Clinical and metabolic implications of obesity in prostate cancer: is testosterone a missing link?

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ABSTRACT

Objectives: To assess sex hormones in men with obesity and prostate cancer (PCa) and to study association between androgens and the pathogenesis biology of PCa *in vitro*.

Subjects and methods: One hundred and eighty-one men older than 45 years selected from of a population attending to Urology departments screening for PCa, (78 participants without PCa and 103 patients with PCa). All participants were assessed for body mass index (BMI), age, Gleason score, and PSA. Endocrine profile was determined for LH, total testosterone (TT), 17β-estradiol (E2), prolactin and leptin. Biochemical profile (HbA1c, triacylglycerols and lipoproteins) was also determined. *In vitro* experiments were also performed, involving the study of 5α-dihydrotestosterone (DHT) and E2 in the presence of adipocyte-conditioned medium (aCM).

Results: All variables were continuous and described a Gaussian distribution unless mentioned. To determine the relation of aggressiveness, variable were transformed into categories. Thus, PCa aggressiveness is associated with the increase of age and BMI ($p < .0001$) but with is decreased with TT and E2 ($p < .05$). Moreover, adipocyte-secreted molecules increase aggressiveness of PCa cells *in vitro*. Lastly, DTH but not E2 enables invasiveness *in vitro*.

Conclusions: It was observed a coexistence of hormone axis profile alteration with sex hormones and BMI in PCa patients, in accordance with the new perspective of PCa pathogenesis.

KEYWORDS
Prostate cancer; obesity; testosterone

Introduction

Across the world, prostate cancer (PCa) is the second most frequent type of Cancer among men [1]. In particular, in the North Portugal it is the more common, and about 22.2% of men who have cancer, have PCa. Moreover, in 2009 it was accounted that 1733 men had PCa and in 2015 the number increased to 2351, representing an increase of 35.7% and an increase of 74.0% is expected from 2009 to 2020 [2].

Evidence has supported obesity as a risk factor for both benign prostate hyperplasia (BPH) and PCa. The World Health Organization and The National Institutes of Health define overweight as a body mass index (BMI) of greater than 25 kg/m^2, and obesity as BMI of greater than 30 kg/m^2 [3].

It is long recognized, that testosterone is a crucial lipid hormone that plays a major role in key metabolic and pathophysiological processes such as obesity and cancer [4–7]. The increased central adiposity (fat mass) and reduced lean mass is related with hypogonadism (low levels of testosterone) [8,9]. These anatomic features are closely related to metabolic dysfunction. Particularly, metabolic features associated to testosterone deficiency include energy metabolism imbalance such as impaired glucose regulation, reduced insulin sensitivity and dyslipidemia [10,11].

Testosterone may be found in different forms. There is "free" testosterone, that is not bound to any proteins or blood transporters and it is recognized to be the most abundant in the body. However, testosterone may also be bound to albumin and to sex hormone-binding globulin (SHBG). In this form testosterone is not active. Testosterone is often converted to dihydrotestosterone (DHT) by a set of widely distributed enzymes, 5α-reductases (EC. 1.3.1.22). DHT is five times more potent than testosterone and is the preferred form used by the cells of prostate, skin, and hair follicles [12].

Androgen levels change with age. It is estimated that testosterone levels decline about 1% per year after the age of 30. When testosterone decreasing

levels in the aging male cause discomfort and loss of quality-of-life such as erectile dysfunction (ED), muscle strength, bone strength [13], and fatigue, hormone therapy must be considered [14,15].

Testosterone low-levels or even deficiency is not only linked with age. In fact, testosterone deficiency is also associated with insulin resistance and the metabolic syndrome (MetS), reduced bone density and increased visceral obesity. Thus, several authors discussed whether restoring testosterone normal physiological levels may exert benefits to elderly men, not only in the metabolic fitness (reduces obesity, increases insulin sensitivity) but also libido improvement and health-related quality of life [16–18].

Regarding the role of testosterone on prostate cancer (PCa) there is still some discussion among the scientific community, since there are several discrepant studies. While numerous longitudinal studies have recognized an association between high levels of TT and subsequent development of PCa [19,20] other smaller, well-designed studies have demonstrated the opposite, that is, increased PCa risk in patients with lower testosterone levels [21,22].

Nevertheless, considered by most scientific and clinical community one of most important and perhaps one of the most robust study on the role of testosterone and PCa relationship comes from the study of the a 5α-reductase inhibitor, dutasteride, the REDUCE trial (Reduction by Dutasteride of Prostate Cancer Events) [23]. The study included two arms, the placebo arm and the Dutasteride arm, and enrolled initially 8231 patients distributed 4126 by placebo and 4105 by dutasteride arms. In analyzing the placebo arm, Muller et al. identified no association of testosterone or DHT with PCa incidence nor Gleason grade [24].

Thus, a large number of scientific evidence have somehow linked serum testosterone levels and/or its carrier (SHBG) to cancer and metabolic disturbances such as metabolic syndrome and diabetes [15,25–30]. In fact, androgen deprivation has been associated to worst glycemic control and increase cardiovascular biochemical risk factors in diabetic prostate cancer patients [31].

However, the exact type of the observed relations remains indeterminate. These ambiguities may be endorsed to the high variability of the associations reported. The most relevant evidence suggests that associations may differ according to age and BMI [32–34].

Aims

Considering these facts, the current study aims to evaluate the association between hormonal and biochemical profile with the occurrence and aggressiveness (Gleason Score) of PCa in the Portuguese obese men from the North of Portugal. In order to achieve that purpose, we have analyzed a set of patients from this region with and without PCa. Also, we have studied the effect of sexual hormones (testosterone and estradiol) on PCa cells *in vitro* within an environment enriched by the secreted signaling molecules of adipose cells.

Material and methods

Sample size and characteristics

The current study presents a cross-sectional design and included 181 men older than 45 years, who were referred to routine urological evaluation. The study protocol was approved by our local ethical committee, and all patients signed the written informed consent.

The indication for prostate biopsy is based on the prostate specific antigen (PSA) blood test, its evolution over time and on prostate changes regarding the consistency or shape detected by direct examination (prostate touch) or both.

Socio-demographic information was obtained, and blood samples were collected for biochemical and hormonal analysis.

Blood collection

Patients who submitted for ultrasound-guided prostate biopsy were given a venous blood sample prior to any prostatic manipulation (rectal examination) and transrectal echography in Vacutainer BD tubes, a tube without any additive, which were subsequently centrifuged at 3500 r.p.m. and the separate serum for the determination of the aforementioned hormones and biochemical markers.

Recommendations for the handling and storage of samples are provided by the National Committee for Clinical Laboratory Standards.

Laboratory tests

For the determination of all the hormones included in the study, commercial reagent kits from Roche (Roche Diagnostics) developed for use in the E170 modular automatic analyzer were used.

The method used for the determination of all hormones was the electrochemiluminescent assay (ECLIA) by means of competitive or sandwich approach.

The reagents used were those indicated by the manufacturer and where necessary calibrations were

carried out and the calibrators used were those recommended by the respective manufacturers.

For each series of determinations, control sera were inserted. These sere were acquired in the market, in three levels of concentration (low, normal and high). The determinations were only made after validating the results of the quality control, which were always within the values defined by the Quality Control platform.

Hormonal and biochemical profile

The hormonal profile in the current study was performed in the patients who underwent the ultrasound-guided prostate biopsy and were defined at the beginning of the study, being constituted by the following hormones: Leptin, Prolactin, Estradiol, Luteinizing Hormone, and Total Testosterone. The normal range considered in the present study were of 0.7–5.3 ng/mL for Leptin, 4.04–15.2 ng/mL for Prolactin, 7.63–42.6 pg/mL for Estradiol (E2), 1.7–8.6 mUI/mL for Luteinizing Hormone, and 2.8–8.0 ng/mL for Total Testosterone. Values below or higher than the normal range, are considered as low or high respectively.

Moreover, the biochemical profile includes glycated hemoglobin HbA1c, triacylglycerols (TAGs), total cholesterol (CHOT) and its fractions, such as high-density lipoproteins (HDL-c), low-density lipoproteins (LDL-c) and very low-density lipoproteins (VLDL). Normal ranges of such biochemical parameters in use in the present study are 4.0–6.0% for HbA1c, 40–160 mg/dL for TAGs, 0–200 mg/dL for CHOT, 35–55 mg/dL for HDL-c mg/dL, 0–130 mg/dL for LDL-c and 3–56 mg/dL for VLDL. As before, we have considered the values inferior or superior to the normal range as low or high respectively.

Cell culture and in vitro *treatments*

For the current work 3T3-L1 (purchased from American Type Culture Collection) cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium with 4.5 g/L Glucose and L-Glutamine, without Sodium Pyruvate. Santa Cruz Biotechnology, Inc.), supplemented with 10% FBS, and 1000 units/mL gentamycin solution, maintained in T-25 tissue culture flasks in 5% CO_2/95% air at 37 °C in a humidified incubator.

Human prostate cancer cell line, PC3 were cultured in RPMI-1640 medium (Gibco, Life technologies, 52400–025, USA) supplemented with 10% of Fetal Bovine Serum (Gibco, Life technologies, 10270, USA) and 1% antibiotic/antimycotic (Gibco, Life technologies, 10270, USA). The cells were maintained in a humidified chamber with 95% air and 5% CO_2 at 37 °C. The cells were used between passages 2 and 8.

Adipocyte differentiation and conditioned medium (CM) collection

3T3-L1 pre-adipocytes were propagated and allowed to reach confluence. After 2 days (day 0), the differentiation was initiated by addition of a hormonal mixture composed of 2 μM insulin, 1 μM dexamethasone and 0.25 mM isobutylmethylxanthine. Three days after (day 3), the induction medium was replaced by complete medium supplemented with insulin only. At day 6 cultures were washed twice in phosphate buffered saline and incubated in serum-free medium. After 24 h (day 7), medium was harvested from the adipocytes cultures, spun for 3000 g for 5 min and the supernatant (mature adipocytes conditioned medium) was stored at −80 °C for the subsequent treatments. This conditioned medium (CM) is rich in adipokines which are globally referred as secretome.

Cell culture treatments

Afterwards, prostate cells were divided in two distinct groups. On one hand, prostate cancer cells PC3 were grown under the influence of the mature adipocytes secretome. On other hand, the control group consisted in the PC3 cells cultured in the absence of 3T3-L1 conditioned medium with low levels of glucose (LG).

Treatments were performed using Dulbecco's Modified Eagle Medium (DMEM), without supplementation. To assay the hormone effects on cell growth, cells were grown for 24 h in DMEM medium (Sigma-Aldrich) supplemented with different concentrations of hormones, testosterone (Fluka), and 17β-estradiol (E2) (Sigma-Aldrich) dissolve in 0,1% of DMSO. The most significant concentration was used to perform an Injury assay.

Cell viability assay

After 24 h incubation of 1×10^5 cells/mL with the different treatments, culture medium was replaced by PBS and 20 μL of the MTT reagent (Abcam, USA), was added into each 96-plate well followed by 3 h incubation period. Color development was determined by measuring absorbance at 550/650 nm.

Table 1. Descriptive statistics for hormonal and biochemical profiles.

	Total participants		Partients with Pca		Participants without Pca	
Variables	Mean	SD	Mean	SD	Mean	SD
Age (y.o.)	66.38	8.53	68.12	8.45	64.06	8.12
BMI (kg/m^2)	26.75	3.88	28.51	3.88	24.41	2.34
tPSA (ng/mL)	17.18	29.58	24.65	37.30	7.19	4.13
LH (mUI/mL)	7.11	6.09	7.94	7.21	5.99	3.92
TT (pg/mL)	3.67	1.90	3.27	1.84	4.21	1.85
E2 (pg/mL)	22.89	10.56	22.93	11.17	22.84	9.75
PROL (ng/mL)	10.33	8.90	8.55	3.83	11.63	11.10
LEPT (ng/mL)	13.84	21.93	15.26	23.60	11.93	19.45
HbA1c (%)	5.72	0.65	5.74	0.70	5.70	0.58
TAG (mg/dL)	157.46	79.54	168.40	90.87	142.82	58.65
CHOT (mg/dL)	192.27	37.82	192.96	38.62	191.35	36.95
HDL (mg/dL)	52.53	14.53	54.02	14.92	50.53	13.83
LDL (mg/dL)	104.15	33.07	101.40	31.52	107.83	34.91
VLDL (mg/dL)	31.24	15.40	32.43	17.66	29.66	11.64

BMI: Body Mass Index; tPSA– total Prostate Specific Antigen; LH: Luteinizing hormone; TT: Total Testosterone; E2: Estradiol; Prol: Prolactin; Lept: Leptin; Hb A1c: Glycated hemoglobin A, fraction 1c; TAG: Triacylglycerols; CHOT: Cholesterol, total fraction; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein; SD: Standard deviation.

Injury assay and computational stereology analysis

Confluent PC3 monolayers on 24-plate well, were wounded with a 10-μl pipette tip. Twenty-four hours after treatment, the migrated distance was photographed under an inverted microscope (Nikon Instruments Inc., Melville, NY) at a 200x magnification and the scratch closure was determined by measuring the injury width by means of bioinformatics analysis using Image J software (U. S. National Institutes of Health, Bethesda, Maryland) with a macro specially designed for injury assays designated as Wound Healing Tool [35].

Statistical analysis

Sample size was calculated *a priori* using G*Power version 3.1.9.3 [36,37], designed to detect statically significant Pearson Correlation Coefficients of 0.5 for level of significance α error of 5% and a power of 90% (1-β) the sample should be at least 86 control individuals and 86 prostate cancer (PCa) patients. The sample was stratified into two groups, according to presence/absence of prostate carcinoma. Student t-test was used to compare means of two different samples. Chi-square test was used to compare categorical data. Kolmogorov–Smirnov and Levene's test were used to determine normality of populations and variances. All the studied variables are normally distributed. Data are presented as mean ± standard deviation of the mean (SEM), unless otherwise stated.

All statistical analyses were performed with IBM SPSS 20 (SPSS Inc., Chicago, IL). All statistical tests were two-sided and $p < .05$ was considered statistically significant and plotted using GraphPad Prism 6.0 (GraphPad Software Inc.).

Results

Clinical and anthropometric characterization

The current study presents a cross-sectional study and included 181 men attending the Urology Department of CHUP (Central Hospital from the University of Porto), 78 participants without PCa and 103 patients with PCa. The age ranges from 46 to 85 years old. The overall mean of ages amongst participants was of 66.4 ± 8.5 (mean ± standard deviation) stratified by 68.1 ± 8.4 versus 64.1 ± 8.1 regarding the participants with and without cancer, respectively (Table 1).

All men consent to be submitted to anthropometric characterization (weight and height) in order to determine the Body Mass Index (BMI) according to WHO recommendations [3]. Thus, the overall BMI was of 26.75 ± 3.88 kg/m2 classifying this sample, generally as overweight. When looking to BMI by groups, it was stablished as 28.51 ± 3.88 kg/m2 versus 24.41 ± 2.34 kg/m2 in the arm with and without cancer respectively (Table 1).

The indication for prostate biopsy was mainly based on the PSA blood test and/or on prostate changes by direct examination. Thus, the global mean of tPSA (total PSA) was of 17.18 ± 29.58 ng/mL, being different amongst participants with and without cancer, varying from 24.65 ± 37.30 ng/mL and 7.19 ± 4.13 ng/mL respectively (Table 1).

Hormonal and metabolic characterization of participants

Several hormones were characterized. The hypothalamus pituitary testicular axis was measured using luteinizing hormone (LH) and total testosterone (TT). Estradiol (E2), an aromatized form of a testosterone was also analyzed. Considering means (±SD) of both arms with or without PCa no significant changes of such hormones were observed. Global levels of LH are of 7.11 ± 6.09 mUI/mL being of 7.94 ± 7.21 mUI/mL and 5.99 ± 3.92 mUI/mL in men with and without PCa respectively. Also, regarding TT, the global levels of this androgen were of 3.67 ± 1.90 ng/mL and the levels in people with and without PCa were of 3.27 ± 1.84 ng/mL and 4.21 ± 1.85 ng/mL respectively. Estradiol (E2) an end product of Aromatase using

testosterone as substrate, was globally of 22.89 ± 10.56 pg/mL but also very similar amongst the two arms varying from 22.93 ± 11.17 pg/mL to 22.84 ± 9.75 pg/mL in patients with and without PCa, respectively. Finally, Prolactin a hormone related to both sexual health and metabolism [38] was also evaluated. Men with PCa have lower levels of prolactin. Thus, these values ranged from 8.55 ± 3.83 ng/mL to 11.63 ± 11.10 ng/mL in patients with and without PCa (Table 1).

In what concerns to metabolic profile of the participants, men with PCa have higher levels of Leptin than men without PCa. In those arms, the mean ± SD of Leptin was of 15.26 ± 23.60 ng/mL in PCa men and of 11.93 ± 19.45 ng/mL in men without PCa. The percentage of glycated hemoglobin HbA1c was determined. The average of this advanced glycation end-product (AGE) was very similar in both groups: 5.74 ± 0.70% and 5.70 ± 0.58% for participants with and without PCa. Regarding TAGs, patients with PCa had higher levels of these lipids. Thus, comparing patients with PCa with people without PCa the ranges of such lipids were 168.40 ± 90.87 mg/dL and 142.82 ± 58.65 mg/dL, respectively (Table 1).

Considering lipoprotein profile, as aforementioned we have measured all the cholesterol fractions. First of all, total cholesterol (CHOT) presents no differences between the two groups in study. CHOT values ranges from 192.96 ± 38.62 mg/dL to 191.35 ± 36.95 mg/dL in men with and without PCa respectively. Additionally, LDL-c fraction was measure and no significant differences were found however people with no cancer presented higher mean values. Patients with PCa presented a mean of values of 101.40 ± 31.52 mg/dL of these lipoprotein and patients with no cancer, presented an average value of 107.83 ± 34.91 mg/dL. Similarly, regarding HDL-c no differences were found amongst the arms in study but this time, patients with PCa presented the higher values. Hence, patients presented a mean of 54.02 ± 14.92 mg/dL of HDL-c, contrasting with healthy participants that presented 50.53 ± 13.83 mg/dL of this lipoprotein. Likewise, VLDL fraction was of 32.43 ± 17.66 mg/dL in people with PCa opposing with the mean of 29.66 ± 11.64 mg/dL belonging to healthy subjects (Table 1).

Correlations between clinical and biochemical profile

Correlation between continuous variables was determined by Pearson Correlation Coefficient or PCC. PCC is represented by the symbol ρ (rho or r). PCC value has a value between +1 and −1, where 1 is total positive linear correlation, 0 is no linear correlation, and −1 is total negative linear correlation. The evaluation of categorical variables (e.g. Gleason Score) were determined by means of crosstabulations and estimation by Chi-square test (χ^2) statistics.

When comparing the categories of negative/positive cases of PCa distributed by Gleason score and BMI stratified by normal weight (NW), overweight (OW) and Obese (OB), the differences were significantly strong ($p < .0001$) estimated by χ^2-test (Figure 1).

Similarly, evidence shows that tumor aggressiveness was correlated with age. Age values were transformed into an ordinal category of five classes of 10 years old each: the first class was of 45 to 54 years old (y.o.), the second of 55 to 64 y.o., and successively until the last class that included all the participants older than 85 years. (Figure 1).

Furthermore, when participants were stratified by normal interval ranges of TT, and divided into three groups named “low”, “normal”, and “high”, strong significantly differences ($p < .0001$) were found when compared to Gleason Score. Lower levels of TT predict higher tumor aggressiveness (Figure 1).

The same approach was followed for several continuous clinical and biochemical variables. Stratification into three groups designated as “low”, “normal”, and “high” were implemented according to normal ranges of such variable. When Chi-square statistics was executed against each variable in study, it demonstrated strong significant differences with Age and BMI, with several hormones, namely Luteinizing Hormone, Testosterone, Estradiol and Prolactin. Also, significance was found in lipoprotein markers LDL and VLDL (Table 2).

Finally, the several continuous clinical and biochemical variables in study were correlated with each other. All variables were normally distributed and Pearson correlation coefficient (PCC) was determined to all variables two-by-two. The results are summarized in Table 3.

Briefly, tPSA (total prostate specific antigen) was inversely correlated ($p < .05$) with total Testosterone (TT) levels. Likewise, BMI was also strongly inversely correlated with TT levels ($p < .0001$). Another negative correlation with BMI was the association between weight status and the levels of Prolactin ($p < .0001$). Nevertheless, BMI was positively correlated with LH ($p < .05$), HbA1c ($p < .05$) and TAGs ($p < .05$).

With age several hormone and metabolic components of the body are known to be deteriorated.

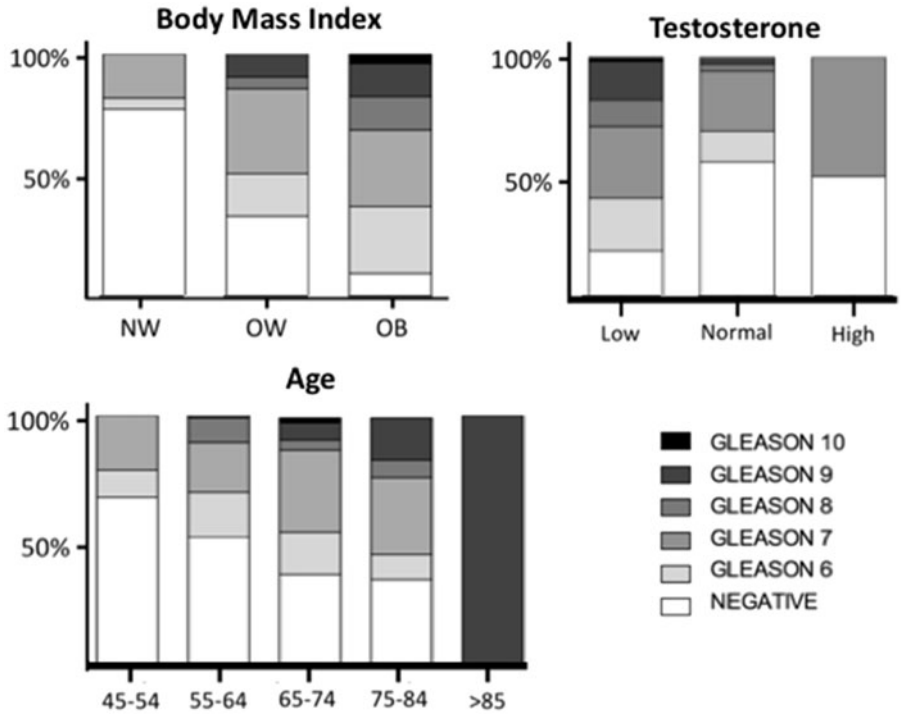

Figure 1. Ponderal profile of Urology patients. More aggressive forms of prostate cancer are associated ($\chi 2$) with overweight and obesity ($p < .0001$). NW: Normal weight; OW: overweight; OB:obesity. Age was also significantly associated ($p < .0001$) to tumor aggressiveness which increases to the age of the patient. Normal levels of testosterone ranging from 2.8–8.0 ng/mL. Inferior and superior levels were considered as low or high respectively. Tumor aggressiveness was significantly ($p < .0001$) associated to low levels of testosterone. Without prostate cancer (white) and with prostate cancer (other colors).

Table 2. Chi-square (χ^2) of Gleason score (tumor aggressiveness) in relation to clinical and biochemical profiles.

				Endocrine profile					Metabolic profile					
Variables (statistics)		Age	BMI	LH	TT	E2	PROL	LEPT	HbA1c	TAG	CHOT	HDL	LDL	VLDL
GLEASON Score	χ^2	**255,3****	**69.29****	**12.26***	**92.41****	**97.41****	**120.01****	8.39	10.7	5.39	8.00	13.34	**11.58***	**13.20***
	p-value	**.000**	**.000**	**.031**	**.000**	**.000**	**.000**	.136	.058	.370	.152	.205	**.041**	**.014**

BMI: Body Mass Index; LH: Luteinizing hormone; TT: Total Testosterone; E2: Estradiol; Prol: Prolactin; Lept: Leptin; Hb A1c: Glycated hemoglobin A, fraction 1c; TAG: Triacylglycerols; CHOT: Cholesterol, total fraction; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein. $^{*}p<.05$; $^{**}p<.001$.

In the present sample of patients with prostate disease, age itself is also associated with increased of LH ($p < .0001$) as well as with decreased TAGs ($p < .05$), CHOT ($p < .0001$) and LDL ($p < .0001$). All outputs of the PCC statistics are summarized in Table 3.

Taking into considerations all data mentioned, we tried to molecularly dissect *in vitro* the role of some parameters that increased aggressiveness of PCa such as BMI and some hormone levels in well stablished cellular models of prostate cancer, namely using PC3 cell lines. Obviously, BMI can't be mimiced *in vitro*. However, a good cellular alternative is to *in vitro* grow adipocytes and mature them to up take and store lipids and then. At this stage, the adipocyte-secreted molecules such as hormones, cytokines, and adipokines, among others is collected (the all bunch of adipocyte-secreted molecules are considered as secretome from now on). This is achieved, as mentioned in materials and methods section, by collecting the adipocyte-conditioned medium (aCM) was add it to cancer cells in culture. In the next section of results, we will reveal the effect of aCM on cell growth and aggressiveness on PC3 was well as the effect of testosterone and E2 *in vitro*.

Adipocyte secretome characterization

Numerous growth-factors, adipokines, cytokines, and angiogenesis-related molecules were secreted by mature adipocytes 3T3-L1 in culture (secretome) into the medium. That adipocyte-conditioned medium was collected (aCM), analyzed and used in PCa cells PC3.

Table 3. Pearson Correlations between clinical and biochemical profiles.

Variables (statistics)		Endocrine Profile					Metabolic Profile					
		LH	TT	E2	PROL	LEPT	HbA1c	TAG	CHOT	HDL	LDL	VLDL
PSA	ρ	0.019	**−0.212***	0.031	0.008	0.177	0.188	−0.038	0.045	−0.073	0.120	−0.036
	p-value	.850	**.032**	.753	.934	.074	.057	.704	.648	.463	.226	.720
BMI	ρ	**0.183***	**−0.323****	0.034	**−0.215****	**0.308****	**0.152***	**0.186***	0.128	−0.042	0.058	0.133
	p-value	**.014**	**.000**	.650	**.001**	**.000**	**.042**	**.012**	.086	.577	.440	.075
Age	ρ	**0.277****	0.018	−0.055	0.051	0.011	−0.047	**−0.154***	**−0.248****	0.078	**−0.220****	−0.138
	p-value	**.000**	.816	.463	.504	.889	.534	**.041**	**.001**	.301	**.003**	.068

BMI: Body Mass Index; tPSA– total Prostate Specific Antigen; LH: Luteinizing hormone; TT: Total Testosterone; E2: Estradiol; Prol: Prolactin; Lept: Leptin; Hb A1c: Glycated hemoglobin A, fraction 1c; TAG: Triacylglycerols; CHOT: Cholesterol, total fraction; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein. $^{*}p<.05$; $^{**}p<.001$.

The hormonal profile of aCM was of 0.257 ± 0.09 mUI/mL for LH, 0.061 ± 0.004 ng/mL for TT, 227.6 ± 30.4 pg/mL for E2 and 0.047 ± 0.000 for Prolactin. Regarding biochemical profiles, adipocyte secretome was composed by 155.1 ± 27.4 mg/dL of glucose, 102.0 ± 35.4 mg/dL of TAGs and 4.0 ± 1.8 mg/dL of CHOT. Our group has also analyzed extensively the secretome of adipocytes in previous studies [39,40] by proteomic approaches including microarrays. Briefly, it is observed in the secretome increased levels of VEGF, TIMP-1, and endocan that control angiogenesis. Also increased levels of the interleukin (IL) family of cytokines: IL-6, IL-11, and leukemia inhibitory factor (LIF) were also significantly overexpressed as well as monocyte chemotactic protein 1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1). Adipose tissue hormones were also identified. Resistin and leptin relative expression levels were higher in aCM. Interestingly though, a significant reduction in adiponectin levels was observed in aCM.

Adipocyte-conditioned medium (aCM) increases viability of PC3

The prostate cells PC3 were treated with aCM. The result was an increased metabolic activity when MTT assay was performed. Also, two dose response curves were determined to 5α-dihydrotestosterone (DHT) and to 17β-Estradiol (E2). The half maximal effective concentration (EC_{50}) for such hormones on PC3 cell lines were of 1 nM for DHT and 0.1 nM for 17β-Estradiol.

When compared to control group (PC3 with complete medium), all experimental groups were significantly different ($p < .0001$). Cancer cells submitted *in vitro* to an environment enriched with adipocyte-related secretome (aCM) demonstrate a metabolic activity of 3,21 ± 0,10 times fold when compared to control group. Also, cells growing with DHT 1.0 nM and E2 0.1 nM increased their metabolic activity to 2.98 ± 0.11 times and 2.04 ± 0.22 times, respectively. Likewise, metabolic activity also increased when compared to control group when aCM was doped with DHT 1.0 nM and E2 0.1 nM to 3.48 ± 0.07 times and 2.98 ± 0.37, respectively (Figure 2(A)).

The injury assay or wound healing assay is an *in vitro* test that is based in the fact that in culture, cells will invade cell-free territories. Experimentally, the territories are created artificially by removing a group of cells of a confluent culture thus creating an injury or wound. The more rapid the wound is closed, the most aggressive are the cells due their invasion capacity. Prostate cancer cells PC3 in culture 24 h after injury were able to close 11.34 ± 4.41%. However, cell under the effect of the aCM that contains all the growth factors, signaling molecules, cytokines derived from adipose tissue were able to close the wound up to 62.08 ± 2.43%. Likewise, PC3 within the same conditions of control arm but supplemented with DHT 1.0 nM were able to repair the injury up to 79.12 ± 3.76%. Finally, the wound closed 23.75 ± 8.76%. When compared to control, the experimental groups aCM and DHT were significantly different and have increased the rate of wound repair of 5.5 ± 0.55 and 7.0 ± 0.85 times. Also, E2 treated cells also increase 2.0 ± 1.9 fold their injury repair but in a non-significant manner (Figure 2(B)).

Discussion

Among the several known risk factors for PCa, age is one of most important factor. Not only the incidence increases but also aggressiveness is higher. Also, age at the diagnosis is related to worst prognosis. In our Portuguese male population attending to urology consultation the age has strong relation (Figure 1, Table 2) not only with its onset and incidence but also with tumor aggressiveness which agrees with most other studies [41–43]. Thus age has major impact on prognosis, response to the treatments and survival rate [44,45].

Obesity has long been suggested to be implicated in the carcinogenesis of PCa through several molecular

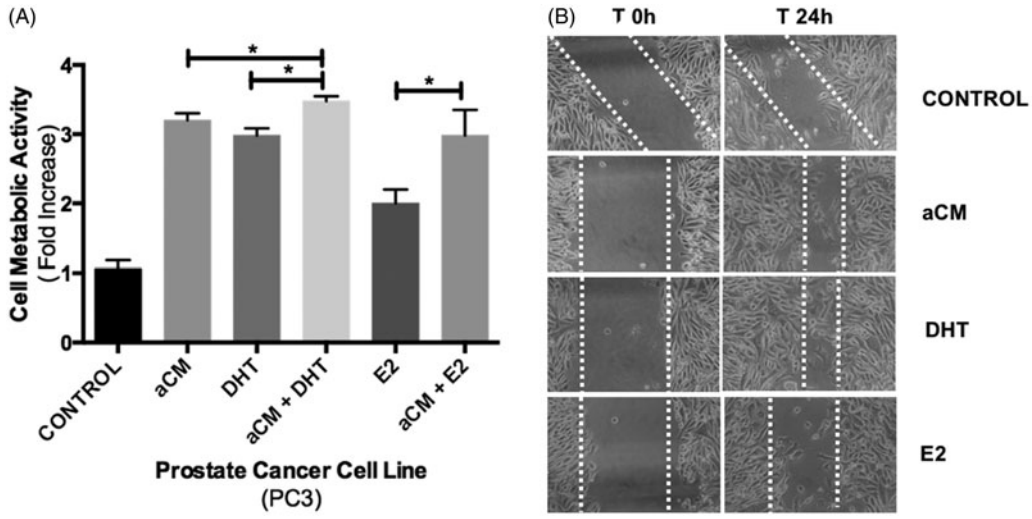

Figure 2. Metabolic activity (A) and Aggressiveness (B) of prostate cancer cells PC3. (A) Metabolic activity was measure with MTT assay (spectrophotometry) by fold increase in relation to the control (PC3 without any treatment). All assays were significantly different by t-test to control group ($p < .0001$). Differences between other groups were indicated by (*) which means $p < .05$. (B) Aggressiveness in vitro was determined by the injury assay. The area was calculated with image J with "Wound Healing Tool" macro and differences were significantly ($p < .0001$) after 24h. aCM: adipocyte-conditioned medium; DHT: 5α-Dihydrotestosterone; E2:17β-Estradiol.

pathways that include sexual hormone axis, adipokines biological signaling among other. Regarding obesity and its contribution to PCa in our population, our results show clear evidence that overweight and obesity play a major role on PCa pathogenesis (Figure 1, Table 2). In our population from the North of Portugal, normal weight patients only 22.4% have PCa and the other patients (77.6%) have some kind of benign prostate pathology such as prostatitis or benign hyperplasia. Interestingly, our sample shows higher scores of Gleason scale increased in overweight patients and again in obese patients with a strong significance ($p < .0001$, χ^2). The contribution of BMI in our population is in agreement to the several studies on the subject [3,46–48].

Grosman et al. [49] have discussed the complexity of sexual hormone axis and Prostate cancer. In the particular case of testosterone this gains a new meaning. As aforementioned Muller et al. [24] identified no association of testosterone or DHT with PCa incidence nor Gleason grade. However, other authors, such as Pierorazio et al. [19] no share the same opinion. Kim et al. [50] in a very recent and critical work on the debate of testosterone levels and PCa using big data have found a significant contribution of total Testosterone (TT) on PCa aggressiveness (Gleason Score). This team has described that low levels of TT have direct relationship with Gleason score. However, the implication of TT is not confined to tumor aggressiveness. Levels of TT also correlate independently with PSA and BMI. In a very interesting recent work, Yassin et al. [51] have found that men receiving testosterone replacement therapy (TRT) reduced the incidence of high risk prostate biopsies [51]. Peskoe et al. [52] have made a nationwide study on this matter and have found a positive correlation between circulating levels of PSA with TT. Otherwise, Aref et al. [53] have evidence circulating levels of PSA correlated inversely with BMI. Our set of results confirm the findings of the authors who share the opinion that low levels of TT correlates with PCa aggressiveness [19,50] (Figure 1, Table 2) and also the findings of Aref et al. [53] since we also found strong significance between low levels of TT and the BMI. However, our results regarding PSA levels and TT we have found an inverse relationship (Table 2) which does not agree with Peskoe et al. study [52]. Nevertheless Rastrelli et al. [54] found that PSA may act as a predictor of testosterone deficiency. In conclusion, low levels of TT correlate with PCa aggressiveness. The low levels of TT may have several causes. The molecular endocrine mechanisms underlying this observation may rely on upstream or downstream hormones, in particular Luteinizing Hormone (LH), at upstream, or estrogens such as 17β-Estradiol (E2) at downstream.

Measuring serum LH is of help, since the level of this hormone indicates where the failure is located: in the testis if serum LH is high (primary), or at the level of the hypothalamus or pituitary if serum LH is low or normal (secondary). In our sample no relationship was

found between the serum levels of TT and LH ($p = .723$, PCC) although inversly related. However, in our patients, LH increased with BMI. This observation may be justified by androgen receptor insensivity and/or hypogonadal function due to obesity [21,55–57].

Due to the fact that testosterone is aromatized to E2 within adipocytes and prostate cells, various preclinical studies support the assumption that estrogen plays an important role in PC development and progression [58,59]. Our sample also evidence that a direct correlation with TT and E2 ($p < .0001$, PCC) and that levels of E2 are associated with higher scores of Gleason scale, thus indicating its role on PCa aggressiveness. Still, no association was found independelty with Age, PSA or BMI.

The physiological role of prolactin in the prostate gland is not clearly understood. It has been associated as a autocrine/paracrine signaling mediator of prostate gland that may act in cancer cells initiation [60,61]. Also, low levels prolactin have associated to metabolic syndrome [62]. In our sample we have found no relation between the two groups of patients (with or without PCa). However, when levels of prolactin were stratified into categories low, normal, and high and compared with Gleason score suggests that low levels of prolactin are related with increase aggressiveness. Also our results show that for this population, the levels of prolactin independetly correlate with obesity which is in aggreement with the finding of Corona et al. [62].

The scientific evidence regarding relationship between prostate health, androgens and obesity comes mainly through studies from HRT [4,11,56,57,63–65]. We have speculated, accordingly to other several scientists that adipose tissue must be seen as another endocrine organ rather than only a mere energy storage organ [66,67]. Likewise, members of our group have confirmed *in vitro* and *in vivo* in a mouse model the influence of adipocyte secreted adipokines on prostate cells [68]. Regarding *in vitro* experimental assays on PCa cell culture it was possible to confirm the role of sexual hormones and the role of the metabolic environment on tumor cells.

Finally, in what concerns to metabolic profile, there were no significant differences to report with some few exceptions. Triacylglycerols (TAGs) and lipoprotein profile no significant different were found when comparing means of the two groups (patients with and without PCa) nor correlations were found between PSA levels and lipids/lipoproteins. Nevertheless, LDL and VLDL levels were correlated with the increase of PCa aggressiveness. In 2010 Grosman et al. have also found some associations between lipoproteins and PCa. In their study, researchers found that PCa patients presented lower CHOT and HDL than BPH and Controls [69].

Regarding metabolic environment studies, on human prostate cancer cell PC3 our results resemble those verified by us previously on mice prostate cancer cell RM1. Comparing both findings, adipocyte secretome-enriched conditioned medium (aCM) increased both cancer cell metabolic activity measured by MTT assay. In the same manner, after injury assay, cancer cells under an environment enriched by adipocyte secretome were able to repair the wound more rapidly (Figure 2). The rate of *in vitro* wound healing is a good cellular biomarker of the ability to invade territories. As mentioned before, *in vitro* studies have also evaluated the individual effects of DHT and E2. Metabolic activity of tumor cells also increased with both DHT and E2, but this effect was more expressive in DHT. Also, significant differences were found in the cells submitted to aCM + DHT in comparison to those with aCM or DHT alone (Figure 2(A)). In a different manner significant differences were found between cells treated with aCM + E2 when compared with E2 alone but no significant differences were found between the cells treated with aCM + E2 and aCM alone. Also comparing the patterns of DHT with E2, E2 is not able to reach a grade of response as achieve by TT or aCM alone. These results are in accordance to the observation of wound healing assay, in the sense that, besides E2 be able to reduce the injury more expressively than the control group, the difference is not significant ($p = .673$). Nonetheless, the aCM and DHT repair the injury significantly faster than control group, but under the effect of DHT alone cell can grow even faster than aCM that contains a broad number of signaling molecules and growth factors secreted by adipocytes. The components existing in the aCM may be related to prostate carcinogenesis and aggressiveness, since they are composed by a high number of low-grade inflammatory cytokines, growth factors and enzymes, that not only promote cancer but also its invasiveness. Some of the component are active chemo-attractive macrophage molecules and matrix metalloproteases that opens a path to cancer cell migration as well as angiogenesis [39,68,70,71]. Supporting this theory, we can refer some of adipokines presented in our cellular model. For example, ESM1 (Endothelial Cell-Specific Molecule 1 or endocan) that is found in aCM was refered as correlates with not only with Gleason score but also with the expression of androgen receptor in PCa [72].

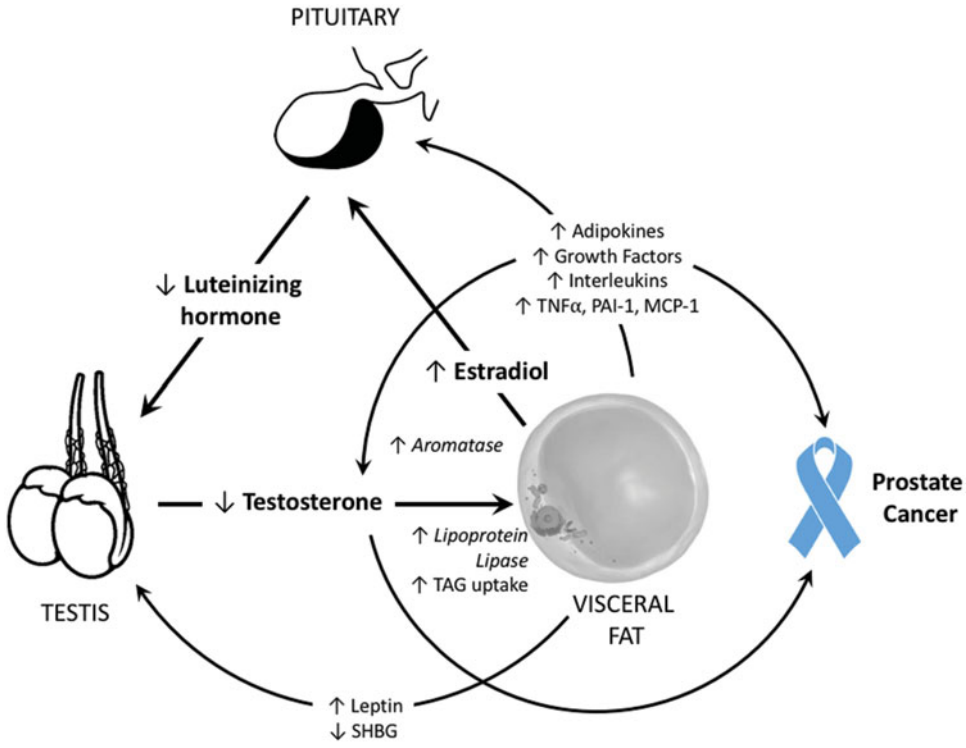

Figure 3. Hypothalamus-pituitary-gonadal axis in obesity and prostate cancer.

Also, our aCM previous proteomic chaterization demonstrated high levels of VEGF, TIMP, MCP-1, PAI-1, IL6 among others that have been extensively associated to PCa pathogenesis not only in humans but also *in vitro* [70,73–75]. This cellular model and microarrays may also be used to address the question whether sex hormones such testosterone are responsible for the phenotypic changes of inflammatory cytokines and acute phase markers as it found in PCa patients [69,73,76].

Conclusions

We have demonstrated in the present work that in the North of Portugal, patients with PCa are mainly overweight and obese. In 2015, in this region 2351 patients were diagnosed. One major limitation of this study is the fact that all participants have prostate disease since were recruited in the Urology Department of a major university central hospital from the region. Although all the participants have prostate disease, the differences between the two arms in study gain a more meaningful significance. Thus, differences observed are linked to cancer specifically and not only to a baseline disease of the prostate gland. Also, the several clinical, biochemical, and metabolic alterations that connect prostate cancer and obesity together may be explain mainly by the alteration of sexual endocrine axis involving testosterone upstream and downstream hormones. In brief, obesity related-hormones and cytokines are responsible for the volume expansion (hyperotrophy) and hyperplasia of both adipocyte and tumor cells. The increase in number of adipose cells increases the activity of aromatase, thus converting testosterone to estrogens (mainly estradiol). Also, adipocytes secrete low-grade inflammatory molecules that, together with increased estradiol are responsible for the decrease of LH reducing even more testosterone levels that also contribute for the pathogenesis of PCa. Besides we have not found significant differences between Leptin and PCa aggressiveness or TT levels we have found significant relation between this hormone and the increase of BMI. Testosterone production by testis are also negatively regulated by Leptin [77,78] thus we may not really exclude their participation in the reduction of testosterone production on testis, based on literature (Figure 3).

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Ethical statement

This study was approved by the institutional ethics committee of Porto University Hospital Centre. No human cells were

used, other than commercially available cell lines, that require prior ethical approval to the corresponding ethic committees.

Disclosure statement

The authors declare that there are no conflicts of interest.

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