Universidade de Aveiro Departamento de Biologia
Ano 2009

Virgínia Filipa Pereira Monteiro da Cunha

Culturas primárias de tecido adiposo humano e impacto no Cancro da próstata

Human adipose tissue primary cultures and impact in Prostate cancer

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Rui Medeiros, Professor Auxiliar Convidado do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, Professor Doutor António Correia, Professor Catedrático, Departamento de Biologia, Universidade de Aveiro e do Dr. Ricardo Ribeiro, Investigador do Grupo de Oncologia Molecular, IPO-Porto

o júri

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À minha avó, com saudade….

resumo

O Cancro da próstata (CaP) é uma das neoplasias mais frequentemente diagnosticadas e a segunda causa de morte por cancro no mundo nos homens. Entre outros factores de risco, a obesidade tem sido frequentemente associada a CaP, embora permaneçam por esclarecer os mecanismos subjacentes a esta associação. Com o presente trabalho pretendeu-se através de estudos *in vitro*, contribuir para a compreensão do impacto do tecido adiposo branco e suas sub-fracções (adipócitos e fracção vascular estromal), com origens anatómicas periprostática e visceral, na proliferação, apoptose, e invasão celular de células de cancro da próstata sensíveis à castração (LNCaP) e resistentes à castração (PC-3). Com o propósito de obter respostas directamente através de estudos em humanos, foram efectuadas culturas primárias de tecido adiposo periprostático e visceral obtido durante cirurgias urológicas (prostatectomia radical e adenomectomia prostática) (n=16). O tecido adiposo foi utilizado para realizar culturas primárias organotípicas (tecido adiposo total fraccionado) e após digestão com colagenase culturas primárias de adipócitos e de células da fracção vascular estromal do tecido adiposo. Foram colhidos sobrenadantes e infranadantes destas culturas de tecido adiposo e utilizados como meios condicionados representativos da produção de adipocinas. As linhas celulares LNCaP e PC-3 foram estimuladas com estes meios e avaliados a apoptose, proliferação celular e invasividade tumoral *in vitro*.

Este modelo de estudo representa um potencial meio para análise do impacto do tecido adiposo nas células tumorais, permitindo avaliar as interacções tecido adiposo-tumor.

Os resultados evidenciam que o tecido adiposo promove a proliferação das células tumorais, que o tecido adiposo periprostático aumenta a apoptose em indivíduos obesos e que os SVF suprimem a invasão das PC-3 através de um efeito directo nas células tumorais.

abstract

Prostate cancer (Pca) is one of the most frequent diagnosed neoplasies and the second cause of cancer-related death in the world, in men. Between others risk factors, obesity has been associated to Pca although the innerent mechanisms to this association remain to be clear. With this work, throuhg in vitro studies, we wish to contribute to the understanding of the impact of white adipose tissue and its sub-fractions (adipocytes and stromal vascular fraction), from visceral and periprostatic anatomic regions, in celular proliferation, apoptosis and invasion of castration sensitivity (LNCaP) and castration resistant (PC-3) prostate cells. With the purpose of obtaining answers directly from humam studies, were performed visceral and periprostatic adipose tissue primary cultures obtained during urologic surgeries (radical prostatectomy and prostatic adenomectomy) (n=16). Adipose tissue was used to make primary organotipical cultures (WAT) and after collagenase digestion adipocytes and SVF primary cultures. Sobrenatants and infranatants of each culture were collect and used as conditioned medium representing adipokines production. LNCaP and PC-3 cell lines were stimulated with these mediums and apoptosis, proliferation and invasion were evaluated, in vitro.

This study model represent a potential form for analyze the impact of adipose tissue in tumor cells, allowing to evaluate adipose tissue-tumor interactions.

The results show that adipose tissue promotes tumor cells proliferation, that periprostatic adipose tissue increase apoptosis in obese individuals and that SVF subfraction suppresses invasion of PC-3 cells through a direct effect in tumor cells.

Abbreviations

ATH – Adipose tissue hipoxia

BMI – Body mass index

CRPCa- Castration resistant prostate cancer

CSPCa- Castration sensitive prostate cancer

CM- Conditioned medium

DMSO – Dimetil Sulphoxide

DNA - Deoxyribonucleic acid

Elisa – Enzyme-linked immunosorbent assay

FITC- Fluorescein isothiocyanate

FBS – Fetal bovine serum

g – gram

IGF – Insulin growth factor

IGFBP – Insulin growth factor binding protein

IL-6 – Interleukin 6

LHRHa- luteinizing hormone releasing hormone agonists

LNCaP - Androgen dependent prostate cancer

mg – miligram

ml – mililitre

n - number of samples

NCS – Newborn calf serum

nm – namometre

PBH – Prostate benign hyperplasia

PBS – Phosphate-buffered saline

PC-3 – Androgen independent prostate cancer

PCa – Prostate cancer

PerCP- Peridinin-chlorophyl-protein

PP- periprostatic adipose tissue

PS – penicil-streptomicin

PSA - Prostate specific antigen

RNA - ribonucleic acid

Rpm – rotations per minute

RT – Room temperature

SHBG - Sex hormone-binding globulin

SPSS – Statistical Package for social sciences

SVF – Stromal vascular fraction

TMB- tetramethylbenzidine

TNF-α – Tumor necrosis factor

TNM- stage of disease

VIS- Visceral adipose tissue

WAT – Whole adipose tissue

WHO – World Health Organization

µl – microlitre

µM – micromol

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INTRODUCTION

1. Introduction

1.1 General aspects about cancer

It is estimated that 1 of each 3 persons is affected by cancer all over the world. Ten million new cases are diagnosed each year, and it is predictable that this number will double in the next 20 years (Pecorino, 2005).

Tumorigenesis is a multistep process that comprises genetic alterations that ultimately lead to progressive transformation of normal into malignant cells. This transformation requires modifications in cell physiology to promote the development of tumor cells, which Hanahan & Weinberg (2000) defined as hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion from programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

In invasive carcinomas, compromise of the basement membrane integrity leads to abnormal epithelial/mesenchymal heterotypic cell crosstalk. Conversely, albeit most studies focused on cancer cell-mesenchymal cell interactions have neglected adipose tissue-derived cells; more concern is being devoted to adipose tissue since it is an excellent candidate influencing tumor behaviour through heterotypic signalling processes and might prove to be critical for tumor survival, growth and metastasis.

Additional research is needed, to help determine novel mechanisms and elaborate new targeted therapies for malignancies treatment and chemoprevention.

1.2 Prostate Cancer: clinics, pathology and molecular mechanisms

Prostate cancer (PCa) is one of the most frequently diagnosed malignancies and the second cause of cancer-related death in men worldwide (Aus et al., 2005). Its incidence and mortality seem to have a geographic trend (Fig. 1), since Eastern countries have the lowest incidence of PCa, contrasting with western countries.

Ethnicity, advanced age and family history are other well established risk factors for this disease (Hsing & Chokkalingam, 2006). Furthermore, circulating androgen levels, chronic prostate inflammation and obesity are also risk factors frequently described in the literature (Anderson et al., 2000; Platz & De Marzo, 2004; Ribeiro et al., 2006).

Prostate cancer is characterized by a long natural history (Nelson et al., 2003). Autopsy studies demonstrated that 30-40% of 60 years-old men and 60-70% of men with 80 years-old had prostate microadenocarcinoma, albeit no previous history of PCa was known (Muir et al., 1991; Whitmore, 1994).

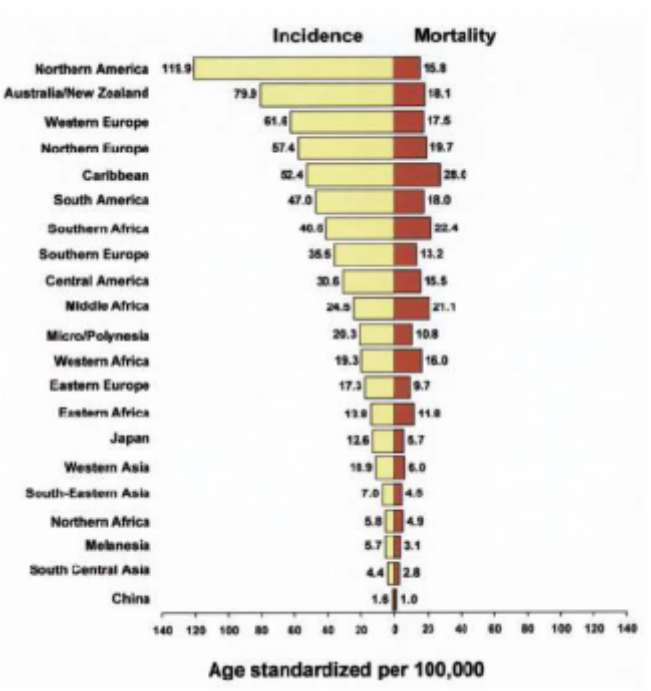

Fig. 1 Geographic distribution of prostate cancer incidence and mortality rates in 2002 (Parkin et al., 2005)

Prostate cancer screening, recommended in men after 45 years of age, include annual measurement of blood PSA (Prostate Specific Antigen) levels and digital rectal examination. Two consecutive rises of PSA value over 0.5 ng/mL or one single value ≥ 4 ng/mL are indications for biopsy (Mistry & Cable, 2003). However, although PSA testing has high sensibility, its specificity is rather low, putting Clinicians under doubts for biopsying, since increased false-positive rates, overdiagnosis and overtreatment have been reported as associated with PSA testing (Nash & Melezinek, 2000; Draisma et al., 2009; Schroder et al., 2009; Welch & Albertsen, 2009).

The biological behaviour of PCa exhibits a high inter-individual variability, and is clinically and pathologically characterized through the percent of tumor volume, histological differentiation and architecture (Gleason grade) and stage of disease (TNM).

Gleason grade is an indicator of aggressiveness and prognosis, evaluating the extension of glandular differentiation and the tumor growth status in prostatic stroma (Gleason, 1992; Humphrey, 2004; Harnden et al., 2007; Heidenreich, 2007).

Due to PCa long natural history, early detection in initial stages has good prognosis. Although initially men diagnosed in initial stages of disease were usually submitted to surgery (radical prostatectomy) and/or radiotherapy, with curative intent (Long et al., 2005; Pronzato & Rondini, 2005), increased concern has been devoted to surgery side-effects (urinary incontinence and sexual impotence) and several cohorts emerged to demonstrate the potential of PCa expectant management (Bill-Axelson et al., 2008; Shappley et al., 2009; Tilling et al., 2009; van den Bergh et al., 2009).

Men diagnosed with advanced stage of disease, not eligible for surgery, are submitted to chemical or surgical castration that consists of androgen blockade (Cabrespine et al., 2004; Pronzato & Rondini 2005), since androgens are important in PCa development (Loblaw et al., 2004; Angelucci et al., 2006). Initially, the neoplasia is sensible to androgen castration (CSPCa). The administration of combined luteinizing hormone releasing hormone agonists (LHRHa) plus antiandrogens is the standard therapy for advanced stage or progressing PCa (Cabrespine et al., 2004; Loblaw et al., 2004). This therapy can delay the tumor growth because it induces apoptosis of tumor hormonal-dependent cells and reduce PSA levels. Nevertheless, despite the initial sensibility, PCa evolves more or less rapidly into an irreversible stage denominated castration-resistant (CRPCa). This is a lethal form of PCa, with a high potential to progression and metastasis (Feldman & Feldman, 2001). Little is known about the mechanisms underlying this transformation. Besides hypothesis focused in androgen receptors expression and mutational profile in castration resistant tumors (Veldscholte et al., 1990; Van der Kwast et al., 1991; Horbisch et al., 1995; Chen et al., 2004), there is also evidence that alternative pathways activated by growth factors and cytokines may modulate or supplant the requirement for androgens to stimulate the androgen receptor (Craft et al., 1999; Culig, 2004), or even growing independently (Feldman & Feldman, 2001).

1.3 Obesity: conceptualisation and pathophysiology of adipose tissue

Obesity has become the most common nutritional disorder and its prevalence is increasing rapidly in most parts of the world (World Health Organization, 2000). The World Health Organization (WHO) defines obesity as "*an abnormal or excessive fat accumulation in adipose tissue, to the extent that health is impaired*". This statement is quite accurate, even more because it has been associated with incidence and mortality of a number of diseases, such as hypertension, coronary heart disease, diabetes, obstructive sleep apnoea, cancer and others (Klein, et al. 2002; Kopelman, 2000; Flegal et al., 2005).

Body Mass Index (BMI) is commonly used in clinical settings for classifying obesity and to provide information about disease risk. BMI is calculated using the individual's weight in kilograms divided by their height in squared meters. For epidemiological purposes, the classification of obesity begins at BMI $\geq$ 30 kg/m^2 (World Health Organization, 2000) (table 1)

Table 1 WHO classification of obesity using BMI. Adapted from Calle & Kaaks (2004)

BMI (Kg/m2)	WHO classification	Description
< 18.5	Underweight	Thin
18.5-24.9	Normal weight	Healthy, normal or acceptable
25.0-29.9	Grade 1 overweight	Overweight
30.0-39.9	Grade 2 overweight	Obesity
≥ 40.0	Grade 3 overweight	Morbid obesity

However, a BMI of 30 kg/m^2 is not always synonym of obesity**.** It is important to note that within each category of BMI there can be substantial individual variation in total and visceral adiposity, and in several related metabolic variables. This is also true within what is at present considered the 'normal' or 'healthy' range of BMI (18.5–24.9). Individuals at opposite ends of the normal range of BMI can experience considerable differences in adiposity-related risks and health outcomes (Calle & Kaaks, 2004).

Obesity is characterized by the increased accumulation of triglycerides in adipose tissue, in subcutaneous and intra-abdominal omental depots, but also in fat surrounding organs such as kidney, epicardium, skeletal muscle and blood vessels (Montani et al., 2004). This is

mainly attributable to adipocyte's hypertrophy, although, to less extent, basal turnover hyperplasia may also occur (Spalding et al., 2008).

White adipose tissue is a heterogeneous tissue containing adipocytes, pre-adipocytes, and a stromal vascular fraction (SVF) that include mainly macrophages, endothelial and mesenchymal cells and fibroblasts (Toda et al., 2009).

Adipose tissue was long known as merely an energy storage organ with the function of triglycerides repository, but emerging data suggest that adipose tissue is a bioactive endocrine organ that affects energy balance and has an important functional role in modulating human disease. Adipose tissue produces several cytokines (such as IL-6, TNF-α, IL-8, ...), hormones (such as adiponectin, leptin, resistin, …) and growth factors (bFGF, VEGF, ...) that play crucial roles in obesity (Furhbeck et al., 2001) and cell proliferation (Onuma et al., 2003) among others (Fig. 2). With only few exceptions, circulating levels of molecules produced in adipose tissue (adipokines) are increased in obesity, and seem to be correlated with total fat mass (Arita et al., 1999). In the contrary, adiponectin levels are negatively correlated with BMI and with visceral fat depot (Arita et al., 1999; Cnop et al., 2003)

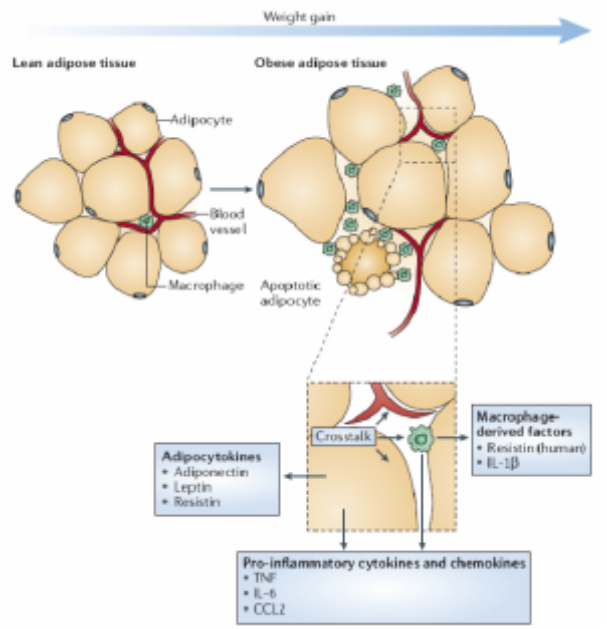

Fig. 2: Adipose tissue and secreted molecules (Tilg & Moschen, 2006)

Adipokines can impact energy balance and production, while playing a relevant role in obesity's pathophysiology. As an example, leptin that is widely expressed during obesity has been found to act as a satiety hormone by interfering with hypothalamic regulatory systems involved in the control of food intake (Friedman & Halass, 1998). Cumulatively, obesity is associated with infiltration of macrophages into adipose tissue, which seem to be responsible for producing inflammatory markers, therefore perpetuating inflammation into a chronic condition (Fain et al., 2004; Trujillo & Scherer, 2006).

Spalding et al. (2008) demonstrated recently that fat cells are highly regulated in the human adult, and because adipocytes are considered differentiated cells the apparent increase of their number must be due to adipogenesis (proliferation/differentiation of pre-adipocytes to adipocytes). This issue highlights the possibility of a feedback effect from tumors on adipose tissue through a paracrine loop. Conversely, Hirano et al. (2008) observed a stimulatory effect of molecules produced by tumor cells in adipogenesis, reflecting a crosstalk between tumor cells and adipose tissue.

Although obesity is a multifactorial disease, ultimately it results from a failure of homeostatic mechanisms that regulate body weight (Blaak, 2009). Positive energy balance develops when energy intake exceeds its expenditure, and excess energy is then stored in the form of triglycerides, predominantly in adipose tissue. Energy imbalance due to excess caloric intake is increasing throughout the world, with increased consumption of processed food and sedentary lifestyles (Ferranti & Mozaffarian, 2008). Therefore, reducing caloric intake can prevent and decrease adiposity and its associated health consequeces (Thompson et al., 2007). In cancer, classic mouse model studies pointed out that caloric restriction was significantly associated with cancer delay and increased lifespan (Weindruch & Waldorf, 1988; Finch, 1990; Hursting et al., 2003). In *vivo* studies with p53-deficient mice subjected to caloric restriction programs, demonstrated carcinogenesis suppression (Berrigan et al., 2002; Patel et al., 2004).

1.4 Obesity and prostate cancer: epidemiology and mechanistic hypothesis

Obesity is a condition that in the last years has become a worldwide public health problem, being related with a number of diseases including cancer (Friedman 2000; Kopelman 2000). It has been associated with increased risk to develop several cancers, such as breast, colorectal, endometrial, or kidney cancer (Bianchini et al., 2002; IACR, 2002; Klein, et al. 2002; Petridou et al., 2002; Calle et al., 2003; Harvie et al., 2003; Stephenson & Rose 2003; Rose et al., 2004; Kuriyama, et al. 2005; Cymbaluk et al., 2008)

Prostate cancer is one of the most diagnosed neoplasies in men, being more frequent in the western countries, mainly in North America, South European countries and United Kingdom (Vercelli et al., 2000). Asian men have had lower rates of PCa, albeit recent studies show a rapid increase, during the last two decades, in Asian countries (Schimizu, et al., 1991; Pu et al.2004).

Epidemiological studies yielded mixed results on the association of obesity and prostate cancer. While some cohort studies demonstrate an association of obesity with PCa (Andersson et al., 1997; Veierod et al. 1997; Engeland et al., 2003;), others failed to find such an association (Nilsen et al., 1999; Schuurman et al., 2000).

Recent studies, suggest that adult obesity is associated with PCa advanced disease and high-grade disease (Dal Maso et al., 2004; Robinson et al., 2005; Freedland et al., 2009). Others established the relation between obesity and higher PCa-associated mortality (Rodriguez et al., 2001) and predisposition for radical prostatectomy in a much younger age (Amling et al., 2001).

Some authors have been arguing that obesity by itself can cause difficulties for PCa detection. In fact, transrectal ultrassonography is more difficult in obese men, eventually resulting in lower capacity for detecting cancer (Buschmeyer & Freedland, 2007). Another emerging problem resides in the fact that obese men tend to have significantly lower PSA values (Gray et al., 2004; Baillargeon et al., 2005; Barqawi et al., 2005), thereby, masquerading higher levels of PSA and indication for biopsy, resulting in more undetected cases. Others studies suggest that obese men have larger prostates (Dahle et al., 2002; Freedland et al., 2006), increasing the difficulty of detecting these cancers through biopsy (Kranse et al., 1999).

In obesity serum concentrations of several hormones and growth factors, including estrogens, insulin, IGF-I, and leptin, are increased, all of which have been linked to prostate cancer (Freedland & Aronson, 2005).

Obesity is correlated with PCa (Fig. 3) through mechanisms that modify the levels of estrogens, inflammatory cytokines, growth factors, insulin and adipokines (Meier and Gressner, 2004; Vendrell et al., 2004; Stattin et al., 2000; Hsing, et al., 2001;Stattin et al., 2001; Chang at al., 2001).

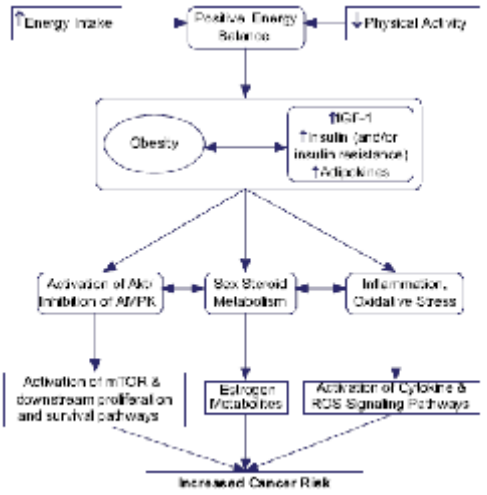

Fig.3:Schematic representation of the potential mechanisms underlying the obesity–carcinogenesis link. Adapted from Hursting et al. (2008)

In obesity the levels of resistin are increased, leading to insulin resistance and reducing the production of IGF binding protein (IGFBP)-1 and IGFBP-2 (usually binds to IGF-I). The reduction in IGFBP-2 increases free IGF-I resulting in cell microenvironment modifications that contribute to cancer development, through apoptosis inhibition and stimulation of cell proliferation (McKeown-Eyssen, 1994 and Giovannucci, 1995) (Fig. 4).

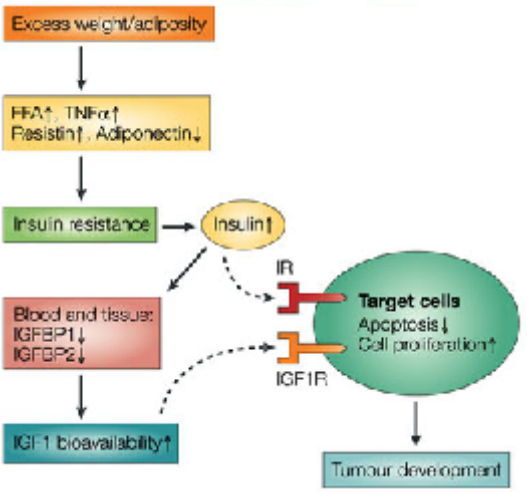

Fig. 4: Insulin and IGF mechanism scheme. Adapted from Calle & Kaaks (2004)

Several previous studies linked adipokines to cancer. As an example, the hormones leptin and adiponectin that are respectively positive and nagatively correlated with adiposity (Lonnqvist et al., 1997; Montague et al., 1997;), have been proposed to be associated with PCa (Chang et al., 2001; Bub et al., 2006; Hoda & Popken, 2008; Mistry et al., 2008; Sher et al., 2008; Arisan et al., 2009; Li et al., 2010), through a direct role in tumor cell migration and expression of growth factors (Frankenberry et al., 2004; Mistry et al., 2008; Tang & Lu, 2009). Other studies support a role for IL-6 production, which is upregulated in obese, in prostate cancer cell migration and invasiveness (Mistry et al., 2007; Finley et al., 2009).

Adipose tissue hypoxia (ATH) seems to be a necessary additional mechanism in the development of systemic insulin resistance, regulation of chronic inflammation and reduction of adiponectin while increasing leptin gene expression, thus contributing to cancer risk (Trayhurn et al., 2008). Additionally, tumor hypoxia is correlated with high mortality and the hypoxia inducible factor-1 alpha (HIF-1α) is associated with an increase in metastatic range (Vaupel & Hoeckel, 1999).

Another candidate mechanism that provides plausible explanation to obesity-prostate cancer association is the role of variants in genes that predispose to obesity or in adipokines' genes for tumor development. Cancer gene maps were derived from a number of SNPs and microarrays genome-wide association studies in prostate (Eeles et al., 2008; Thomas et al., 2008; Moore et al., 2009; Wang et al., 2009). Other gene-targeted studies

have been published concerning genetic variants in several adipokine's pathways (Kote-Jarai et al., 2003; Ribeiro et al., 2004; Tan et al., 2005; Danforth et al., 2008; Kesarwani et al., 2008; Langsenlehner et al., 2008).

A recent line of research supports the association obesity-cancer through migration of adipose stromal cells since the adipose tissue to the tumor. In fact, tumor progression depends on the establishment of a functional neovasculature, which can rely on progenitor mesenchymal stromal cells. There is increasing evidence that besides bone marrow-derived progenitor cells, mesenchymal stromal cells may arise from white adipose tissue and promote tumor growth (Yu et al., 2008; Zhang et al., 2009).

1.5 Functional impact of adipose tissue in tumor development: animal models and in vitro studies

Obesity is manifested by overgrowth of white adipose tissue, and it has been proposed that the tissue itself may have a direct effect on cancer progression (Rehman et al., 2003; Vona-Davis et al., 2007).

In vitro studies can help us understand the relation between obesity and PCa. It was recently reported that mature adipocytes co-cultured with breast and colon cancer cells can promote tumor growth (Manabe et al., 2003; Amemori et al., 2007).

The few studies using PCa cells, demonstrated that co-cultured adipocytes with PCa cell line resulted in cancer cell proliferation (Tokuda et al., 2003). Another study in prostate cancer, investigated the association of molecules produced by the periprostatic adipose tissue with pathological features of aggressive PCa, and found that the over-expression of IL-6 protein (375 times higher expression in local adipose tissue compared with serum levels) was responsible for the modulator role of periprostatic adipose tissue in PCa aggressiveness (Finley et al., 2009).

In a recent elegant study, Zhang et al., 2009 evaluated the recruitment of white adipose tissue derived cells by tumors and the effect of their engraftment on tumor growth. They observed migration of stromal and vascular progenitor cell (mesenchymal stromal cells) to

the tumor (also prostate tumors), with resulting tumoral neoangiogenesis and accelerated cancer progression. Nevertheless, contradictory results were obtained in other cancer models, such as pancreatic cancer (Cousin et al., 2009), suggesting a tumor-type specific effect. In such models human adipose tissue-derived mesenchymal stem cells is being studied as a promising source of autologous stem cells for personalized cell-based therapies, including their role as mediators of prodrug-converting enzymes to inhibit tumor growth (Kucerova et al., 2007; Cavarretta et al., 2009).

In this emerging field of research, we believe many questions remain to be answered: what is the result of adipose tissue and its subfractions-derived conditioned medium in PCa cells growth, apoptosis and invasiveness. Additionally, it is necessary to understand the effect of conditioned medium from different anatomical fat depots, pre-peritoneal and periprostatic, which is the extra-prostatic ground for tumor cells after invading the organ capsule.

OBJECTIVE

2. Objective

2.1. General objective

To get mechanistic insight on the association of obesity with prostate cancer through human adipose tissue primary cultures experiments. The objective is to evaluate the impact of molecules produced by the adipose tissue and its sub-fractions in prostate tumor cells (castration-resistant PC-3 cell line and castration sensible LNCaP cell line).

2.2. Specific objectives

1) To study the effect of molecules produced in primary cultures of human adipose tissue and sub-fractions (conditioned medium assays) in the proliferation of prostate tumor cell lines (PC-3 and LNCaP);

2) To analyse the effect of molecules produced in primary cultures of human adipose tissue and sub-fractions (conditioned medium assays) in the apoptosis of prostate tumor cell lines (PC-3 and LNCaP);

3) To evaluate the stimulatory effect of conditioned medium from human adipose tissue cultures in tumor cells' invasiveness;

4) To understand the impact of anatomical fat depot origin in tumor cell proliferation, apoptosis and invasiveness.

MATERIAL AND METHODS

3. Material and Methods

3.1. Donors of white adipose tissue samples

Human visceral and periprostatic adipose tissues were obtained during the dissection to reach the prostate in prostate cancer patients elective for radical prostatectomy or prostatic adenomectomy in IPO-Porto, Military Hospital of Porto, Terço Order and São Francisco Order. A total of fifty nine men took part in this study after signing informed consent. The study was approved by the Institute's Ethics Committee.

During surgery, after anaesthetic induction and initial dissection to reach the prostate, samples of visceral pre-peritoneal and anterior-lateral periprostatic fat depots (1-10 g each) were obtained by a urological surgeon and immediately processed within the surgery room.

Only samples from 16 individuals, choosen randomly, where effectively used. This fact was due to, time and samples limitations. This group of patients (mean age, 63.2±7.4 years; mean BMI, 25.5±3.3 $Kg.m^{-2}$) included individuals with benign prostate hyperplasia (BPH) (n=2) and with prostate cancer (PCa) (n=14).

For proliferation, apoptosis and invasion experiments, patients were divided according to quartiles of BMI. Patients were stratified in three groups: quartile 0-25 corresponds to BMI < 24 (n=3); quartiles 25-75 corresponding to a BMI 24-28 (n=10); and quartile 75-100 corresponds to a BMI > 28 (n=3).

3.2. White adipose tissue processing and adipose tissue primary cell cultures

The adipose tissue samples were sectioned and cleaned from macroscopic blood and vessels and rinsed with phosphate buffer saline plus 1% penicillin and streptomycin (PBS+PS1%). Adipose tissues from visceral and periprostatic depots (1-5g) were kept in PBS+PS1% solution at 37ºC for further processing within two hour and used for primary adipose tissue cell culture.

- **Primary cultures of adipose tissue explants**

Adipose tissue samples were transported to the lab in PBS+PS1% at 37ºC and further processed under a laminar flow hood. A portion of whole adipose tissue (~1-2 g) from each anatomical location (visceral and periprostatic) was placed in a sterile plastic cell culture Petri dish for further dissection into 1-2 cm pieces. Then, the adipose tissue fragments were weighted and placed in 6-well plates (2.5 mL medium per well) at a quantity of 0.3g explant/mL of culture medium DMEM/F12 (Gibco) suplemented with biotin 16µM, panthotenate 18 µM, ascorbate 100 µM, PS 1%, and minced with scissors into 5-10 mg pieces.

The explants were incubated overnight at 37ºC with 5% CO_2 atmosphere. In the next morning (~16-24 hours) medium was removed with a pipette tip of 1000 µL and fresh medium added to reach the concentration of 0.3 g tissue/mL. The time that fresh medium was added was referred to as time zero for time-course experiments. At 24 and 48h the undernatant was collected (1ml each), centrifuged (20000g, 3 min) and stored at -80ºC.

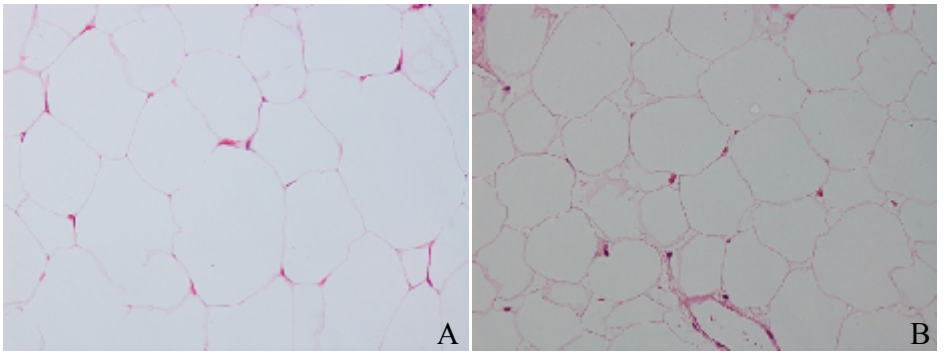

Fig. 5: Visceral (A) and periprostatic (B) adipose tissue, stained with hematoxilin/eosin (400x)

- **Separation of whole adipose tissue into adipocytes and stromal fractions**

Whole adipose tissue (WAT) pieces (visceral and periprostatic) were weighted and placed in a 50 mL Falcon tube with collagenase solution (2 mg/mL) (Collagenase A, Roche Diagnostics). The tissue was minced with scissors and the tube incubated for 60 minutes at 37ºC with agitation (120 rpm).

- **Primary cultures of adipocytes**

After collagenase digestion, the solution was pipeted through a 100 µm cell strainer (BD Falcon) for removing undigested tissues, and the enzyme activity stopped by adding DMEM/F-12 supplemented medium with 10% NCS.

The solution was then centrifuged at 1800 rpm for 10 minutes at room temperature (RT). Next, the floating adipocyte layer was removed with a Pasteur pipette, placed in a new tube and ressuspended in DMEM/F-12 supplemented (without NCS), followed by a centrifugation at 1100 rpm for 5 minutes at RT. The underneath medium was removed with a Pasteur pipette and the remaining adipocytes pellet was seeded in a 6 well plate with 2 mL/well DMEM/F-12 supplemented medium. For conditioned medium experiments 1 ml of cell culture medium was removed at 24 and 48 h, centrifuged at 20000g for 3 minutes at RT and the undernatants stored at -80º C.

- **Primary cultures of adipose tissue-derived stromal vascular fraction cells**

After removal of adipocytes layer, the supernatant was discarded. The SVF pellet was then resuspended in DMEM/F-12 supplemented medium with 10% NCS and filtered through a 40 µm cell strainer for removing remaining adipocytes. After centrifuging at 1500 rpm for 5 minutes, the supernatant was discarded and the pellet resuspendend and incubated (15 min at RT) in erythrocyte lysis buffer (QIAgen).

Afterwards the sample was centrifugated at 1500 rpm for 10 minutes at RT and the supernatant discarded. SVF were ressuspended and seeded in a 6 well plate with 2 mL DMEM/F-12 supplemented medium with 10% NCS.

Plates used for culturing SVF, were coated with 1 mL of gelatine 0.2% (Gellatine 2%, Sigma) and then incubated at 37ºC. After SVF cells adhered to the bottom of plates (Fig.6) and reached confluence (~48 hours), the medium was removed, the cells washed with PBS and fresh medium added. This time was referred to as time zero for time-course experiments. Conditioned medium (1 mL) was collected at 24 and 48 hours, centrifuged at 20000g for 3 minutes at RT and the supernatant stored at –80ºC.

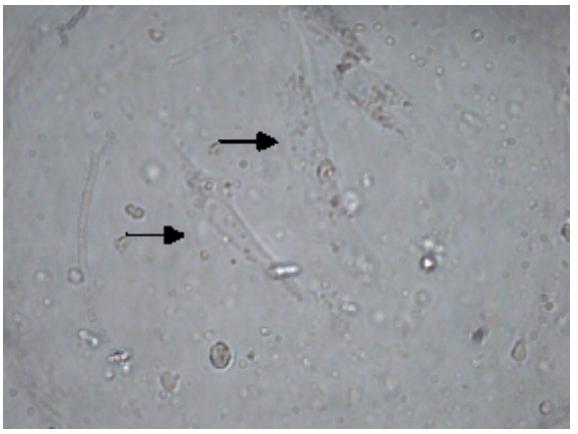

Fig. 6: In vitro primary culture of SVF (400x)

3.3. Culture of prostate cancer cell lines (LNCaP and PC-3)

Studies were performed in two established and well characterized prostate cancer lines, representative of different phases of prostate cancer, androgen-dependent or castration-sensible (LNCaP) and androgen-independent or castration-resistant (PC-3). PC-3 cells were obtained directly from ECCAC, while LNCaP cells were gently given by Dr Mariana Freitas from The General Pathology Institute, Faculty of Medicine, University of Coimbra.
The LNCaP cell line was isolated in 1977, from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate carcinoma.

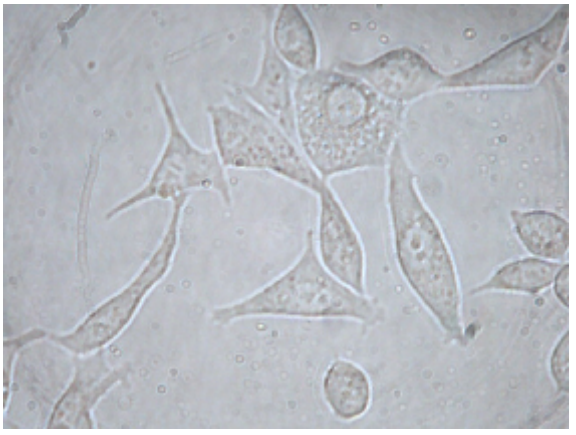

Fig. 7: In vitro culture of LNCaP cells (400x)

The base medium used for this cell line was RPMI 1640 supplemented with L-glutamine and Hepes (Gibco), 10% FBS (Gibco) and 1% PS (Gibco). Cells were incubated at 37ºC with 5% CO2. For conditioned medium experiments, cells were seeded in 12 wells plate ($3x10^5$ cells/well) and allowed to grow until 80% confluence. Then the medium was removed and replaced by RPMI 1640 + PS (1%) without FBS for 24h until the administration of conditioned medium.

The PC-3 cell line was derived from a 62-year-old Caucasian male and initiated from a bone metastasis of a grade IV prostatic adenocarcinoma in 1979 (Fig.8). RPMI 1640 supplemented with L-glutamine and Hepes (Gibco), 10% FBS (Gibco) e 1% PS (Gibco) was used as basal medium. Cells were incubated at 37ºC with 5% CO2. For conditioned medium experiments, cells were seeded in 12 well plates (1.5×10^5 cells/well) and allowed to grow until 80% confluence. Then we removed the medium and replaced it by RPMI 1640 + PS (1%) without FBS.

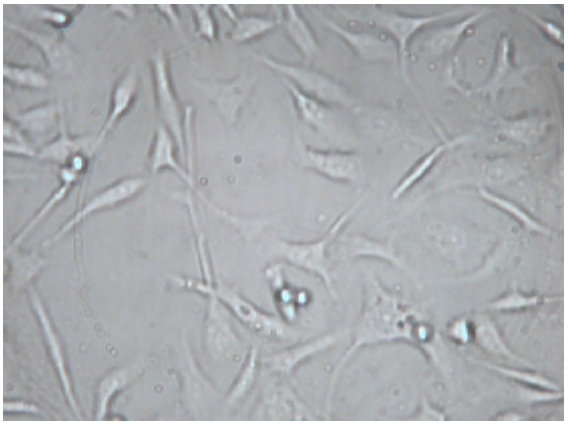

Fig. 8: PC-3 cells culture (200x)

The time and cell concentration for conditioned medium experiments was determined previously in our lab. Proliferation was assessed through a fluorescent proliferation assay (Cell Proliferation Assay Kit, Invitrogen), read in a Fluorimeter plate reader.

3.4. Leptin dosage in WAT Conditioned medium

In order to determine which medium to use in our experiments, leptin levels were measured by ELISA (BLK) in supernatants of explants collected at 24 and 48h. Samples from thirteen different subjects were analysed in duplicate.

3.5. Conditioned medium experiments in PCa cell lines

- **Proliferation Experiments**

Tumor cells were seeded into 96-well plates ($5x10^3$ PC-3 cells/well and $10x10^3$ LNCaP cells/well, 0.2 mL medium) and incubated 24 hours (PC-3) and 48 hours (LNCaP) with RPMI 1640 medium with 10% FBS to allow cell attachment. After removal of medium, fresh cell medium with variable percentages of adipose tissue-derived conditioned medium (from periprostatic and visceral fat from 10 normal weight patients, quartiles BMI 2 and 3) were added to the culture (RPMI 1640 medium without FBS plus 50% CM and without CM). In these experiments conditioned medium from WAT 48hours, adipocytes 24hours and SVFs 48hours, were used. All samples were performed in duplicate.
After 24h of incubation at 37 ºC and 5% CO2, the supernatant was removed and the cells were washed with PBS to remove the phenol red from the medium and then freezed at -80ºC for a minimum of 30min. A mixture of CyQuant dye with cell-lysis buffer was added to the wells and fluorescence measured at 480nm excitation and 520 nm emission. A standard curve was previously obtained for each cell type and readings were transformed in cell counts using this curve.

- **Apoptosis Experiments**

Tumor cells were seeded into 24-well plates (1×10^5 cells/well, 500µl medium) and incubated 24 hours (PC-3) and 48 hours (LNCaP) with RPMI 1640 medium with 10% FBS to allow cell attachment. After supernatant removal, fresh medium, with variable

percentages of visceral and periprostatic WAT 48h, adipocytes 24h conditioned medium (from three men in quartile BMI 1 and three men within quartile 4), was added to the culture (RPMI 1640 medium with no FBS plus 50% CM or no CM). Additionally, a pro-apoptotic agent (sodium selenite, Na_2SeO_3) (Menter et al., 2000) was added to the medium at a 5mM concentration. Twenty four hours later, supernatants and cells were removed from the wells (adherent cells detached with trypsin 0.05%), centrifuged at 2500 rpm for 3min, washed with cold PBS and centrifuged again. This process was done twice to remove phenol red of the cells.

Cells were then simultaneously stained with annexin-V-FITC (3µg/sample) and PI (1µg/mL) (both from BD Pharmingen). Before the samples were incubated in the dark for 15min, a binding buffer solution (1x) was added (from a 10x stock solution with 0.1M Hepes, pH 7.4; 1.4M NaCl and 25mM CaCl2).

All samples were analyzed in a flow cytometer (BD FACSCanto II, BD Bioscience, San Jose, CA), and nuclei gated in a forward/side scatter plot (FSC-A vs SSC-A), to capture the cells of interest. Propidium Iodide flourescence was read at FL-2 and Anexin V conjugated with FITC at FL-1. Early apoptotic cells are characterized by high annexin binding and low PI staining, whereas late apoptotic and necrotic cells stain strongly for both annexin and PI

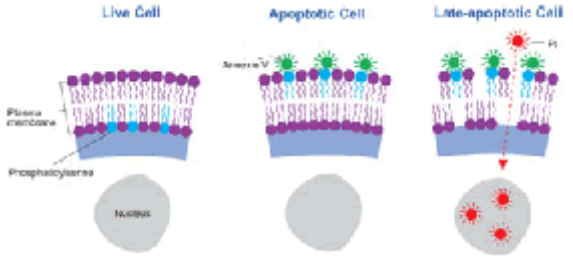

Fig.9: Apoptosis detection with Annexin V and Propidium Iodide (adapted from www.biomol.de)

Controls used were: unstained cells with sodium selenite, unstained cells without sodium selenite, only Annexin V-FITC, and only Propidium Iodide.

Up to 10.000 cells were detected in each sample and data acquired using BD FACSDiva V6.1.1 software (BD Biosciences). All experimental data were analyzed using Infinicyt V1-1.0 software (Cytognos S.L., Salamanca, Spain). To outline the different populations, quadrants were set by the unstained cells without sodium selenite.

- **Invasion Assay**

Prior to the experiment, PC-3 cells were starved for 24h (RPMI 1640 without FBS) and incubated at 37ºC with 5% CO2. Pre-warmed serum free medium was added to the interior of the inserts coated with ECMatrix (Cell Invasion Assay, QCM 24-Well, Millipore), and allowed to rehydrate the ECM layer for 15-30min at RT. After rehydration, 250µl of medium was removed from the insert and tumor cells seeded inside the inserts in 24-well plates ($5x10^4$ PC-3 cells/well). Conditioned medium used in these experiments were from SVFs 48hours, from men within quartile BMI 1 (n=3) and men within quartile BMI 4 (n=3).

1) conditioned medium inside the insert

Inside the insert 50% or 0% visceral and periprostatic adipose tissue-derived conditioned medium (with RPMI 1640 medium without FBS). In the lower chamber was added tumor cells medium with 10% FBS as chemoattractant. The plate was incubated for 24 hours at 37 ºC with 5% CO2.

Afterwards, the medium and the remaining cells from the top side of the insert were carefully removed, and the inserts were placed into a clean well containing pre-warmed Cell Detachment Solution and incubated for 30 min at 37 ºC. After the insert was removed, a mixture of Lysis Buffer and Dye Solution was added to the well containing the detached cells, and incubated for 15min at RT (Fig. 10). Two-hundred microliters of each condition were transferred to a 96-well plate and fluorescence read at 480/520 nm.

This experiment was designed to evaluate the influence of the molecules produced by SVFs directly in tumor cells invasive potential.

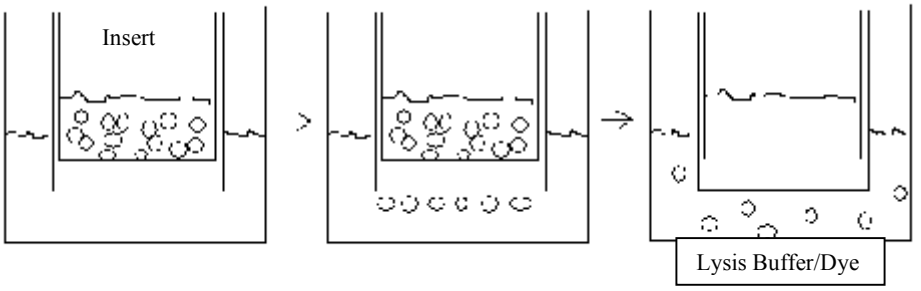

Fig. 10: Representation of the invasion assay.

2) conditioned medium in the well (lower chamber)

Inside the insert tumor cells were cultured in cell medium without FBS. In the lower chamber 50% and 0% conditioned medium from visceral and periprostatic SVFs. The plate was incubated for 24 hours at 37 ºC with 5% CO2. The subsequent steps of the protocol were identical to 1). With this experiment we intended to analyse the chemoattractive potential of the molecules produced by SVFs.

3.6. Statistical Analysis

The results are expressed as mean ± standard error (SE). Differences in cell growth, apoptosis and invasion distributions between CM treated and corresponding untreated controls were tested using Student's *t* tests. Non-parametric variables were analysed by Mann-Whitney U test. To analyse differences between independent variables such as BMI quartile, anatomical origin of CM (periprostatic vs. Visceral) and adipose tissue fractions (WAT, adipocytes and SVF), adequate Student's *t* test or ANOVA analysis were performed. In proliferation experiment for cell quantification: we created a reference cell number standard curve for converting sample fluorescence values into cell numbers. Linear regression for PC-3 standard curve was r2=0.946 and for LNACaP standard curve r2=0.971.

The significance level was set at $P \leq 0.05$.

Statistical analysis was carried out using SPSS 17.0.

RESULTS

Measurement of leptin levels

The leptin levels in supernatants from visceral WAT, at 24h and 48h of in vitro culture are presented in Figure 11. Leptin was significantly higher in supernatants collected at 48hours (p=0,006).

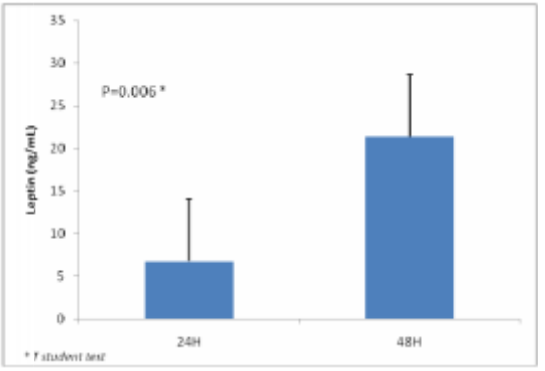

Fig.11: Leptin levels in visceral WAT conditioned medium (n=13) at 24h and 48h (Independent Student t test)

Experiment 1: Influence of conditioned medium in PC-3 and LNCaP proliferation

PC-3

The impact of CM from periprostatic and visceral WAT, adipocytes and SVF fractions in PC3 proliferation are described in Table 2. A trend towards increased proliferative potential of CRPCa cells PC-3 with CM was found for WAT from visceral and periprostatic depots (p=0,056 and p=0,057, respectively).

Table 2: PC-3 cells proliferation under the influence of conditioned medium

		0% cond. medium	50% cond. medium	*p**
WAT	PP	9006±1976,98	11205±2758,81	***0,057***
	Vis	9739±2845,98	12819±3801,47	***0,056***
Adipocytes	PP	9909±3806,32	11307±3481,50	0,403
	Vis	10551±2462,04	11955±4980,39	0,439
SVF	PP	12102±5479,27	13972±4283,69	0,407
	Vis	11619±4293,53	13904±1813,73	0,147

PP – periprostatic depot; Vis – visceral depot; SVF – stromal vascular fraction; WAT – whole adipose tissue explants. Mean differences between no CM and 50% CM by Independent Students *t* test. Values represent mean cell count ± SE, from Q2-Q3 normal weight patients (n=10).

PC-3 cell proliferation after stimulation with CM considering the adipose tissue anatomic region of origin showed that periprostatic and visceral adipose tissue do not influence differently castration-resistant tumor cell growth (Fig. 12).

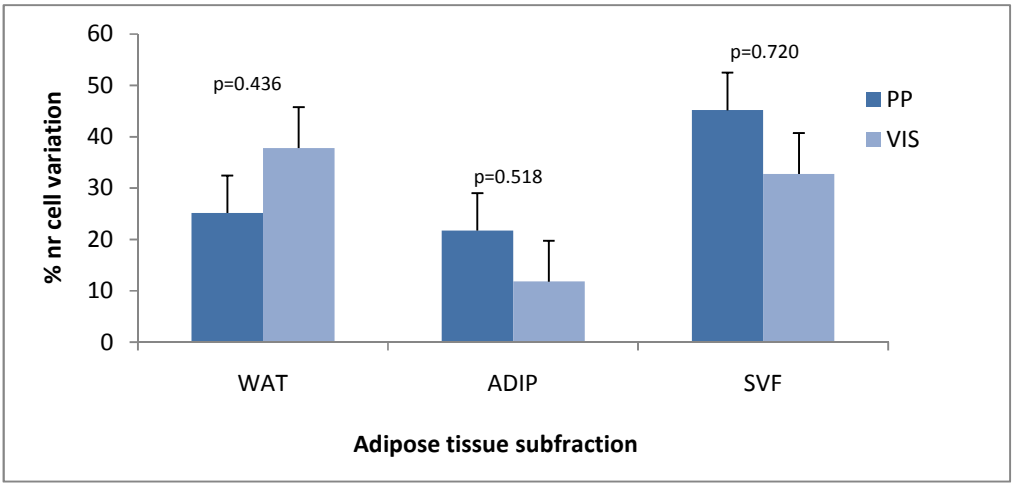

Fig. 12: PC-3 proliferation considering adipose tissue anatomic region. In each subfraction the % of cell count difference between 50% CM and 0% CM was used (CM from 10 patients within Q2-Q3 of BMI). Differences were computed by independent Student *t* tests. PP, periprostatic adipose tissue; VI, visceral adipose tissue; WAT, whole adipose tissue explants; ADIP, adipocytes; SVF, stromal vascular fraction

Furthermore, we aimed to look at the influence of subfractions within each of the two anatomic fat depots in PC-3 proliferation. We observed lack of significant differences between WAT, adipocytes and SVF in cell growth (Fig. 13).

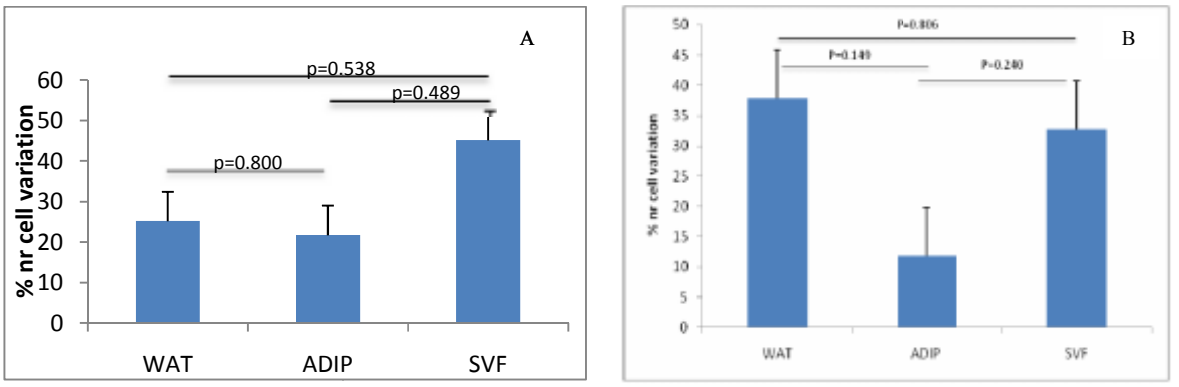

Fig. 13: In PC-3 cells, cell growth associated with the subfractions WAT, adipocytes and SVF were not different in CM from periprostatic (A) and visceral (B) origin. Factorial ANOVA and post-hoc LSD was used to determine differences in cell proliferation between subfractions. In each fat depot and subfraction the % of PC-3 cell count difference between 50% CM and 0% CM was used (CM from 10 patients within Q2-Q3 of BMI). WAT, whole adipose tissue explants; ADIP, adipocytes; SVF, stromal vascular fraction.

LNCaP

When we analyzed the differences in LNCaP cell proliferation between stimulation with 50% CM and no CM, a significant proliferative input seem to be added by influence visceral SVF CM, compared with no CM (p=0,002) and a trend to significant growth with periprostatic SVF CM (p=0,056) (Table 3).

Table 3: Influence of conditioned medium in LNCaP proliferation

		0% cond. medium	50% cond. medium	*p**
WAT	PP	352906±140525	309406±109576	0,451
	Vis	470906±167495	330156±160883	0,071
Adipocytes	PP	474656±133968	392406±95468	0,133
	Vis	408156±142062	418156±115925	0,865
SVF	PP	480906±157398	606406±111520	***0,056***
	Vis	386406±113825	642656±132115	***0,002***

PP – periprostatic depot; Vis – visceral depot; SVF – stromal vascular fraction; WAT – whole adipose tissue explants. Mean differences between no CM and 50% CM by Independent Students *t* test. Values represent mean cell count ± SE, from Q2-Q3 normal weight patients (n=10).

In the castration-sensible LNCaP cell line proliferation we observed a trend to significant growth after stimulation with CM of SVF from visceral origin (p=0.059) (Fig. 14).

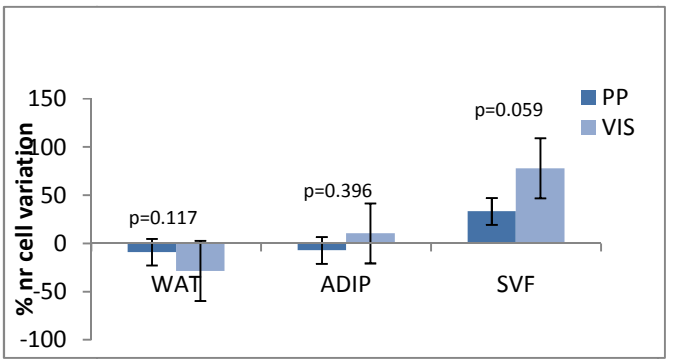

Fig. 14**:** LNCaP cell proliferation considering the origin of adipose tissue (PP vs. VIS). In each subfraction the % of cell count difference between 50% CM and 0% CM was used (CM from 10 patients within Q2-Q3 of BMI). Differences were computed by independent Student *t* tests. PP, periprostatic adipose tissue; VI, visceral adipose tissue; WAT, whole adipose tissue explants; ADIP, adipocytes; SVF, stromal vascular fraction

The influence of CM from adipose tissue subfractions in each of the two anatomic regions (VIS and PP) in LNCaP cell proliferation is presented in Figure 15. Significant differences were observed between WAT and SVF (p=0.003) and between adipocytes and SVF (p=0.043), in cells stimulated with CM from adipose periprostatic tissue. In visceral tissue we observed significant differences between the stimulation of LNCaP cells with CM derived from all subfractions (WAT vs. SVF, p=0.003; WAT vs. Adipocytes, p=0.027; adipocytes vs. SVF, p=0.011).

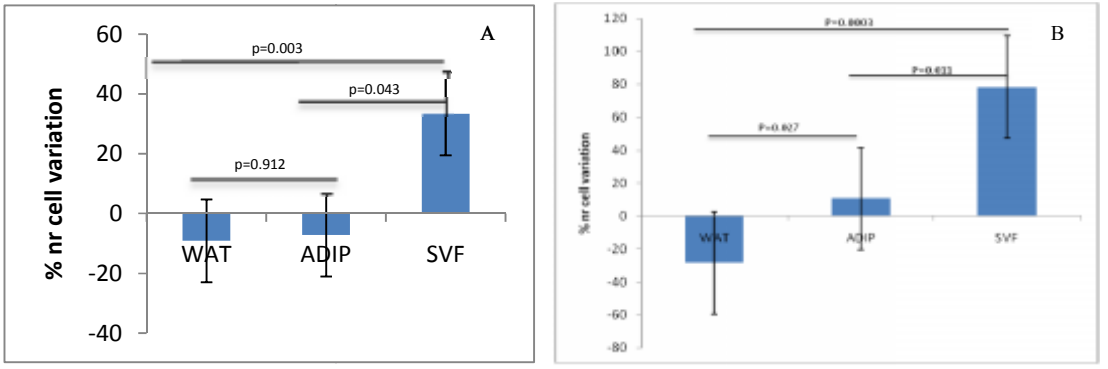

Fig. 15: Influence of CM from subfrations of PP (A) and VIS (B) in LNCaP cell proliferation. Factorial ANOVA and post-hoc LSD was used to determine differences in cell proliferation between subfractions. In each fat depot and subfraction the % of LNCaP cell count difference between 50% CM and 0% CM was used (CM from 10 patients within Q2-Q3 of BMI). WAT, whole adipose tissue explants; ADIP, adipocytes; SVF, stromal vascular fraction.

.

Experiment 2: Influence of conditioned medium in tumor cell apoptosis

The analysis of CM of subfractions (adipocytes and WAT) derived from periprostatic and visceral adipose tissues in PC-3 and LNCaP cell apoptosis was made through flow cytometry with concomitant administration of a pro-apoptotic compound (sodium selenite) to the culture medium.

The percents of tumor cells in early apoptosis or all the apoptotic (early plus late apoptosis), after stimulation with adipose tissue-derived CM are described in Tables 4 and 5.

The analysis according to subfractions (adipocytes vs. WAT) showed a trend toward more total apoptosis in WAT compared to adipocytes alone in castration-resistant PC-3 cells (p=0.092).

Table 4: Percent of variation in early apoptosis and cells in early/late, considering the differences between LNCaP and PC-3 cells stimulated with CM of WAT and adipocytes from periprostatic origin.

		Subfraction	% of difference between 50%-0%CM	p*
LNCaP	%Early apoptosis	Adipocytes	82,49±137,07	
		WAT	1,10±60,76	0,226
	%All apoptosis	Adipocytes	8,35±79,24	
		WAT	15,19±24,91	0,847
PC-3	%Early apoptosis	Adipocytes	-8,15±59,21	
		WAT	25,45±71,45	0,397
	%All apoptosis	Adipocytes	15,32±31,40	
		WAT	70,46±62,44	0,092

LNCaP and PC-3 percent of variation in apoptosis after stimulation with CM from adipocytes and SVF of periprostatic origin. The % of difference in apoptotic cells between 50% and 0% CM was calculated through the following formula: apoptotic cells (50% CM) – apoptotic cells (0% CM)*100 / apoptotic cells (0% CM). Mean differences±SE are from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Differences were computed by independent Student t tests. WAT, whole adipose tissue explants. Early apoptosis refers to cells positive for Annexin-V but negative for IP, while in all apoptosis we consider all cells positive for Annexin-V.

When we analysed the influence of visceral adipose tissue-derived CM in LNCaP and PC-3 apoptosis phenotype, no significant differences were observed.

Table 5: Percent of variation in early apoptosis and cells in early/late, considering the differences between LNCaP and PC-3 cells stimulated with CM of WAT and adipocytes from visceral origin.

		Subfraction	% of difference between 50%-0%CM	*p**
LNCaP	%Early apoptosis	Adipocytes	32,18±109,27	
		WAT	110,01±316,72	0,589
	%All apoptosis	Adipocytes	-8,07±32,15	
		WAT	28,03±54,74	0,201
PC-3	%Early apoptosis	Adipocytes	29,20±61,04	
		WAT	2,62±44,27	0,410
	%All apoptosis	Adipocytes	4,69±25,87	
		WAT	31,07±30,64	0,139

LNCaP and PC-3 percent of variation in apoptosis after stimulation with CM from adipocytes and SVF of visceral origin. The % of difference in apoptotic cells between 50% and 0% CM was calculated through the following formula: apoptotic cells (50% CM) – apoptotic cells (0% CM)*100 / apoptotic cells (0% CM). Mean differences±SE are from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Differences were computed by independent Student *t* tests. WAT, whole adipose tissue explants. Early apoptosis refers to cells positive for Annexin-V but negative for IP, while in all apoptosis we consider all cells positive for Annexin-V.

When we evaluated if the origin of adipose tissue (periprostatic vs. visceral) could influence apoptosis, after stimulation with conditioned medium from adipocytes (Table 6) and WAT (Table 7), no significant differences were observed both in LNCaP and PC-3 cell lines.

Table 6: Percent of variation in early apoptosis and cells in early/late, considering the differences between PP and VIS in adipocytes' CM on LNCaP and PC-3 apoptosis

		Adipocytes	% of difference between 50%-0%CM	*p**
LNCaP	%Early apoptosis	PP	82,49±137,07	
		Vis	32,18±109,27	0,499
	%All apoptosis	PP	8,35±79,24	
		Vis	-8,07±32,15	0,653
PC-3	%Early apoptosis	PP	-8,15±59,22	
		Vis	29,20±61,04	0,307
	%All apoptosis	PP	15,32±31,40	
		Vis	4,69±25,87	0,537

LNCaP and PC-3 percent of variation in apoptosis after stimulation with CM of adipocytes from periprostatic and visceral origin. The % of difference in apoptotic cells between 50% and 0% CM was calculated through the following formula: apoptotic cells (50% CM) – apoptotic cells (0% CM)*100 / apoptotic cells (0% CM). Mean differences±SE are from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Differences were computed by independent Student *t* tests. PP, periprostatic adipose tissue; Vis, visceral adipose tissue; WAT, whole adipose tissue explants. Early apoptosis refers to cells positive for Annexin-V but negative for IP, while in all apoptosis we consider all cells positive for Annexin-V.

Table 7: Percent of variation in early apoptosis and cells in early/late, considering the differences between PP and VIS in WATs' CM on LNCaP and PC-3 apoptosis

		WAT	% of difference between 50%-0%CM	*p**
LNCaP	%Early apoptosis	PP	1,10±60,76	
		Vis	110,01±316,72	0,443
	%All apoptosis	PP	15,19±24,92	
		Vis	28,03±54,74	0,617
PC-3	%Early apoptosis	PP	25,45±71,45	
		Vis	2,62±44,23	0,524
	%All apoptosis	PP	70,46±62,44	
		Vis	31,07±30,64	0,206

LNCaP and PC-3 percent of variation in apoptosis after stimulation with CM of WAT from periprostatic and visceral origin. The % of difference in apoptotic cells between 50% and 0% CM was calculated through the following formula: apoptotic cells (50% CM) – apoptotic cells (0% CM)*100 / apoptotic cells (0% CM). Mean differences±SE are from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Differences were computed by independent Student *t* tests. PP, periprostatic adipose tissue; Vis, visceral adipose tissue; WAT, whole adipose tissue explants. Early apoptosis refers to cells positive for Annexin-V but negative for IP, while in all apoptosis we consider all cells positive for Annexin-V.

The percent of variation in apoptosis of CM 50% from WAT versus no CM, according to the patient's obesity profile is described in Tables 8 and 9.

Table 8: Percent of variation in apoptotic tumor cells, considering the differences between obesity profiles of adipose tissue donors, when stimulated with visceral WAT conditioned medium

		Obesity	% of difference between 50%-0%CM	*p**
LNCaP	%Early apoptosis	Q1	-30,45±42,53	
		Q4	250,48±435,63	0,380
	%All apoptosis	Q1	4,97±23,89	
		Q4	51,10±72,97	0,391
PC-3	%Early apoptosis	Q1	-21,83±23,34	
		Q4	27,07±50,61	0,232
	%All apoptosis	Q1	16,58±33,99	
		Q4	45,55±23,70	0,300

LNCaP and PC-3 percent of variation in apoptosis after stimulation with CM of WAT from visceral origin, according to the obesity phenotype of patients. The % of difference in apoptotic cells between 50% and 0% CM was calculated through the following formula: apoptotic cells (50% CM) – apoptotic cells (0% CM)*100 / apoptotic cells (0% CM). Mean differences±SE are from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Differences were computed by independent Student *t* tests. Q1, quartile 1 of BMI; Q4, quartile 4 of BMI; WAT, whole adipose tissue explants. Early apoptosis refers to cells positive for Annexin-V but negative for IP, while in all apoptosis we consider all cells positive for Annexin-V.

Lack of influence of the background obesity phenotype of patients was observed when visceral and periprostatic WAT explants-derived CM was used for testing apoptosis.

We can observe that in LNCaP cells, the conditioned medium of individuals with the obesity profile Q4 cause increased early apoptosis (p=0,029), demonstrating a pro-apoptotic effect of periprostatic WAT-derived CM in obese individuals (Table 9).

Table 9 Percent of variation in apoptotic tumor cells, considering the differences between obesity profiles of adipose tissue donors, when stimulated with periprostatic WAT conditioned medium

		Obesity	% of difference between 50%-0%CM	*p**
LNCaP	%Early apoptosis	Q1	-48,57±18,85	
		Q4	50,77±38,37	***0,029***
	%All apoptosis	Q1	28,37±30,21	
		Q4	2,02±10,91	0,267
PC-3	%Early apoptosis	Q1	-17,59±51,77	
		Q4	68,48±67,28	0,159
	%All apoptosis	Q1	53,54±61,05	
		Q4	87,37±71,84	0,569

LNCaP and PC-3 percent of variation in apoptosis after stimulation with CM of WAT from periprostatic origin, according to the obesity phenotype of patients. The % of difference in apoptotic cells between 50% and 0% CM was calculated through the following formula: apoptotic cells (50% CM) – apoptotic cells (0% CM)*100 / apoptotic cells (0% CM). Mean differences±SE are from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Differences were computed by independent Student *t* tests. Q1, quartile 1 of BMI; Q4, quartile 4 of BMI; WAT, whole adipose tissue explants. Early apoptosis refers to cells positive for Annexin-V but negative for IP, while in all apoptosis we consider all cells positive for Annexin-V.

The percentage of variation in apoptosis after stimulation with visceral adipocyte's CM, according to the obesity profile is described in Table 10. Lack of significant differences between obese and thin individual's visceral adipocytes-derived CM were found.

Table 10: Percent of variation in apoptotic tumor cells, considering the differences between obesity profiles of adipose tissue donors, when stimulated with visceral adipocyte's CM

		Obesity	% of difference between 50%-0%CM	*p**
LNCaP	%Early apoptosis	Q1	-19,88±13,95	
		Q4	84,24±146,72	0,344
	%All apoptosis	Q1	-21,77±10,95	
		Q4	5,63±43,61	0,391
PC-3	%Early apoptosis	Q1	33,61±83,50	
		Q4	24,79±47,81	0,883
	%All apoptosis	Q1	5,47±36,29	
		Q4	3,91±18,83	0,951

LNCaP and PC-3 percent of variation in apoptosis after stimulation with CM of adipocytes from visceral origin, according to the obesity phenotype of patients. The % of difference in apoptotic cells between 50% and 0% CM was calculated through the following formula: apoptotic cells (50% CM) – apoptotic cells (0% CM)*100 / apoptotic cells (0% CM). Mean differences±SE are from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Differences were computed by independent Student *t* tests. Q1, quartile 1 of BMI; Q4, quartile 4 of BMI. Early

apoptosis refers to cells positive for Annexin-V but negative for IP, while in all apoptosis we consider all cells positive for Annexin-V.

We can observe a pro-apoptotic effect of periprostatic adipocytes-derived CM in PC-3 cells in obese (p=0,022), and a tendency toward a significant pro-apoptotic influence in Q4 individuals (p=0,080) (Table 11).

Table11: Percent of variation in apoptotic tumor cells, considering the differences between obesity profiles of adipose tissue donors, when stimulated with periprostatic adipocyte's CM

		Obesity	% of difference between 50%-0%CM	p*
LNCaP	%Early apoptosis	Q1	-16,16±64,56	
		Q4	181,13±116,65	***0,080***
	%All apoptosis	Q1	-5,99±38,18	
		Q4	22,70±116,72	0,719
PC-3	%Early apoptosis	Q1	-51,14±12,85	
		Q4	34,84±55,29	0,108
	%All apoptosis	Q1	-11,16±7,19	
		Q4	41,80±17,56	***0,022***

LNCaP and PC-3 percent of variation in apoptosis after stimulation with CM of adipocytes from periprostatic origin, according to the obesity phenotype of patients. The % of difference in apoptotic cells between 50% and 0% CM was calculated through the following formula: apoptotic cells (50% CM) – apoptotic cells (0% CM)*100 / apoptotic cells (0% CM). Mean differences±SE are from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Differences were computed by independent Student t tests. Q1, quartile 1 of BMI; Q4, quartile 4 of BMI. Early apoptosis refers to cells positive for Annexin-V but negative for IP, while in all apoptosis we consider all cells positive for Annexin-V.

Experiment 3: Influence of conditioned medium in tumor cell (PC-3) invasion

We analyzed the invasive capacity of tumor cells after stimulation with SVF-derived CM inside the insert, to evaluate the direct impact of SVF-produced molecules in tumor cells' invasive behaviour, and inside the well, to ascertain the chemoattractant potential of SVF-derived molecules for inducing tumor cells migration. We observed significant differences in tumor cells stimulated with CM from visceral SVF inside the insert (Fig. 16). The tumor cells invaded less when stimulated with visceral SVF CM independently of obesity phenotype (all cases, p=0.0004; Q1 thin subjects, p=0.013; Q4 obese subjects, p=0.047). A

trend towards a lower invasive potential was observed in periprostatic SVF-derived CM in the well only on obese patients.

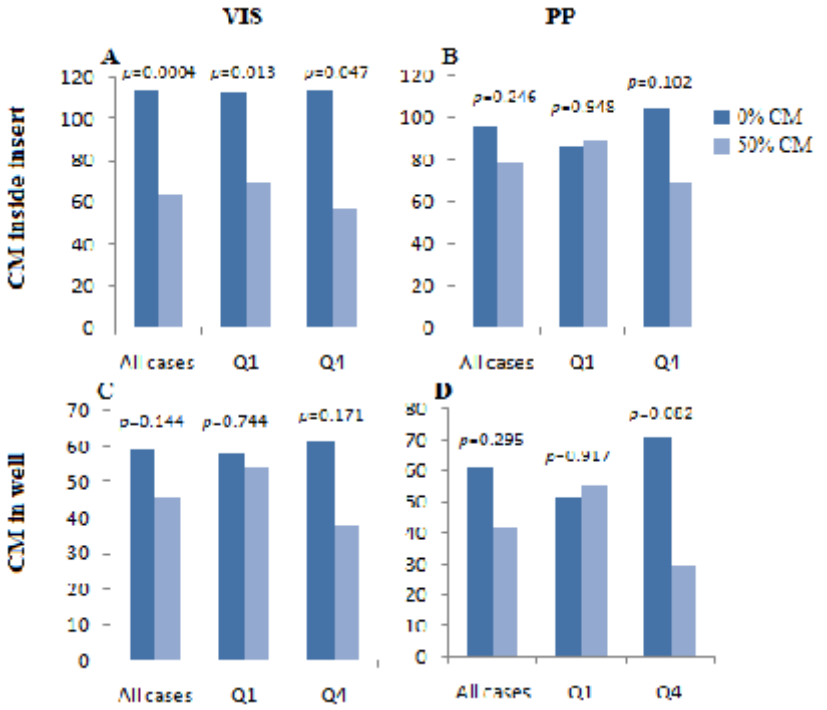

Fig.16: Invasive behaviour of tumor cells in the presence or absence of SVF-derived conditioned medium, considering obesity profile. A) Visceral SVF-derived CM inside the insert, B) Periprostatic SVF-derived CM inside the insert, C) Visceral SVF-derived CM in the well. D) Periprostatic SVF-derived CM in the well. Differences among mean values were computed through independent Student *t* tests. Bars are representative of the mean number of invasive cells, from experiments with CM from 6 patients (3 thin within Q1 of BMI and 3 obese in the Q4 of BMI). Q1, quartile 1 of BMI; Q4, quartile 4 of BMI.

No significant differences in invasiveness of tumor cells were observed when we analyzed results according to the localization of SVF-derived conditioned medium in the invasion chamber (inside insert vs. in the well) and according to the patients obesity phenotype (Q1 vs. Q4 of BMI), in adipose tissue from periprostatic or visceral fat depots (Table 12).

Table 12: Analysis of chemoattractive vs. direct migratory effect and obesity phenotype (BMI quartiles) in tumor cells invasion phenotype

	CM-associated invasiveness ratio (50% : 0% CM)			
Chamber	PP	*p**	Vis	*p**
insert	0,86±0,15		0,58±0,06	
well	0,88±0,45	0,109	0,81±0,13	0,109
Obesity				
Q1	1,18±0,42		0,80±0,11	
Q4	0,56±0,09	0,150	0,59±010	0,337

Invasive behaviour of tumor cells, considering the CM site in the invasive chamber and patient's obesity profile. Differences among mean values±SE of CM-associated invasiveness ratio were computed through non-parametric Mann-Whitney U tests. Q1, quartile 1 of BMI; Q4, quartile 4 of BMI; PP, periprostatic adipose tissue; Vis, visceral adipose tissue.

In Table 13 is represented the influence of the obesity profile the invasion, as well as the anatomic region, considering the localization of the conditioned medium, but we didn't observe any significant difference.

Table13: Influence of the obesity profile and adipose tissue anatomic region conditioned medium in the invasion of tumor cells.

	CM-associated invasiveness ratio (50% : 0% CM)			
Obesity	insert	*p**	well	*p**
Q1	0,82±0,14		1,16±1,42	
Q4	0,62±0,10	0,200	0,53±0,09	0,150
Anatomic region				
PP	0,86±0,15		0,88±0,45	
Vis	0,58±0,06	0,200	0,81±013	0,262

Invasive behaviour of tumor cells, considering the obesity phenotype of patients and the origin of fat depots. Differences among mean values±SE of CM-associated invasiveness ratio were computed through non-parametric Mann-Whitney U tests. Q1, quartile 1 of BMI; Q4, quartile 4 of BMI; PP, periprostatic adipose tissue; Vis, visceral adipose tissue.

DISCUSSION

5. Discussion

During the last decades it has become evident that not only do cell-autonomous signals regulate the behaviour of malignant cells, but also that heterotypic mechanisms can be involved. Heterotypic mechanisms involve soluble secreted factors from cells that surround tumors, extracellular-matrix components and interactions between stromal cells and tumor cells, which create a specific and local peritumoral microenvironment. This process involves constant bidirectional crosstalk between normal cells and partially or fully transformated malignant cells (Hanahan & Weinberg, 2000).

The adipose tissue received little attention up to a few years ago, since it was viewed as a merely energy-storing organ, relatively inert to its surrounding. Work by many different laboratories over the course of the past few years has, in fact, shown that the adipose tissue is a highly active endocrine organ that secretes numerous factors, including growth factors, cytokines, hormone-like molecules and many other molecules (Ahima & Flier, 2000). Several studies have demonstrated that the abundant number of growth factors and cytokines released from adipose tissue can exert a substantial impact on the progression and outcome of many human diseases, including prostate cancer (Baillargeon & Rose, 2006; Housa et al., 2006; Ribeiro et al., 2006; Mistry et al., 2007). For instance, adipose tissue surrounding prostate, i.e. periprostatic adipose tissue, is frequently invaded by prostate tumor cells, although its contribution to the tumor microenvironment is largely unknown. Only a recent study demonstrated that periprostatic adipose tissue produced locally IL-6 at levels significantly higher than those in circulation (Finley et al., 2009).

Different fat depots contribute differently to disease and function. It has been appreciated that fat tissue is regionally heterogeneous with respect to the content of stem cells and metabolic function (Kirkland & Dax, 1984; Arner, 1997).

In the present study, we tried to reproduce *in vitro*, the interaction between adipose tissue-produced molecules and castration-resistent and -sensible prostate tumor cells. Our purpose was to find out the impact of periprostatic and visceral-derived molecules and its subfractions (whole adipose tissue, adipocytes and stromal vascular fraction) in key mechanisms, which are hallmarks of malignant phenotype in prostate cancer cells.

The release of adipokines by adipose tissue has already been the subject of study in samples from subcutaneous and visceral fat depots (Fain et al., 2004). It was demonstrated that *in vitro* primary cultures of adipose tissue were a good model to reproduce specific differences in endocrine function of fat from different anatomical locations (Boquest et al., 2006; Thalmann et al., 2008). Thus, conditioned medium obtained after primary culture of adipose tissue and its subfractions, may contain the adipokines usually produced in vivo (Toda et al., 2009).

Our results showed that the conditioned medium of WAT cultures, derived both from periprostatic and visceral origin, might promote proliferation of PCa castration-resistant cells (PC-3) (WAT Vis p=0,056; WAT PP p=0,057). Visceral fat is associated with systemic inflammatory activity (Fernandez-Real & Ricart, 2003), with lower differentiation capacity of adipose stem cells (Toyoda et al., 2009) and with increased circulating levels of pro-tumoral adipokines such as VEGF, bFGF, IGF, IL-6 (Mistry et al., 2007). Since visceral fat metabolic activity seems to be more prone to favour tumor development and has been associated with prostate cancer (Hsing et al., 2000; von Hafe et al., 2004), the similarity of results between visceral- and periprostatic-induced growth of prostate cancer cells, supports a role for periprostatic fat in prostate cancer growth. The local effect of pro-inflammatory cytokines may be more relevant for periprostatic adipose tissue, which was shown to produce significantly higher levels of IL-6 compared with circulating levels (Finley et al., 2009).

Furthermore, examining whole-tissue production of proteins does not account for the heterogeneous cellular components of adipose tissue. Results show a unique effect of WAT in tumor cell growth, suggesting that the combined production of molecules from many cell types within adipose tissue, may contribute differently to an expression pattern favourable to castration-resistant tumor cells growth. In fact, interactive crosstalk between signalling mechanisms have been described in adipose tissue (Toda et al., 2009).

Other studies that evaluated the influence of adipose tissue in cancer cells proliferation, studied only subfractions and found that adipocyte secreted factors promote the growth of PCa cells (Onuma et al., 2003; Tokuda et al., 2003) and other cancer cell types (Schnäbele et al., 2009; Manabe et al., 2003; Amemori et al., 2007).

For PCa castration-sensible cells (LNCaP), the conditioned medium from SVFs promoted cell proliferation (SVF Vis p=0,002; SVF PP p=0,056). These results agree with others, which demonstrated that stromal cells may support subcutaneous tumor growth when co-injected with tumor cells (Zhu et al., 2006; Hall et al., 2007; Cavarretta et al., 2009). This effect can be result of the transcriptome profile of adipose-derived adherent stromal cells, in which genes involved in matrix proteins, growth factors and receptors and proteases were shown to be highly transcribed (Katz et al., 2005; Peroni et al., 2008). However, caution must be taken when analyzing these results, since it was observed that on *in vitro* culture, transcripts associated with cell cycle quiescence, stemness and cytokines production were down-regulated (Boquest et al., 2005).

When we analyze the proliferation considering the adipose tissue anatomic region of origin, results demonstrate that LNCaP growth can be more influenced by SVF independently of the origin of adipose tissue (in periprostatic adipose tissue SVF vs. WAT, p=0,003 and SVF vs. adipocytes, p=0,043; in visceral adipose tissue SVF vs. WAT, p=0,003 and SVF vs. adipocytes, p=0,011). In proliferation experiments we excluded the obesity factor, by using conditioned medium from individuals with obesity profile within the normal quartile 2-3. Future experiments should use conditioned medium from individuals with obesity profile within Q1 vs. Q4 quartiles in order to elucidate the influence of the background obesity phenotype in PCa tumor cell growth.

In apoptosis experiments we used conditioned medium from WAT and adipocytes cultures to stimulate prostate tumor cells. Our results show no evidence of a significant role of factors produced by adipose tissue from periprostatic or visceral origin in apoptosis of PC-3 and LNCaP cells. However, we found that in PCa castration-sensible cells, periprostatic WAT-derived CM from obese patients induced apoptosis (p=0,029). Furthermore, in the same cell line, periprostatic adipocytes-derived CM from obese individuals have a trend toward a pro-apoptotic effect (p=0,080). Additionally, in castration-resistant cells, results show that periprostatic adipocytes-derived CM from obese individuals also causes more apoptosis (p=0,022).

Hoda & Popken (2008) observed that leptin, and adipocyte-derived adipokine, had anti-apoptotic characteristics in castration resistant prostate cancer cell lines. Concordantly,

others authors demonstrated similar results (Somasundar et al., 2004). However, these studies used different methods for evaluating apoptosis and didn't administer an apoptosis inducer. One used the nucleosomal fragmentation assay (Somasundar et al., 2004), and the other used an ELISA apoptosis assay (Hoda & Popken, 2008), which are methods that analyze cell populations, while in our study a single cell approach was made through flow cytometry quantification. Nevertheless, flow cytometry of adherent cells raise concerns about the impact of trypsinization in cell apoptosis analysis.

These authors have stimulated the prostate tumor cells only with human recombinant leptin, and a more complex interpretation arises from studying the impact of all adipokines produced at the same time. A study on breast cancer cells stimulated with adipose tissue derived-conditioned medium showed a synergistic role of adipokines in the induction of anti-apoptotic transcriptional programs (Iyengar et al., 2003).

Sodium selenite induces cell death and apoptosis through the production of superoxide in mitochondria and activation of the mitochondrial apoptotic pathway (Xiang et al., 2009). In our apoptosis experiments, the cells were treated with sodium selenite. We hypothesize that if the adipokines use an anti-apoptotic mechanism distinct from the mitochondrial pathway, adipokine's anti-apoptotic effect may not overcome apoptosis induced by selenite. Studies using other methods to induce and measure apoptosis are needed to clarify if these results are truly due to the adipose tissue-derived conditioned medium.

Prostate cancer cells have a striking tendency to metastasis (Van´t Veer & Weigelt, 2003; Robinson et al., 2008), and this is the major cause of mortality for cancer patients. The development of metastasis involves cellular transformation and growth, angiogenesis, invasion of new organs and proliferation (Hanahan & Weinberg, 2000).

The adipose tissue stromal vascular fraction is known to produce several growth factors with a relevant role in tumor cell migration and invasion, such as vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGFb1), basic fibroblast growth factor (bFGF), plasminogen activator inhibitor -1 (PAI-1) (Festuccia et al., 1998; Sugamoto et al., 2001; Qi et al., 2003; Ao et al., 2006).

The invasion study was made only in PC-3 cells, since they are more aggressive and therefore more likely to invade. The experiment was done with stimulation by CM from

SVFs, since previous articles showed that adipose tissue-derived stromal cells migrate towards the tumor and produce factors that have the potential to induce migration (Cavarreta et al., 2009; Zhang et al.,2009). We tested two hypotheses: the first to evaluate the direct influence of CM in PC-3, by adding CM inside the insert; and the second to analyse the chemoattractive potential of molecules in CM, through using CM in the well.

Results show a suppression of PC-3 cells invasive potential when directly stimulated with CM from visceral SVF cultures independently from patient's BMI (all cases, p=0,0004; Q1, p=0,01; Q4, p=0,05). These results contrast with published studies that report that mesenchymal stem cells induce invasion in tumor cells (Yamada et al., 1999; Cavarreta et al., 2009; Walter et al., 2009; Zhang et al.,2009). One possible explanation for these contrasting results may be the short time of SVF culture used in our experiments, which could no allowed a full potential production of growth factors. We cultured the SVF cells until ~50% confluence (~48h), and then CM was removed after 48h, in a total culture time of 4 days. Other reports using primary cultures of adipose tissue-derived stromal cells harvested the CM after 6-14 days of culture (Rehman et al., 2004; Katz et al., 2005; Toyoda et al., 2009).

The administration of CM in well to analyze chemoattraction yielded null results. This can be due to lack of molecules produced by SVFs with capacity to attract tumor cells. For instance, leptin, an adipokine exclusively expressed in adipocytes, seems to have a significant role in monocyte and macrophages chemotaxis. (Gruen et al., 2007). Further studies are warranted using conditioned medium from WAT and adipocytes.

The adipose tissue is therefore an excellent candidate organ to play an important role in influencing tumor behaviour through heterotypic signalling processes and may prove to be critical for tumor survival, growth and metastasis of prostate cancer. This study adds to the growing body of evidence that individual biological factors of the host (the medium in which the tumor develops) plays an important part in disease development or progression.

CONCLUSION AND FUTURE PRESPECTIVES

6. Conclusion and future perspectives

In this study we observed that molecules produced in the adipose tissue influence castration-resistant and -sensible prostate tumor cells' proliferation, albeit each type of tumor cell is influenced by different adipose tissue subfractions. Our results also demonstrated that in obese individuals the factors produced by periprostatic adipose tissue increase apoptosis of neoplasic cells, causing an unexpected pro-apoptotic effect. Additionally, invasion assay experiments showed that the adipose tissue SVF subfraction, suppresses invasion of PC-3 cells through a direct effect in tumor cells.

Future studies should address the following issues:

Increase the number of adipose tissue samples and replicate results from the present study;

Appreciate the activity of matrix metalloproteases in conditioned medium from all subfractions and the expression of MMP2, MMP9 and MMP11 in tumor cells after stimulation with CM from adipose tissue cultures (through luciferase reporter assays);

Evaluate adipocytes size from all samples of adipose tissue by microscopy and use the information as an indirect metabolic phenotype to understand the differences encountered in experiments;

Analyze gene expression of adipokines receptors in tumor cells when stimulated with conditioned medium from adipose tissue culture;

Analyze gene expression of adipokines after stimulation of adipose tissue (WAT and subfractions) with tumor cells-derived conditioned medium.

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