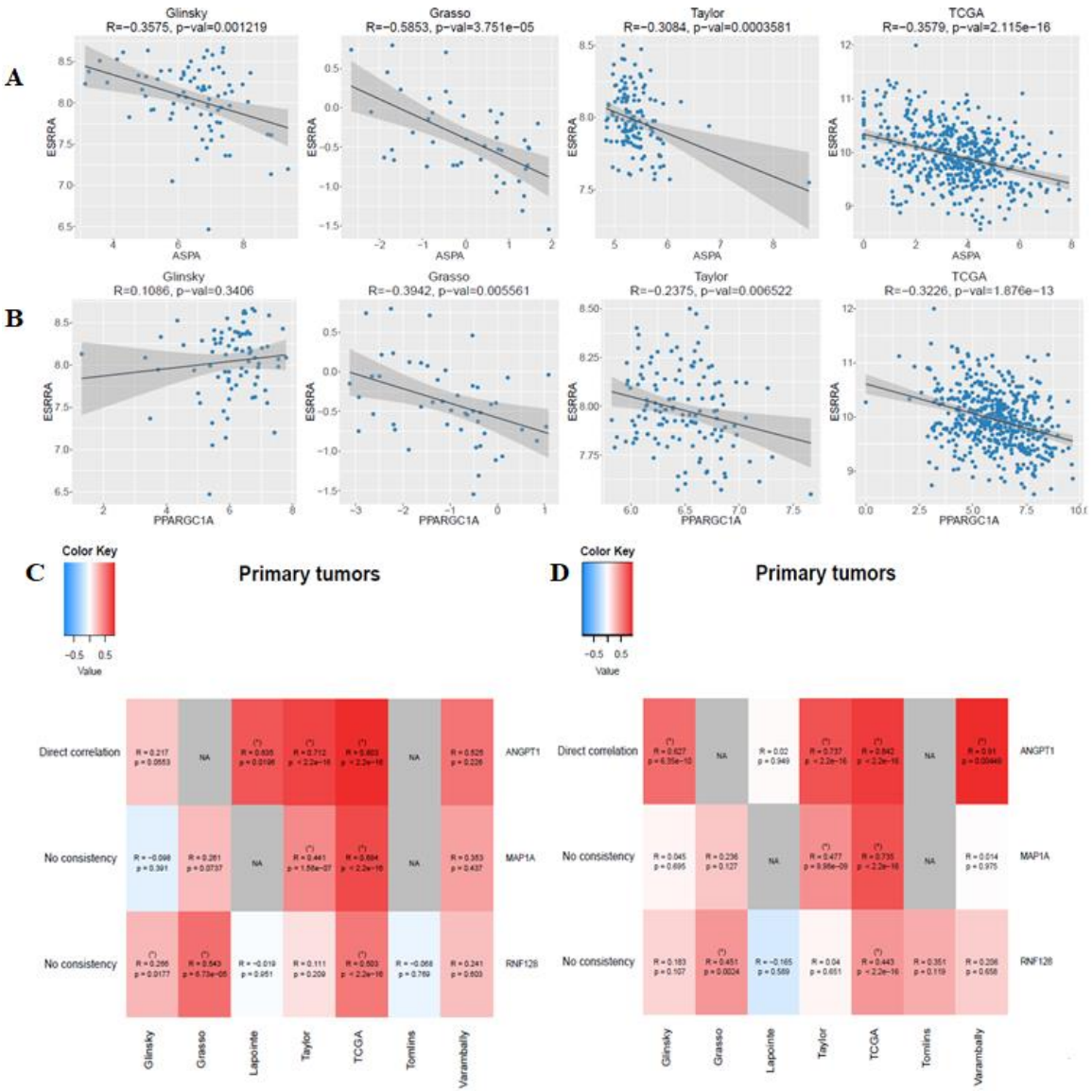

Figure 7. A) Correlation analysis between ASPA and ERRα expression in primary tumours in prostate cancer datasets. B) Correlation analysis between PGC1α and ERRα expression in primary tumours in prostate cancer datasets. C) Correlation analysis between PGC1α and ERRα's targets ANGPT1, MAP1A and RNF128 expression in primary in prostate cancer datasets. D) Correlation analysis between ASPA and ERRα's targets ANGPT1, MAP1A and RNF128 expression in primary in prostate cancer datasets. NA = Non Applicable; datasets contain insufficient number of samples to correctly perform the analysis.

5. DISCUSSION

PGC1α expression has been proved to be altered in several chronic diseases and it has showed critical control function in cancer development[18]. PGC1α has dualistic character in changing metabolic states to maximise metastatic processes. This is reflected with the diverse expression dependent in tumour-type and the regulation of those expression levels[10]. The coregulator has also been employed as an efficient target in anticancer therapy[12,27]. As proved with studies till the day, PGC1α acts as a tumour suppressor through ERRα in PCa, ceasing disease progression till metastatic stages[8,10]. PGC1α-ERRα axis regulates the transcriptional programme, targeting a gene of interest and activating transcription[28] and this has great relevance in the metabolic reprogramming of cancer cells[13]. In fact, in the case of PCa, this axis is responsible for the invasive phenotype[17].

Preliminary data from our lab (RNAseq and mRNA correlation analysis) suggested ASPA was one of the clinically relevant genes associated with PGC1α whose expression was affected in PCa too. Other studies in the field have also emerged ASPA's interest in PCa, especially as a novel anticancer therapy based on stromal cells targeting[29,30]. According to bioinformatical analyses executed by *Sun* and colleagues, ASPA, together with other genes, seems to be involved in PCa and they could be used as stromal molecular signature, due to their nature of stromal feature gene. Nonetheless, experiments were not performed in the study. Thus, even though the findings laid bare on the molecular mechanisms applied in cancer, it was recognised that further studies were needed[30]. Our study could be profitable in that aspect, as the basis of ASPA's deregulation in PCa have been investigated.

In our study the possible molecular mechanisms of ASPA's regulation came to light. Prior to the study, it was observed that ASPA expression was downregulated as the disease progressed, suggesting its tumour suppressor activity. In addition, it was observed that ASPA was directly correlated to PGC1α expression. This led to the hypothesis of ASPA expression being driven by PGC1α-ERRα, which also operates as suppressor of tumours. That is to say, proliferation of cancerous cells would decrease with the rise of ASPA expression.

In vitro was observed that, ASPA is regulated by PGC1α through ERRα. Nonetheless, deletion of ERRα by itself induced downregulation of ASPA in comparison to the control cells (not expressing PGC1α) (fig. 4), meaning that the only condition in which ASPA is increased is when both, PGC1α and ERRα, are expressed. Thus, this study reveals that in cell lines either at mRNA level and protein level, ASPA is regulated by ERRα, instead of being directly regulated by PGC1α expression.

When data was inferred to *in vivo*, results were conforming to cell lines' in regard to mRNA. However, when analysing protein expression, there was no consistency, as the results showed the opposite of thought. Induction of PGC1α instead of upregulating ASPA's expression, it decreased it. Furthermore, ERRα knock-outs had no effect; indeed, when comparing PGC1α induced xenograft samples, the knocked out samples overexpressed ASPA, which would make us reject our hypothesis.

There may be several reasons for this difference to arose *in vivo* at mRNA and protein levels. On the one hand, the stabilisation of the protein coded by ASPA, aspartoacylase, may be altered in xenograft samples. It has been proved ASPA possesses a glycosylation motif which is engaged in the maintenance of the protein's

stability and the catalytic activity[31]. Nevertheless, all the outcomes regarding ASPA are linked to brain-tissue, since its expression is predominant in the kidney and the white matter[31]; hence, the lack of tissue specific information hinders to break into the mentioned inconsistency regarding stabilisation.

On the other hand, the samples themselves could be the explanation for achieving different expression levels of ASPA at qPCR and Western Blot experiments. It is essential to bear in mind the way xenografts samples were obtained. Engrafting cultured prostate cancer cells into immunocompetent mice produced cell line xenograft models[32]. The cells of interest, in this case sgcontrol ERRα and knocked out cell line sg69 ERRα, were injected subcutaneously[33] and not directly in the prostate.

The fact of injecting prostate cancer subcutaneously entailed other type of cells could be attached to PCa cells when the tumour was extracted. This denotes our samples would be contaminated with other tissue specific cells. This could be a possible reasoning for the difference in the xenograft samples concerning ASPA's expression. Given the case mouse cancer cells were extracted together with the human prostate cancer in the tumour, when ASPA's expression was analysed, both mouse and human cells' ASPA would be measured at protein level. This happens owing to the lack of species specificity of the antibodies. The primary antibody of ASPA (Abcam #223269) reacts with mouse, rat and human; therefore, in the event that samples were contaminated with mouse cells, apart from observing human ASPA (coming from prostate cancer cells), we would also see mice's ASPA.

On the contrary, at mRNA level the same did not occur. The primers employed were specific for human ASPA, thus, mouse ASPA was neither amplified nor measured. Thus, we claim the expression of ASPA is PCa cell line specific. Therefore, Western Blot results at protein level may not be accurate to human ASPA.

Other possibility could be that the hypothesis is not correct and the regulation of ASPA follows another mechanism. As for the molecular mechanisms of ASPA's regulation at mRNA level (fig 6.), it is observed deletion of ERRα affects negatively in ASPA's expression, as it was hypothesised. Even though ERRα is mostly a transcriptional activator, with just a few reports of it suppressing target gene expression[17], ERRα may not be implicated in ASPA regulation as thought. When observing ERRα's effect on its own, it is visible ASPA's expression decreases significantly. However, when the effect of ERRα is analysed together with PGC1α overexpression, the drop-off in expression of ASPA is also significant. This could suggest that ASPA instead of being regulated by ERRα, it could be regulated by PGC1α overexpression.

Correlation analysis between ASPA and ERRα in primary tumour would be in line with the mentioned possibility, as they are negatively correlated (fig. 7A). Nevertheless, ERRα's other targets, which together with ASPA appeared directly correlated to PGC1α in the RNAseq performed by the lab, were directly correlated to ASPA in several PCa datasets' primary tumours (fig. 7B). Therefore, this could mean that ERRα itself affects in a specific manner, but when acting with PGC1α, the initial hypothesis would be plausible. Regarding this fact, the regulation of ASPA through ERRα could not be dismissed.

It is of relevance to mention that opposed to what we hypothesised, ASPA might not to be involved in the proliferation of the cells. It was thought that increasing in ASPA's expression would lead to a decrease in proliferation, as it happens with PGC1α. However, those cell lines expressing less ASPA did not proliferate significantly more than non-knocked out cells (fig. 1). It seems proliferation of cells is not sensitive to

ASPA. Therefore, the direct implication of ASPA in the proliferation has not been proved with this study; hence, more studies should be carried through to correlate ASPA with PCa cells proliferation. Silencing or knock-out of ASPA would prevent from its upregulation and in consequence, ASPA's effect in proliferation could be tested.

6. CONCLUSIONS

On the whole, this study has identified ASPA's expression is regulated by PGC1α-ERRα axis. *In vitro,* PGC1α regulates expression of ASPA through ERRα, either at mRNA level or protein level. Besides, ERRα itself regulates the expression of ASPA; as a general rule, deletion of ERRα leads to ASPA's downregulation. However, *in vivo*, expressions of ASPA at mRNA and protein level do not correlate. Hence, this study provides opportunities for ASPA to be used as a biomarker and to be a target for future therapies; anyhow, more research efforts are needed to disclose the precise molecular mechanisms underlying ASPA's regulation and to expand its applicability.

7. BIBLIOGRAPHY

1. Cancer Today. Accessed June 14, 2021. https://gco.iarc.fr/today/home
2. Merriel SWD, Funston G, Hamilton W. Prostate Cancer in Primary Care. *Adv Ther*. 2018;35(9):1285-1294. doi:10.1007/s12325-018-0766-1
3. Balk SP, Ko YJ, Bubley GJ. Biology of prostate-specific antigen. *J Clin Oncol*. 2003;21(2):383-391. doi:10.1200/JCO.2003.02.083
4. Castillejos-Molina RA, Gabilondo-Navarro FB. Prostate cancer. *Salud Publica Mex*. 2016;58(2):279-284. doi:10.21149/spm.v58i2.7797
5. Ilic D, Djulbegovic M, Jung JH, et al. Prostate cancer screening with prostate-specific antigen (PSA) test: A systematic review and meta-analysis. *BMJ*. 2018;362. doi:10.1136/bmj.k3519
6. Mansinho A, Macedo D, Fernandes I, Costa L. Castration-resistant prostate cancer: Mechanisms, targets and treatment. In: *Advances in Experimental Medicine and Biology*. Vol 1126. Springer New York LLC; 2018:117-133. doi:10.1007/978-3-319-99286-0_7
7. Bost F, Kaminski L. The metabolic modulator PGC-1α in cancer. *Am J Cancer Res*. 2019;9(2):198-211. Accessed June 14, 2021. http://www.ncbi.nlm.nih.gov/pubmed/30906622
8. Torrano V, Valcarcel-Jimenez L, Cortazar AR, et al. The metabolic co-regulator PGC1α suppresses prostate cancer metastasis. *Nat Cell Biol*. 2016;18(6):645-656. doi:10.1038/ncb3357
9. Luo C, Lim JH, Lee Y, et al. A PGC1α-mediated transcriptional axis suppresses melanoma metastasis. *Nature*. 2016;537(7620):422-426. doi:10.1038/nature19347
10. Luo C, Widlund HR, Puigserver P. PGC-1 Coactivators: Shepherding the Mitochondrial Biogenesis of Tumors. *Trends in Cancer*. 2016;2(10):619-631. doi:10.1016/j.trecan.2016.09.006
11. Klimcakova E, Chénard V, McGuirk S, et al. PGC-1α promotes the growth of ErbB2/neu-induced mammary tumors by regulating nutrient supply. *Cancer Res*. 2012;72(6):1538-1546. doi:10.1158/0008-5472.CAN-11-2967
12. LaGory EL, Wu C, Taniguchi CM, et al. Suppression of PGC-1α Is Critical for Reprogramming Oxidative Metabolism in Renal Cell Carcinoma. *Cell Rep*. 2015;12(1):116-127. doi:10.1016/j.celrep.2015.06.006
13. Deblois G, St-Pierre J, Giguère V. The PGC-1/ERR signaling axis in cancer. *Oncogene*. 2013;32(30):3483-3490. doi:10.1038/onc.2012.529
14. Di W, Lv J, Jiang S, et al. PGC-1: The energetic regulator in cardiac metabolism. *Curr Issues Mol Biol*. 2018;28:29-46. doi:10.21775/cimb.028.029
15. Giguère V. To ERR in the estrogen pathway. *Trends Endocrinol Metab*. 2002;13(5):220-225. doi:10.1016/S1043-2760(02)00592-1
16. Fujimura T, Takahashi S, Urano T, et al. Differential

expression of estrogen-related receptors β and γ (ERRβ and ERRγ) and their clinical significance in human prostate cancer. *Cancer Sci*. 2010;101(3):646-651. doi:10.1111/j.1349-7006.2009.01451.x

17. Valcarcel-Jimenez L, Macchia A, Crosas-Molist E, et al. PGC1a suppresses prostate cancer cell invasion through ERRA transcriptional control. *Cancer Res*. 2019;79(24):6153-6165. doi:10.1158/0008-5472.CAN-19-1231
18. Tan Z, Luo X, Xiao L, et al. The role of PGC1α in cancer metabolism and its therapeutic implications. *Mol Cancer Ther*. 2016;15(5):774-782. doi:10.1158/1535-7163.MCT-15-0621
19. Home - Gene - NCBI. Accessed June 14, 2021. https://www.ncbi.nlm.nih.gov/gene/
20. Roscoe RB, Elliott C, Zarros A, Baillie GS. Non-genetic therapeutic approaches to Canavan disease. *J Neurol Sci*. 2016;366:116-124. doi:10.1016/j.jns.2016.05.012
21. Francis JS, Wojtas I, Markov V, et al. N-acetylaspartate supports the energetic demands of developmental myelination via oligodendroglial aspartoacylase. *Neurobiol Dis*. 2016;96:323-334. doi:10.1016/j.nbd.2016.10.001
22. Kaul R, Gao GP, Balamurugan K, Matalon R. Canavan disease: Molecular basis of aspartoacylase deficiency. *J Inherit Metab Dis*. 1994;17(3):295-297. doi:10.1007/BF00711811
23. German Collection of Microorganisms and Cell Cultures GmbH: Details. Accessed June 14, 2021. https://www.dsmz.de/collection/catalogue/details/culture/ACC-465
24. SYBR Green for qPCR | Bio-Rad. Accessed June 14, 2021. https://www.bio-rad.com/featured/en/sybr-green-for-qpcr.html
25. ECL western blotting substrates for horseradish peroxidase (HRP) detection. | Enhanced Luminol | Life Science Research | Bio-Rad. Accessed June 14, 2021. https://www.bio-rad.com/featured/en/ecl-western-blotting-substrates.html
26. Cortazar AR, Torrano V, Martín-Martín N, et al. Cancertool: A visualization and representation interface to exploit cancer datasets. *Cancer Res*. 2018;78(21):6320-6328. doi:10.1158/0008-5472.CAN-18-1669
27. Vazquez F, Lim JH, Chim H, et al. PGC1α Expression Defines a Subset of Human Melanoma Tumors with Increased Mitochondrial Capacity and Resistance to Oxidative Stress. *Cancer Cell*. 2013;23(3):287-301. doi:10.1016/j.ccr.2012.11.020
28. Schreiber SN, Knutti D, Brogli K, Uhlmann T, Kralli A. The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor α (ERRα). *J Biol Chem*. 2003;278(11):9013-9018. doi:10.1074/jbc.M212923200
29. Van Der Heul-Nieuwenhuijsen L, Hendriksen PJM, Van Der Kwast TH, Jenster G. Gene expression profiling of the human prostate zones. *BJU Int*. 2006;98(4):886-897. doi:10.1111/j.1464-410X.2006.06427.x
30. Sun C, Gu Y, Chen G, Du Y. Bioinformatics Analysis of Stromal Molecular Signatures Associated with Breast and Prostate Cancer. *J Comput Biol*. 2019;26(10):1130-1139. doi:10.1089/cmb.2019.0045
31. Sommer A, Sass JO. Expression of aspartoacylase (ASPA) and Canavan disease. *Gene*. 2012;505(2):206-210. doi:10.1016/j.gene.2012.06.036
32. Lin D, Xue H, Wang Y, et al. Next generation patient-derived prostate cancer xenograft models. *Asian J Androl*. 2014;16(3):407-412. doi:10.4103/1008-682X.125394
33. Wu X, Gong S, Roy-Burman P, Lee P, Culig Z. Current mouse and cell models in prostate cancer research. *Endocr Relat Cancer*. 2013;20(4). doi:10.1530/ERC-12-0285

SUPPLEMENTARY INFORMATION

Supplementary T1. Composition of the buffers employed for experiments

Buffer	Composition
RIPA lysis buffer	50 mM TrisHCl pH 7'5, 150 mM NaCl, 1 mM EDTA, 0'1% SDS, 1% Nonidet P40, 1% sodium deoxycholate, 1mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM betaglycerophosphate and protease inhibitor cocktail (Roche)
Laemli loading buffer 5x	10% SDS, 50 mM Tris pH 6'8, 10% water, 50% glycerol, 1% β-mercaptoethanol, 10 mM DTT and 0'2 mg/mL bromophenol blue
Transfer buffer	10% Transfer buffer 10x (200 mM glycine, 25 mM Tris), 20% ethanol and MilliQ water up to volume
TBST	1% Tween20 10x, 10% TBS 10x and MilliQ water up to volume

Supplementary T2. Primer sequences used for amplification of GAPDH, ASPA and PGC1α genes by qPCR

Gene name	Species	Forward sequence	Reverse sequence
GAPDH	Human	5'-ACATCGCTCAGACACCATG-3'	5'-TGTAGTTGAGGTCAATGAAGGG-3'
ASPA	Human	5'-CGTTCCATAGCCAAGTATCCTG-3'	5'-TCAGCTCTCAGAACCCCTTG-3'
PGC1α	Mouse	5'-GAAAGGGCCAAACAGAGAGA-3'	5'-GTAAATCACACGGCGCTCTT-3'