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Tumor Cell-educated Periprostatic Adipose Tissue Acquires an Aggressive Cancer-promoting Secretory Profile

Ricardo J.T. Ribeiro[a,b,c], Cátia P.D. Monteiro[a,c], Virginia F.P.M. Cunha[a,c], Andreia S.M. Azevedo[a,c], Maria J. Oliveira[d,k], Rosário Monteiro[e], Avelino M. Fraga[a,b,f], Paulo Príncipe[f], Carlos Lobato[g], Francisco Lobo[h], António Morais[h], Vitor Silva[h], José Sanches-Magalhães[h], Jorge Oliveira[h], João T. Guimarães[e,i], Carlos M.S. Lopes[b,j] and Rui M. Medeiros[a,b,c]

[a]Molecular Oncology Group-CI, Portuguese Institute of Oncology, Porto, [b]Abel Salazar Biomedical Sciences Institute, University of Porto, Porto, [c]LPCC, Research Department-Portuguese League Against Cancer (NRNorte), [d]INEB, Biomaterials Division, NEWTherapies Group, Porto, [e]Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, Porto, [f]Urology Department, Porto Hospital Centre, Porto, [g]Urology Department, Porto Military Hospital, Porto, [h]Urology Department, Portuguese Institute of Oncology, Porto, [i]Clinical Pathology Department, São João Hospital, Porto, [j]Pathology Department, Porto Hospital Centre, Porto, [k]Department of Pathology and Oncology, Faculty of Medicine, University of Porto, Porto

Key Words

Adipokines • Periprostatic adipose tissue • Prostate cancer

Abstract

Background/Aims: The microenvironment produces important factors that are crucial to prostate cancer (PCa) progression. However, the extent to which the cancer cells stimulate periprostatic adipose tissue (PPAT) to produce these proteins is largely unknown. Our purpose was to determine whether PCa cell-derived factors influence PPAT metabolic activity. Methods: Primary cultures of human PPAT samples from PCa patients (adipose tissue organotypic explants and primary stromal vascular fraction, SVF) were stimulated with conditioned medium (CM) collected from prostate carcinoma (PC3) cells. Cultures without CM were used as control. We used multiplex analysis and ELISA for protein quantification, qPCR to determine mitochondrial DNA (mtDNA) copy number and zymography for matrix metalloproteinase activity, in order to evaluate the response of adipose tissue explants and SVFs to PC3 CM. Results: Stimulation of PPAT explants with PCa PC3 CM induced adipokines associated with cancer progression (osteopontin, tumoral necrosis factor alpha and interleukin-6) and reduced the expression of the protective adipokine adiponectin. Notably, osteopontin protein expression was 13-fold upregulated. Matrix metalloproteinase 9 activity and mitochondrial DNA copy number were higher after stimulation with cancer CM. Stromovascular cells from PPAT in culture were not influenced by tumor-derived factors. Conclusion: The modulation of adipokine expression by tumor CM indicates the pervasive extent to which tumor cells command PPAT to produce factors favorable to their aggressiveness.

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Dr. Ricardo Ribeiro, MD
Portuguese Institute of Oncology, Porto Centre
Molecular Oncology Group - CI, Edifício Laboratórios - Piso 4,
Rua Dr. António Bernardino Almeida, 4200-072 Porto (Portugal)
Tel. +351 912 157 736, Fax +351 225 084 001, E-Mail oriebir.r@gmail.com

Introduction

The association between obesity and prostate cancer (PCa) is complex. Obese men are more likely to develop metastasis or die from PCa and are at greater risk of progression after radical prostatectomy [1, 2], supporting that obesity is associated with a more aggressive biology.

In recent years, several studies underline the essential role of adipose tissue in tumor progression, since it produces molecules with oncologic potential, such as leptin, adiponectin, tumoral necrosis factor alpha (TNFα), interleukin-6 (IL-6), osteopontin (OPN), monocyte chemoattractant protein-1 (MCP-1), RANTES (CCL5), among others [3, 4].

Nevertheless, although adipose tissue effect on cancer has been investigated, the direct effects of cancer cells on adipose tissue function have received little attention. Previous findings using other cancer models, suggest that cancer-derived factors induce adipogenesis, modulate stromelysin-3 production by adipocytes and induce adipose tissue-derived stem cells differentiation into carcinoma-associated fibroblast-like cells [5-7].

The adipokine expression profile has been shown to be characteristic of fat depots anatomical localization [8]. Recent findings showed that periprostatic adipose tissue (PPAT) thickness and the increased local production of IL-6 are associated with PCa aggressiveness [9-11]. However, the functional roles of PPAT, which can be relevant for the obesity-PCa association because of the localization and eventual crosstalk in extra-prostatic disease, remains to be determined. Hence, we investigated the impact of PCa cell-derived media on the functional activity of PPAT, to test the hypothesis that tumor-stimulated adipose tissue increases metabolic activity and the production of adipokines with oncologic potential, further modulating cancer development and aggressiveness.

Materials and Methods

Patients and collection of human PPAT

Twelve men diagnosed with PCa and eligible for retropubic radical prostatectomy, without other major co-morbidities, were included in this study after informed consent agreement. Patient's characteristics are described in Table 1. The project was approved by the ethics committees of the participating Hospitals. Human anterior-lateral samples of PPAT were collected during surgery and immediately processed.

Primary cultures and conditioned medium (CM) experiments

For explants culture, periprostatic adipose tissue fragments were processed using a modified protocol from Thalmann et al. [12]. Briefly, after overnight incubation of explants in culture medium DMEM/F12 (Gibco) supplemented with biotin 16 μM, panthotenate 18 μM, ascorbate 100 μM and penicillin-streptomycin 1% (sDMEM/F12), fresh medium was added, which was referred to as time zero for time-course experiments. At this time-point, medium from PC3 cell line (prior to treatment of PPAT) was added to the explants (0% CM and 25% CM).

To obtain the stromal vascular fraction (SVF) of PPAT, another portion of PPAT was incubated with collagenase (2 mg/mL) (Collagenase A, Roche) for 60 minutes at 37°C with agitation (120 rpm). Afterward, the SVF cell pellet was resuspended in sDMEM/F-12 with 10% Newborn Calf Serum (NCS) and filtered through a 40 μm cell strainer (BD Falcon, BD Biosciences). After washing and erythrocyte lysis, SVFs were resuspended and seeded (500 μL of cell suspension) in wells coated with 0.2% gelatin (Gelatin, Sigma Aldrich) in sDMEM/F-12 medium with 10% NCS. After 48 hours, fresh medium without NCS was added. This time was referred as time zero for time-course experiments. At this time-point supernatant from PC3 cell line (prior to treatment of PPAT) was added to the culture medium (0% CM and 25% CM).

Cultures were stimulated with CM for 48h. After this time, media was collected, centrifuged (20000g, 3 min), aliquoted and stored at -80°C. Explants and SVFs were included in Tripure reagent (Roche) and stored at -80°C.

Human PC3 cell line

PC3 cell line was obtained directly from ECCAC. RPMI 1640 supplemented with L-glutamine and Hepes (Gibco), 10% FBS (Gibco) and 1% PS (Sigma Aldrich) was used as basal medium. Cells were incubated at 37ºC with 5% CO_2. For CM experiments, cells were seeded and grown to confluence. Thereafter, medium was replaced by RPMI 1640, 1%PS, without FBS and maintained for 24 h. After, supernatant was collected, centrifuged at 20000g for 3 minutes (RT), aliquoted and stored at -80°C.

Mitochondrial DNA quantification

DNA was isolated through QIamp DNA mini kit (QIAGEN), according to the manufacturer instructions. PCR and cloning procedures to synthesize standard DNA and quantitative real-time PCR protocol were done as described [13]. Each PCR run included efficiency, melting curve, and standard curve correlation coefficient analysis. Relative mtDNA copy number was calculated as *16SrRNA* copy number/*GAPDH* copy number.

Protein assay

Supernatants derived from PPAT-derived explants and SVF cultures following PC3 CM treatment and from PC3 (prior to treatment of PPAT) were used for adipokine measurements. Multiplex for TNFα, MCP-1 and IL-6 (Human cytokine/chemokine Milliplex MAP kit, 3-plex, Millipore, USA) was

	Men with prostate cancer (n=12)
Age (years) [a]	60.9 (57.5-64.3)
PSA at diagnosis (ng/mL) [a]	9.9 (5.9-14.0)
Body mass index (Kg/m^2) [a]	26.4 (24.2-28.6)
Prostate weight (g) [a]	47.9 (40.2-55.6)
Pathological stage [b]	
Localized	58.3
Locally advanced	41.7
Gleason score [b]	
≤ 7 (3+4)	66.7
≥ 7 (4+3)	33.3

Table 1. Clinical characteristics of patients included in the study. [a]Mean (95% confidence interval), [b]Percentage of individuals

performed for both explants and SVF supernatants, whereas multiplex for leptin and adiponectin (Human adipocyte Milliplex MAP kit, 2-plex, Millipore, USA) was only run in media from explants, according to the manufacturer's instructions. Since these adipokines are exclusively produced by adipocytes, and the SVF fraction does not include adipocytes, their concentration was not determined in SVF supernatants. Sample concentrations were measured using Luminex 200 (Luminex, Arium, Lisboa, Portugal) and analysed with Luminex 100 IS v2.2 software. A 5-parameter logistic method was used for calculating adipokine concentrations in samples. The intra- and inter-assay precisions were adequate, according to the manufacturer. The minimum detectable concentration was 0.4 pg/mL for IL-6, 1.2 pg/mL for MCP-1, 0.2 pg/mL for TNFα, 27.4 pg/mL for leptin and 80.3 pg/mL for adiponectin.

Osteopontin and CCL5 levels were determined in supernatants using commercially available human ELISA kits (R&D Systems), according to manufacturer's instructions. A 4-parameter logistic formula (GraphPad Prism 5 software) was used to calculate concentrations. The intra- and inter-assay precisions were <4% and <7% for OPN and <3% and <7% for CCL5, respectively, and the minimum detectable amount was 0.011 ng/mL for OPN and 2.0 pg/mL for CCL5. For all samples, median fluorescent intensity or absorbance was subtracted from the readings obtained for PC3 CM (prior to treatment of PPAT) sample.

Zymography

Matrix metalloproteinases 2 and 9 (MMP2 and MMP9) gelatinolytic activities were determined on substrate impregnated gels. Briefly, total protein from supernatants of primary cultures incubated or not with PC3 cell CM (12 µg/well), were separated on 10% SDS-PAGE gels containing 0.1% gelatin (Sigma-Aldrich). Gels were washed (2% Triton X-100) and incubated for 16-18h at 37°C in substrate buffer (50mM Tris-HCl, pH7.5, 10mM $CaCl_2$). Gels were stained in a solution with Comassie Brilliant Blue R-250 (Sigma-Aldrich), 40% methanol and 10% acetic acid for 30 minutes. The correspondent MMP2 and MMP9 clear lysed areas were identified based on their molecular weight and measured with a densitometer (Quantity One, BioRad).

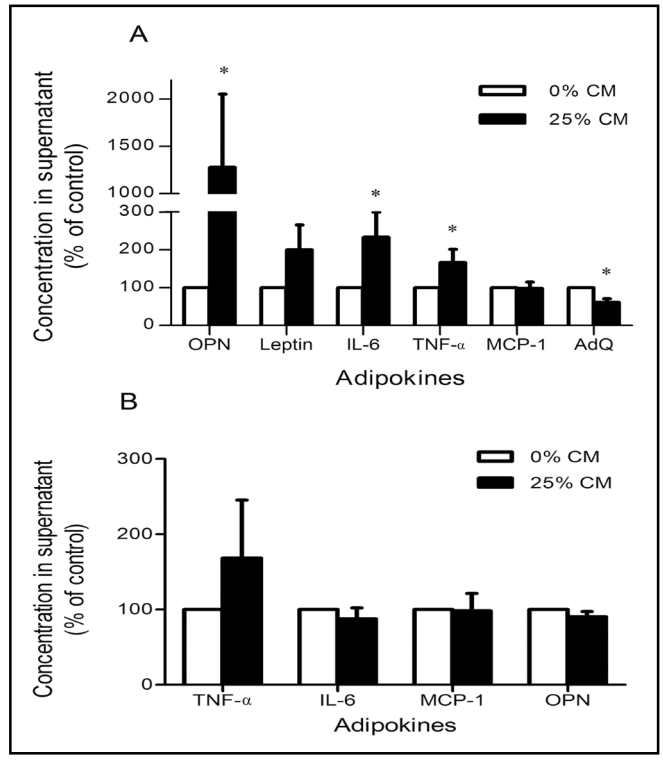

Fig. 1. Percent increase in adipokines concentration measured in supernatants of adipose tissue explants (A) and stromal vascular fraction (SVF) (B), after stimulation with conditioned medium (CM) from PC3 prostate tumor cells (mean ± SE). OPN, osteopontin; IL-6, interleukin-6; TNFα, tumoral necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; AdQ, adiponectin; CCL5, RANTES. * $p<0.05$, compared with control (0% CM)

Statistical analysis

Data are presented as mean ± SE. Measurements of mtDNA, protein and MMPs activity were adjusted per gram of PPAT before statistical analysis. Values for mtDNA copy number and MMPs activity were $\log_{10}$-transformed to become normally distributed. Unpaired t-test or Mann-Whitney U tests were used for statistical comparison. Analyzes were performed using SPSS 17.0 and $p<0.05$ was considered significant.

Results

After 48h of stimulation with CM from a PCa cell line, PPAT-derived explants increased protein expression of TNFα by approximately 1.7-fold (p=0.030), IL-6 by 2.3-fold (p=0.044) and OPN by 13-fold (p=0.015), whereas the expression of adiponectin decreased by 0.6-fold (p=0.045) (Fig. 1). The effects on MCP-1, leptin and CCL5 expression (data not shown) were not statistically significant (p=0.728, p=0.146 and p=0.482, respectively). In contrast, we found no effect of CM from cancer cells on adipokine expression by SVF. Stratified

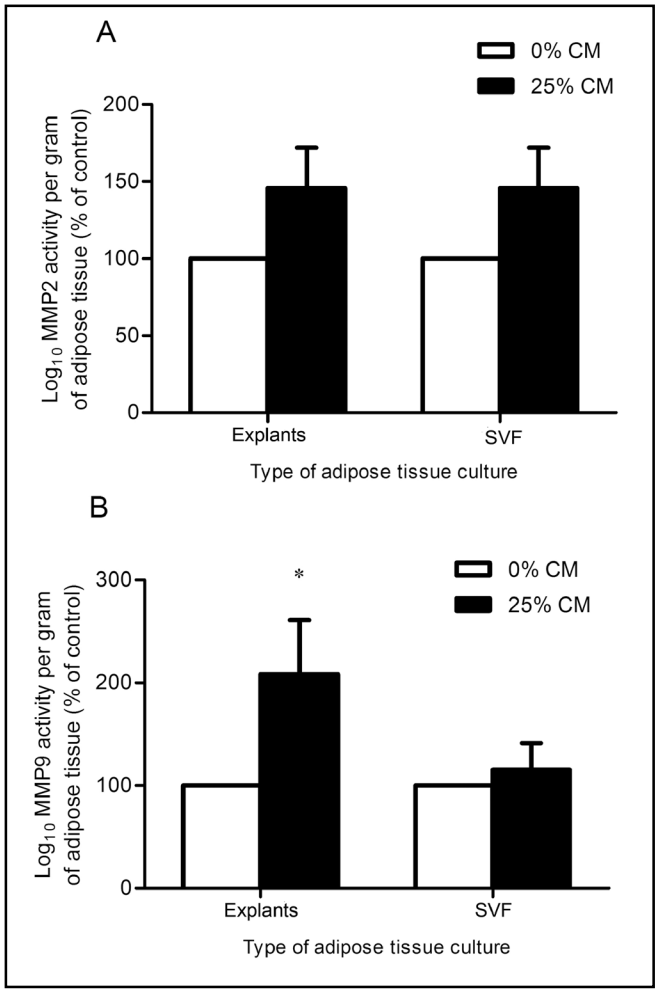

Fig. 2. Log-transformed (Log_{10}) matrix metallo-proteinase-2 (MMP-2) (A) and matrix metalloproteinase-9 (MMP-9) (B) activity per gram of tissue in adipose tissue explants and in stromal vascular fraction (SVF) after stimulation with PC-3 tumor cells conditioned medium (CM) (mean±SE). * $p<0.05$, compared with control (0% CM)

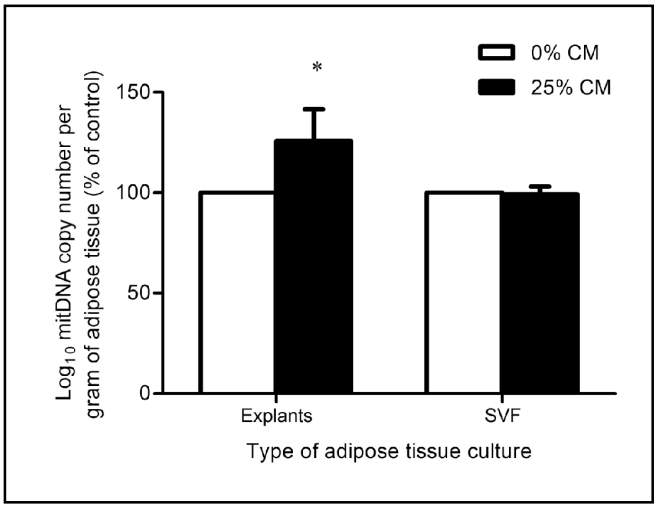

Fig. 3. Log-transformed mitochondrial DNA (Log_{10} mitDNA) copy number adjusted per gram of adipose tissue in adipose tissue explants and stromal vascular fraction (SVF) after stimulation with conditioned medium (CM) from PC3 prostate cancer cell line (mean ± SE). * $p<0.05$, compared with control (0% CM)

analysis concerning obesity and stage of disease, showed that obesity did not influence the production of adipokines, despite the increased expression of TNFα, IL-6 and OPN in PPAT in overweight/obese group (data not shown). Interestingly, following treatment with PC3 CM, the expression of TNFα, IL-6 and OPN protein was higher for PPAT-derived explants from patients with locally advanced disease (p=0.016, p=0.004 and p=0.030, respectively). Adipokine levels, MMPs activity or mtDNA copy number of PPAT explants prior to treatment with PC3 CM were not influenced by patients obesity (normoponderal versus obesity/overweight), Gleason grade (below 3+4 versus above 4+3), PSA at diagnosis (PSA < 10 ng/mL versus PSA = 10 ng/mL) and prostate capsule invasion (yes versus no) (data not shown).

The *in vitro* MMP9 activity of PPAT was higher in stimulated explants (p=0.043), whereas a trend for higher activity of MMP2 was observed in SVF cultures (p=0.088) (Fig. 2). Noteworthy, we observed an increased MMP2 activity following PC3 CM treatment in explants according to the obesity background of patients (lean vs. overweight/obese, p=0.013). Furthermore, stimulation with PCa CM increased mitochondrial DNA (mtDNA) copy number in explants but not in SVF (p=0.033 and p=0.687, respectively) (Fig. 3).

Discussion

In PCa, tumors grow in the peripheral zone and frequently progress towards the posterior surrounding PPAT. The proximity between both tissues promotes adipose tissue-tumor cells crosstalk. Concordantly, recent studies reported the association of PPAT thickness and increased local production of IL-6 with aggressive PCa phenotype [9-11].

One of the mechanisms that causally invoke obesity with cancer progression includes deregulation of adipokine

production [14]. The only study on PPAT, evidenced increased IL-6 local concentration, and its correlation with tumor aggressiveness [11]. Studies of IL-6 circulating levels suggest that IL-6 is involved in PCa progression and has prognostic significance [15, 16]. Interestingly, in this study we demonstrated that after exposure to PCa cell CM, the PPAT explants increased their production of IL-6. Likewise, adipocytes cocultured with breast cancer cells overexpressed IL-6 [17]. The increased IL-6 availability may induce neuroendocrine differentiation, intra-prostatic androgen production and activation of androgen receptor [18, 19].

The adipokine OPN is an instigator of tumor growth and progression, contributing to disease aggressiveness [20, 21]. It has been implicated in tumor cells malignancy in vivo, in tumor cell dormancy, and as a plasma marker for aggressive disease [20, 22], even though its role as a PPAT-derived molecule with paracrine effect in locally advanced prostate cancer has not been evaluated so far. In the present study, we observed a near 13-fold increased production of OPN by PPAT in response to PCa cell CM, suggesting a significant contribution to OPN local production, further eliciting tumor progression.

Another adipose tissue secretory product is TNFα, a mediator of cancer-related inflammation [23], which has a direct effect causing genetic damage to cells, but also enhances malignant cell survival and induces epithelial-to-mesenchymal transition [24, 25]. Its circulating levels correlate with the extent of disease in patients with PCa [16]. We found increased TNFα expression by stimulated PPAT explants, further involving this fat depot in tumor progression.

Conversely, although adiponectin is exclusively produced in adipose tissue, its levels are inversely related to adiposity degree [26]. Adiponectin modulates the AMP-activated protein kinase (AMPK) pathway, acting as a direct endogenous inhibitor of inflammation, angiogenesis and cancer invasiveness [4]. Additionally, a 25-year prospective study demonstrated a protective effect of adiponectin serum levels for developing high-grade PCa and dying from the cancer [27]. Thus, the observed reduction of adiponectin production by PPAT explants following stimulation agrees with a favorable tumor microenvironment orquestrated by tumor cell-derived factors. Interestingly, the expression of other candidate adipokines (MCP-1, CCL5 and leptin) was not influenced by PCa cell CM, although they have a role in tumor growth and metastasis [28, 29]. We hypothesize that these molecules might be modulated by other clinical factors (e.g. obesity), or alternatively, by other cell types usually involved in cancer, such as macrophages and fibroblasts. Otherwise, the PC3 cell line may not produce the factors that could influence the expression of CCL5, leptin and MCP-1.

Recent studies have shown that basal adipokine expression vary on PPAT explants with respect to Gleason grade and stage of the tumor [11]. Our results showed increased adipokine (TNFα, IL-6 and OPN) expression by the PPAT from men with locally-advanced PCa, after treatment with PC3 CM, suggesting that adipose tissue from advanced disease patients, whether modulated by the tumor or genetically predetermined, produces higher levels of adipokines that are associated with cancer progression. However, in our study base levels of adipokines before stimulation were not determined by obesity, Gleason grade, PSA and stage of disease of the patients. These findings may suggest the periprostatic adipose tissue has plasticity to be metabolically more active (not only by adipocyte hyperthrophy), after induction by unknown specific factors produced by PC3 cell line, and that this tissue exerts its modulating effects through an adaptive and responsive potential, further supporting the relevant role of a multidirectional dynamic tumor microenvironment.

Matrix metalloproteinases, are known to be involved in extracellular matrix remodeling and angiogenesis [30-32], which are common mechanisms to both obesity and cancer. Both gelatinases (MMP2 and MMP9) have the ability to proteolytically degrade denatured collagen in the vascular basal membranes, implicating them in angiogenesis [32]. Moreover, while MMP2 also facilitates cell migration by direct degradation of the basement membrane, MMP9 has been shown to release growth factors and to elicit the development of epithelial-to-mesenchymal transition in tumor cells [33, 34]. In PCa, expression of gelatinases has been associated with aggressive and metastatic disease [35-37]. Higher circulating levels and tumor expression of MMP2 and MMP9 were characteristic of PCa patients, compared with non-cancer individuals [38-40]. In this study, we found that upon stimulation of PPAT with PCa cell CM, explants start to present higher MMP9 activity. Since inflammatory stimuli induces MMP expression [41], the inflammatory mediators produced by tumor cells, such as IL-6, may explain MMP activity. Further studies, involving silencing of IL-6 expression would be required to confirm such hypothesis. Cumulatively, the observation that MMP9 expression in adipocytes increases over 100-fold when cocultured with macrophages [42], suggests that adipocytes have a great plasticity for

increasing MMP9, possibly explaining our findings on explants. Interestingly, the level of MMP9 activity was not modulated by obesity, whereas MMP2 was increased in obese patients. Catalan et al. [43] also showed higher MMP2 activity in the visceral adipose tissue of obese individuals, whereas no changes were observed for MMP9 activity.

Mitochondria are key players in energy turnover. Recent research indicate that mtDNA biology may be relevant for adipose tissue function [44], and that increasing numbers of mtDNA copies are associated with increased number of mitochondria per cell and with mitochondrial function [45, 46]. It has been suggested that mtDNA levels in adipocyte mitochondria may influence nuclear gene expression through retrograde signaling (via reactive oxygen species or AMPK activity) [44], thus affecting overall adipocyte physiology and influencing the synthesis and release of adipokines. Since we observed increased metabolic activity of PPAT after stimulation with PCa cell CM, the increased mtDNA content may reflect an adaptive mechanism of adipose tissue to the increasing demand for synthesis of adipokines, an important energy-consuming process. Therefore, it seems plausible that adipokines synthesis need to be paralleled by mitochondrial function of adipose tissue. Additionally, the observed increase of mtDNA content may be attributed to the adipogenic effect of tumor-derived factors [5], since adipocyte differentiation has been associated with increased mitochondrial content in adipocytes [47, 48]. White adipocyte differentiation entails a large stimulation of mitochondrial proliferation, upregulation of components of the mtDNA replication (of the deoxynucleotide metabolism) and transcription (e.g. TFAM) machinery [44, 49]. In agreement, drugs that induce adipocyte differentiation *in vitro*, increase mtDNA content in human adipocytes [50].

In this study, we show that factors secreted by PCa cells have the ability to modulate adipose tissue explants (pre-adipocytes, mature adipocytes, monocytes, macrophages, lymphocytes, endothelial cells, adipose stem cells and fibroblasts), but not SVF (all the cell types such as in explants except for the adipocytes that were removed) [12]. In fact, such organ (explants) culture preserve the paracrine signals by maintaining the existing crosstalk among the different cell types [12, 51]. On the other hand, the conditions of SVF culture might have influenced the proportion of cellular populations and their function.

We cannot rule out that other aspects of adipose tissue functionality or even CM from different PCa cell lines might lead to distinct findings. However, it is not possible to address all features in the present clinical setting with only small amounts of tissue at hand.

This study examines alteration of PPAT from cancer patients by prostate cancer cell CM. In a clinicopathological setting it seems relevant to understand the challenge that prostate tumor cells may impose over PPAT and the repercussion in cancer progression and aggressiveness. Nevertheless, future studies using PPAT from non-cancer patients may contribute to further elucidate the role of PPAT in benign conditions. Although the influences of patients' tumors in our results are unknown, prostate cancer cells are frequently found in the posterior fat pad and in our population less than half (5 of 12 individuals) had extraprostatic disease. Concomitantly, the small size of the studied cohort of patients advises cautious interpretation of conclusions after stage- and obesity-stratified analysis. Further work should be performed in larger samples with wider BMI values. It will also be interesting to address the relative expression of adipokines (such as OPN, TNFα, adiponectin and IL-6) by PPAT in prostate cancer patients compared with non-cancer.

Our results are in line with reports on reactive stroma and inductive fibroblasts [52], indicating the tumor microenvironment as a potential inducing factor in prostate cancer progression. In conclusion, we found that tumor factors can regulate the expression of adipokines with oncologic potential (OPN, TNFα, IL-6 and adiponectin), MMP activity and mitochondrial DNA in PPAT. Our data suggest that in the context of extra-prostatic disease, PPAT may acquire a phenotype favorable to aggressive PCa.

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