

OBESITY AND PROSTATE CANCER: EPIDEMIOLOGY, GENETICS AND MOLECULAR AND CELLULAR MECHANISMS

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Para a Beatriz

"To my mind, science is a means of generating new knowledge through the application of the scientific method. The attributes that distinguish the true scientist are curiosity, the power of observation (which involves a degree of skill as well as the patience and effort to make accurate and reliable observations), objectivity, and a form of humility that allows one to subject one's observations to the scrutiny of others and to accept the fact that one may be wrong. The scientific method provides us with a means to solve problems. Thus the physician-scientist is one who displays the attributes above in his daily work whether he is engaged in formal investigative endeavors in the laboratory and clinic or in the full-time care of patients."

Frederick C. Robbins, MD

"The Physician-Scientist: Reality or Myth?"

1981 Merrimon Lecture, University of North Carolina

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I - Ribeiro R, Lopes C, Medeiros R. The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives. *Prostate Cancer Prostatic Dis* 2006; 9 (1): 19-24.

II – Ribeiro R, Lopes C, Medeiros R. Porquê a preocupação com o estilo de vida e obesidade dos homens idosos? *Geriatrics* 2007; 3 (14): 57-66.

III – Ribeiro R, Araújo A, Lopes C, Medeiros R. Immunoinflammatory Mechanisms in Lung Cancer Development: Is Leptin a Mediator? *J Thorac Oncol* 2007; 2:105–108.

IV - Teixeira AL, Ribeiro R, Cardoso D, Pinto D, Lobo F, Fraga A, Pina F, Calais-da-Silva F, Medeiros R. Genetic polymorphism in EGF is associated with prostate cancer aggressiveness and progression-free interval in androgen blockade-treated patients. *Clin Cancer Res* 2008; 14 (11): 3367-71.

V - Teixeira AL *, Ribeiro R *, Morais A, Lobo F, Fraga A, Pina F, Calais-da-Silva FM, Calais-da-Silva FE, Medeiros R. Combined analysis of EGF+61G>A and TGFB1+869T>C functional polymorphisms in the time to androgen independence and prostate cancer susceptibility. *Pharmacogenomics J* 2009; 9 (5): 341-6.

VI – Ribeiro R, Monteiro C, Azevedo A, Cunha V, Ramanakumar A, Fraga A, Pina F, Lopes C, Medeiros R, Franco E. Performance of an adipokine pathway-based multilocus genetic risk score for prostate cancer risk prediction (submitted).

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VIII - Ribeiro R, Monteiro C, Ramanakumar AV, Guedes A, Francisco N, Ferreira AL, Fraga A, Sousa M, Cunha V, Azevedo A, Maurício J, Lobo F, Pina F, Calais-da-Silva FM, Calais-da-Silva FE, Lopes C, Franco EL, Medeiros R. Inherited variation in adipokine

pathway genes may determine prognosis for prostate cancer patients receiving androgen-deprivation therapy (submitted).

IX - Ribeiro R, Monteiro C, Cunha V, Azevedo A, Oliveira MJ, Monteiro R, Fraga A, Príncipe P, Lobato C, Lobo F, Morais A, Silva V, Magalhães JS, Oliveira J, Guimarães JT, Lopes C, Medeiros R. Tumor cell-educated periprostatic adipose tissue acquires an aggressive cancer-promoting secretory profile. *Cell Physiol Biochem* 2012; 29: 233-40.

X – Ribeiro R, Monteiro C, Cunha V, Oliveira MJ, Freitas M, Fraga A, Príncipe P, Lobato C, Lobo F, Morais A, Silva V, Sanches-Magalhães J, Oliveira J, Pina F, Mota-Pinto A, Lopes C, Medeiros R. Human periprostatic adipose tissue promotes prostate cancer aggressiveness *in vitro*. *J Exp Clin Cancer Res* 2012, 31:32.

XI – Ribeiro R, Monteiro C, Catalán V, Hu P, Cunha V, Rodríguez A, Gómez-Ambrosi J, Fraga A, Príncipe P, Lobato C, Lobo F, Morais A, Silva V, Magalhães JS, Oliveira J, Pina F, Lopes C, Medeiros R, Frühbeck G. Obesity and prostate cancer: gene expression signature of human periprostatic adipose tissue (submitted).

XII – Ribeiro R *, Monteiro C *, Silvestre R, Castela A, Coutinho H, Fraga A, Príncipe P, Lobato C, Costa C, Cordeiro-da-Silva A, Lopes JM, Lopes C, Medeiros R. Human periprostatic white adipose tissue is rich in stromal progenitor cells and a potential source of prostate tumor stroma (submitted).

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LIST OF ABBREVIATIONS

ACM	All-cause mortality
ADT	Androgen deprivation therapy
AR	Androgen receptor
ASC	Adipose-derived stem cell
AUC	Area under the curve
BFP	Body fat percent
BMI	Body mass index
BPH	benign prostate hyperplasia
CCL5	Chemokine (C-C motif) ligand 5 or RANTES
CM	Conditioned medium
CRPCa	Castration-resistant prostate cancer
DHT	Di-hydrotestosterone
DRE	Digital rectal examination
FGF	Fibroblast growth factor
FRDO	Final relative distance to origin
GRS	Genetic risk score
GWAS	Genome-wide association study
HC	Hormonal castration
HGPCa	High-grade prostate cancer
HRPCaMtx	High-risk prostate cancer for metastasis
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
LNCaP	Hormone-sensitive prostate cancer cell line
MCP-1	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinase
MS	Mean speed
mtDNA	Mitochondrial DNA
NP	Normoponderal
OB/OW	Obesity/overweight
OPN	Osteopontin
OR	Odds ratio
aOR	Adjusted odds ratio

OS	Overall survival
PCa	Prostate cancer
PC-3	Hormone-refractory prostate cancer cell line
PIA	Proliferative inflammatory atrophy
PIN	Prostate intraepithelial neoplasia
PP	Periprostatic
PSA	Prostate specific antigen
ROC	Receiver operating characteristic
SNP	Single nucleotide polymorphism
SVF	Stromal-vascular fraction
TGFb	Transforming growth factor beta 1
TNFa	Tumoral necrosis factor alpha
VEGF	Vascular endothelial growth factor
VIS	Visceral, pre-peritoneal
95%CI	95% confidence interval

ABSTRACT

Prostate cancer (PCa), a highly incident neoplasia, is biologically heterogeneous and presents a wide spectrum of clinical manifestations. Increasing evidence suggests a link between obesity and PCa, although the molecular mechanisms underlying this association are poorly understood.

Through a series of basic research studies we aimed to uncover the uniqueness and mechanisms of the previously unrecognized periprostatic adipose tissue role in PCa progression. Translational studies were undertaken to evaluate, in elderly men before diagnosis by prostatic biopsy, the incapacity of body mass index to discriminate fat mass, against whole body adiposity as a measure of obesity and to assess the value of inherited variants from adipokine pathways genes in the prediction and prognosis of PCa.

By analyzing periprostatic adipose tissue, we added valuable new insights into the role of adipose tissue in PCa pathophysiology. Noteworthy, we demonstrated that there was a two-way crosstalk between adipose tissue and prostate tumor cells that ultimately favors tumor progression, and where adipokines and tumor factors are key mediators. In agreement, the transcriptomic analysis of periprostatic adipose tissue revealed that obesity modifies gene expression to ultimately foster fat mass growth, whereas PCa induces hypercellularity and reduced immunoinflammatory activity, which are liable to the promotion of a favorable environment to support PCa progression. Furthermore, we presented previously unrecognized findings on adipose stem cells in humans, which provide an innovative mechanistic link between adipose tissue, adipose stem cells and PCa.

We found that body adiposity is associated with PCa, and that body mass index classification misses subjects with disease because of low accuracy to identify obesity defined by body fat percent. These findings may urge the redefinition of the methodologic landscape for evaluating obesity-PCa association and serve as reference for redesigning future studies. In addition, using functional genetic polymorphisms from adipokine pathways, we developed a multilocus genetic risk score that outperformed the predictive value of prostate specific antigen and age in estimating absolute risk for PCa and high-grade disease, which may prove clinically useful particularly for obese patients; and in a cohort of PCa patients receiving androgen-deprivation therapy where we found that some of these polymorphisms were determinants of PCa prognosis end points (resistance to hormonal castration, all-cause mortality and development of metastasis), therefore with a potential role as molecular predictors and as indicators for targeted therapy.

Results from the present work extended our understanding about the molecular links between adipose tissue and PCa, and provided experimental evidence of the applicability of molecular markers in adipokine pathways for establishing predictive models of risk and prognosis in PCa. In an increasingly obese population, recognizing the mechanistic association of adipose tissue may provide an opportunity for preventive and therapeutic strategies to reduce PCa morbidity and mortality.

RESUMO

O cancro da próstata, uma neoplasia de elevada incidência, é biologicamente heterogênea e apresenta um amplo espectro de manifestações clínicas. Evidências crescentes sugerem uma associação entre obesidade e cancro da próstata, embora os mecanismos moleculares subjacentes a esta associação permaneçam mal compreendidos.

Através de uma série de estudos investigação básica, com o objetivo descobrir a singularidade e mecanismos ainda não reconhecidos sobre o papel do tecido adiposo periprostático na progressão do cancro da próstata. Foram efetuadas investigações de translação para avaliar, em homens idosos antes do diagnóstico por biópsia prostática, a incapacidade do índice de massa corporal para discriminar a massa gorda, usando a adiposidade como medida da obesidade, e para apreciar o valor de variantes de linha germinativa em genes de vias de adipocinas na predição e prognóstico do cancro da próstata.

Ao analisar o tecido adiposo periprostático, adicionamos novos dados sobre o papel do tecido adiposo na fisiopatologia do cancro da próstata. Digno de nota, demonstrou-se que havia uma interação em ambos os sentidos, entre o tecido adiposo e células tumorais da próstata, que em última análise favorece a progressão do tumor, e onde adipocinas e factores do tumor são mediadores importantes. De acordo, a análise transcriptômica do tecido adiposo periprostático revelou que a obesidade modifica a expressão genética em favor do crescimento da massa de gorda, enquanto o cancro da próstata induz hiper-celularidade e atividade imuno-inflamatória reduzida, os quais são responsáveis pela promoção de um ambiente favorável de suporte à progressão do cancro da próstata. Além disso, mostramos pela primeira vez resultados sobre células progenitoras do tecido adiposo em humanos, que fornecem uma ligação inovadora e mecanística entre o tecido adiposo, células progenitoras do tecido adiposo e cancro da próstata.

Observamos que a adiposidade corporal está associada com cancro da próstata, e que usando o índice de massa corporal ficam indivíduos com doença por classificar corretamente, devido à baixa precisão para identificar obesidade definida pela percentagem de gordura corporal. Estes achados poderão provocar a redefinição de metodologias a usar para avaliação da associação obesidade-cancro da próstata e servir como referência para redesenhar estudos futuros. Além disso, utilizando polimorfismos genéticos funcionais em vias de adipocinas, desenvolvemos uma pontuação de risco genético que superou o valor preditivo do antígeno específico da próstata e idade na estimativa do risco absoluto para cancro da próstata e doença de alto grau, o que pode

ser clinicamente útil, particularmente para pacientes obesos; e numa coorte de pacientes com cancro da próstata tratados com privação androgénica, onde verificamos que alguns destes polimorfismos eram determinantes para o seu prognóstico (resistência à castração hormonal, mortalidade e desenvolvimento de metástases), portanto, com um papel potencial como preditores moleculares e indicadores de eventuais terapia-alvo.

Os resultados deste trabalho adicionaram ao nosso conhecimento sobre os mecanismos moleculares envolvidos na associação entre o tecido adiposo e o cancro da próstata, e proporcionaram evidência experimental sobre a aplicabilidade de marcadores moleculares em vias de adipocinas com o intuito de estabelecer modelos preditivos de risco e prognóstico em cancro da próstata. Em uma população cada vez mais obesa, reconhecer a associação mecanística do tecido adiposo poderá proporcionar uma oportunidade para desenvolver estratégias preventivas e terapêuticas promovendo a redução da morbidade e mortalidade por cancro da próstata.

1. INTRODUCTION

1.1. PARTICULARITIES OF PROSTATE CANCER AS A RESEARCH MODEL

Prostate cancer (PCa) is widely known as one of the most important medical problems in men. In Europe, PCa is the most common solid neoplasm, with an incidence rate of 214 cases per 1000 men, outnumbering lung and colorectal cancer ¹. Furthermore, it is the second most common cause of cancer death in men ². Indeed, a trend for higher mortality from PCa has been observed during the last 30 years, even in regions where it is not common ³.

The main screening tools used to look for evidence of PCa include digital rectal examination, serum concentration of prostate specific antigen (PSA) and transrectal ultrasound-guided biopsies ⁴. As a result of widespread use of screening PSA, most cancers are now discovered in a localized stage, even though the impact in PCa mortality is still debatable ^{5, 6}. Nevertheless, the low specificity of the test for predicting cancer ⁷ and its inability to distinguish latent from aggressive cancer means that a substantial number of men are overdiagnosed with and overtreated for PCa ⁸. Currently, PCa is being overdiagnosed, and the number of patients needed to treat is 20 to prevent only one death ⁹⁻¹¹. Thus, the current challenge is to identify which men have disease that may be cured with treatment, and which men do not require treatment and therefore should not be exposed to the morbidities associated with therapy.

Several efforts have been made to find markers for early detection of PCa, other than total serum PSA. Some interesting markers to use in men with elevated PSA include free PSA, kallikrein markers in blood, urinary PCA3 and urinary detection of *TMPRSS2-ERG* gene fusion ¹²⁻¹⁵. However, a recent consensus report indicates that total serum PSA is still the best marker ¹⁶, since it is strongly associated with clinically relevant PCa endpoints ¹⁷ and highly informative of long-term risk. Furthermore, the risk for a PCa to be metastasized is closely associated with a locally advanced PCa, a poorly differentiated tumor (grade > 4) or a high serum PSA concentration (> 20 ng.mL⁻¹). Prostate carcinomas present as different grades based on a histologic pattern that is scored by the Gleason grading system. In this system the most prominent histologic pattern is assigned a grade 1-5, and the second most common is assigned another grade; the sum of both grades are reported as total Gleason score.

Autopsy studies showed that at the time of death, approximately 70% of men have cancer in the prostate, even though these cancers are not clinically relevant. It has been estimated that 15-30% of males over age 50 and about 80% of males over age 80 harbor

microscopic, undiagnosed PCa¹⁸. Despite this, PCa is typically a slow growing tumor that appears to develop over 20-30 years or more¹⁹. PCa arise in differentiated epithelial cells and/or progenitor cells as a result of a complex interplay between genes, cellular microenvironment, the macroenvironment of the host, and the host environment. Early prostate tumorigenesis appears to be associated with dysplasia that starts as proliferative inflammatory atrophy (PIA) and may progress to prostatic intraepithelial neoplasia (PIN) that can lead to carcinoma²⁰. These early lesions seem to be initiated by inflammation. Therefore, tumor-microenvironment interaction has a relevant controlling role in local PCa growth, invasion and distant metastasis. As previously suggested²¹, genetic alterations in PCa cells alone are not enough to confer an aggressive and metastatic phenotype without interaction with supporting tumor microenvironment. Further evidence of the molecular mechanisms and cellular intervenient in the tumor-stromal interaction may yield improved management of aggressive PCa.

Initial treatment of presumable organ-confined cancer is prostatectomy or radiotherapy with potential curative intent. However, a significant percentage of patients are not cured through these therapies and recurrence occurs, or they are diagnosed after cancer spreading. The mainstay therapy for progressive as well as for advanced PCa is androgen deprivation therapy (ADT), which despite disease regression will eventually fail in most cases. Androgens are considered central for prostate cells growth and proliferation. Testosterone is converted into dihydrotestosterone (DHT) by the enzyme 5 α -reductase and binds to the androgen receptor (AR) triggering the transportation to the nucleus, where it transactivates genes with androgen responsive elements. Resistance to hormonal castration develops and is modulated by several factors and through multiple pathways²², progressing to lethal metastasized disease. The progression of metastatic castration-resistant PCa is the final stage of this disease and constitutes a substantial threat of morbidity and mortality. At present, in this stage there is no established effective therapy with significant impact in survival besides chemotherapy with docetaxel, despite recent advances accomplished with autologous active cellular immunotherapy and abiraterone^{23, 24}.

PCa is common, biologically heterogeneous, and variable in its clinical manifestations. Given the limitations of PSA as a screening test, new paradigms for PCa detection are needed. The substantial risk of overdiagnosis and overtreatment of latent PCa implies that new methods should increase specificity of PCa screening, and specifically its ability to indentify high-risk disease. For men with a diagnosis of PCa, algorithms based on clinical variables, PSA kinetics and, hopefully, new biological markers should help clinicians choose the adequate treatment.

1.2. OBESITY AND PROSTATE CANCER: WHAT TYPE OF ASSOCIATION?

Over the past few years, there has been increasing interest in understanding the influence of obesity on cancer. A landmark paper published in 2003²⁵ identifying an association between obesity and increased risk for death in several cancers surged interest for research on this field.

Obesity is currently considered epidemic, mostly in Western countries, with a trend for increasing prevalence in the near future^{26, 27}. The Organization for Economic Co-operation and Development Health Data 2004²⁸ shows that in Europe (19 countries with repeated measures of body mass index since 1980 until 2003), the obesity prevalence is increasing. Among the United States of America population, there were about 20% of obese individuals²⁹. Asian countries, historically nonobese populations, also had pronounced increase in obesity prevalence during the last decade's³⁰. Concomitantly, PCa incidence has also been increasing. In the United States, PCa incidence annual percent change increased 2.7 in the time period of 1973–1988, 16.2 in 1988–1992, and 11.7 in 1992–1995³¹. It seems that PCa incidence follows obesity incidence (Figure 1), except for the years 1992–1995 when after a peak of PCa incidence attributable to implementation of PSA screening, there was an expected decrease to normal detection rate.

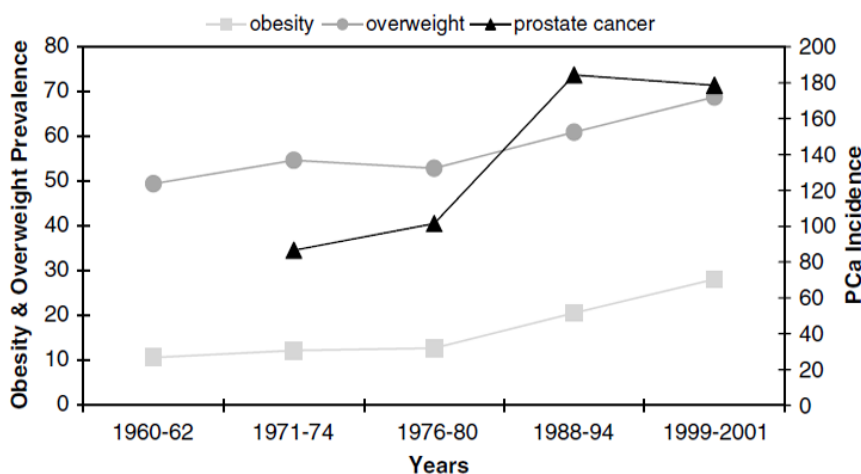


Figure 1. Similar trends in obesity and prostate cancer in the last 40 years in the USA.

The increase in obesity and overweight prevalence is accompanied by incidence increase in prostate cancer, in several time periods since 1962 through 2001, in the USA population (extracted from PAPER I).

It has long been hypothesized that obesity influence the risk of PCa, albeit studies assessing the association between obesity and PCa yielded apparently conflicting results³²⁻³⁵. Obese men are more likely to develop metastasis or die from PCa and are at greater

risk of progression after radical prostatectomy ^{33, 35}. Moreover, recent findings showed increased risk of upstaged disease with obesity still in low risk patients eligible for active surveillance ³⁶, and that obesity was related to higher grade and larger prostate tumors ^{37, 38}. The reasons for the observed link with advanced disease and controversy for nonaggressive PCa risk likely are multifactorial.

A potential impact of obesity is in the detection of PCa. Difficulties in PCa detection in obese men may arise from the potential to influence digital rectal examination (DRE) ³⁹, from the inverse correlation of body mass index (BMI) and body fat percent (BFP) with PSA serum levels ⁴⁰⁻⁴², and from the link with increased prostate size ^{43, 44}. In fact, even though obese men are more likely than normal weight men to be screened for PCa through PSA testing ^{45, 46}, obesity was associated with lower PSA-driven biopsy rates ⁴⁷. Another potential explanation includes differences in testosterone levels. A low testosterone environment as seen in obese men ⁴⁸, among other hormonal and signaling pathway changes ⁴⁹, may lower the likelihood of diagnosing low grade PCa. An additional justification resides in the fact that most studies of PCa and obesity relied on weight, height and BMI to define obesity ^{33, 34}. However, BMI and other classical anthropometric measures are imperfect estimates of adiposity, particularly in men because of greater lean mass. Body fat quantity and regional fat accumulation measures, as opposed to BMI that fails to appropriately discriminate fat mass ⁵⁰⁻⁵³, have been seldom used to evaluate the association between obesity and PCa ⁵⁴⁻⁵⁶.

Obesity represents the overgrowth state of an influential and responsive endocrine and paracrine tissue, adipose tissue, which has been proposed to produce mediators and cells that may affect PCa progression and partially explain the causally invoked association between obesity and PCa ⁵⁷. Several candidate mechanisms have been described in the literature as possible mediators between obesity and PCa. Among the most studied are insulin and insulin-like growth factors axis, sex steroids system and adipokines ⁵⁷⁻⁵⁹. Other, less explored, potential explanatory mechanisms include the obesity-related mild chronic inflammation, altered immune response, oxidative stress and the recently proposed migratory capacity of adipose stem cells towards tumors ⁶⁰⁻⁶².

Evidence suggests that obesity may promote the progression of established PCa. However, the lack of strong epidemiological evidence implicates the need to develop novel methods to better define obesity and to unravel the mechanisms involved in this association.

1.3. GENETIC POLYMORPHISMS IN ADIPOKINE PATHWAYS AS RISK PREDICTORS FOR DIAGNOSIS AND PROGNOSIS IN PROSTATE CANCER

The individual genome represents the starting point for prostate epithelial cells and microenvironment cells, and, based on their particular profile of germline polymorphisms, the lifetime risk of developing PCa can vary widely. In fact, the cumulative effect of genetic polymorphisms may provide a hospitable environment for prostate tumor initiation and progression. Only a small subpopulation of individuals with PCa has true hereditary PCa, which is defined as three or more affected relatives or at least two relatives who have developed early onset disease, i.e. before age 55 ⁶³. The application of genomic analyses has demonstrated that germline polymorphisms, gene expression, and protein expression can be used to anticipate PCa behavior. By using such molecular markers to anticipate behavior provides the opportunity for targeted interventions.

Single-nucleotide polymorphisms (SNPs) identified as loci associated with PCa in genome-wide association studies (GWAS) are common but confer only small increases in risk and the mechanisms underlying the association with PCa risk remains unknown ^{64, 65}. Recently, selected SNPs from GWAS were analyzed and converted into a genetic risk score, which was shown to reduce the number of biopsies although it did not discriminate aggressive cases ⁶⁶. In the context of variants in genes encoding components of adipokine pathways, a few studies have been evaluated for PCa risk ⁶⁷⁻⁷⁰. Promising candidates have been identified among the adipokines with oncologic potential in which functional polymorphisms were described. These candidate genes code for molecules found to be over- or under-expressed in obesity ⁷¹⁻⁷³ and are involved in several biological mechanisms that modulate tumor proliferation, apoptosis, angiogenesis, motility, migration, and immunity ^{57, 74}, i.e., traits that ultimately influence tumor behavior. Thus, common functional polymorphisms in adipokine pathways are plausible candidates that may help predict PCa susceptibility. However, no studies have examined PCa risk in the context of multi-loci SNPs in different adipokine pathways.

Patients diagnosed in advanced stages are frequently submitted to endocrine manipulation with ADT. However, these patients inevitably relapse with androgen refractory tumors and often suffer from side effects caused by ADT ^{7, 75, 76}, thus meriting the effort of finding new strategies to help decide which patients benefit more of this therapy. Albeit mechanisms responsible for PCa cells survive after ADT are not entirely understood, there is evidence that AR reactivation and AR-independent pathways may be implicated ^{22, 77}. Recent studies associating DNA polymorphisms with response to ADT were performed with emphasis on SNPs of genes involved in biosynthesis and metabolism of steroids and androgens ⁷⁸⁻⁸¹. In addition, recent findings showed that

susceptibility SNPs might also improve outcome prediction following ADT ⁸²⁻⁸⁵. Nevertheless, progression into advanced PCa and incurable forms has also been associated with the activation of other cascades mediated by growth factors responsible for the balance between cell growth rate and apoptosis. Several pathways have been proposed to be involved in development of castration-resistant PCa (CRPCa), and their understanding will pave the way to more effective therapies ²². In fact, prostate cell growth and differentiation in the absence of androgens may be due to alternative intracellular signaling pathways ²², which implicates several adipokines as probable ligands or in the cross-talk between signaling transduction pathways (e.g., leptin, interleukin 6, transforming growth factor, vascular endothelial growth factor or osteopontin, among others) ^{86, 87}. Genetic variants may encode products with altered functional characteristics; therefore functional polymorphisms in genes encoding molecules of adipokine pathways, such as enzymes, binding proteins, receptors and transcriptional activators can affect bioavailability, cellular uptake and cellular response to peptides and hormones, providing insight into important biological pathways.

Predicting the individual's response to treatment remains the ultimate objective of predictive genomic research. In personalized medicine, understanding an individual's inherited genetic traits and the somatic biology of their tumor will be important to formulate informed treatment decisions. However, the ability to tailor risk assessment, prevention, diagnosis, and therapy of PCa to the individual patient is likely to evade the application on simple algorithms. Thus, more empirical-based analyses with multiple factors, including functionally tested genetic variants are required to confer increased predictive value together with traditional variables.

1.4. ADIPOSE TISSUE-SECRETED MOLECULES WITH ONCOLOGIC POTENTIAL IN PROSTATE CANCER

Currently, the adipocyte is no longer considered a standby component of human metabolism. A continuously growing body of evidence supports the concept of adipose tissue as an endocrine and paracrine organ, with multiple biological effects, through production of growth factors, polypeptides and cytokines ⁸⁸. These biologically active molecules secreted by adipocytes and stromal cells in the adipose tissue were named adipokines, which were defined as “molecules exclusively or partially produced in adipose tissue” ⁸⁹.

Relevant adipokines for oncology include adiponectin, leptin, hepatocyte growth factor, resistin, tumoral necrosis factor alpha (TNFa), monocyte chemoattractant protein 1 (MCP-1), interleukin 6 (IL-6), fibroblast growth factor (FGFs), among others, that are

significantly increased in the circulation of obese subjects ^{71-73, 90, 91}. This list is continuously expanding due to an ever-growing list of factors known to be produced by the adipose tissue. Many of these have been extensively studied and reviewed and are known to exert biologic effects on PCa cells, regulating cellular differentiation, apoptosis, proliferation, chemotaxis, migration and angiogenesis ^{57, 92-95}, and as recently found also influence cancer stem cell growth ⁹⁶. Besides *in vitro* demonstration of the growth effects of individual adipokines over tumor cells, some studies uncovered the synergistic impact of adipokines ⁹⁷⁻¹⁰⁰, which seems a more rational explanation for the influence of adipose-derived factors in PCa development.

Matrix metalloproteinases (MMPs) are proteolytic enzymes that regulate many cell mechanisms prominent in cancer biology ¹⁰¹. Their expression in prostate tumors is related with disease progression and metastasis ¹⁰², whereas MMP9 was shown to increase growth factors bioavailability and to elicit epithelial-to-mesenchymal transition in tumor cells ^{103, 104}, therefore promoting an aggressive phenotype. A recent report indicated that oesophageal tumors from obese patients express more MMP9 and that co-culture of visceral (VIS) adipose tissue explants with tumor cells up-regulated MMP2 and MMP9 ¹⁰⁵. The expression and activity of these MMPs are well correlated with body adiposity ^{106, 107}, implying excess body fat in the modulation of extra-capsular cancer cells' microenvironment. Hitherto no studies on this subject have been published in PCa.

Molecules that have a relevant role in cancer but only recently found to be produced in adipose tissue, and adipokines known to be expressed by adipose tissue newly implicated in cancer merit further investigation. One such case is osteopontin (OPN), which is a well established instigator of tumor cell aggressiveness ¹⁰⁸ that only recently was found to be expressed by the adipose tissue and to a larger extent in obesity ⁷². Another example is visfatin, a previously established adipokine, found to have a novel role in prostate carcinogenesis and angiogenesis ^{109, 110}. Moreover, other promising molecules upregulated in obesity are starting to be analyzed for their promising role as mediators between adipose tissue and PCa, such as thrombospondin ¹¹¹, macrophage inhibitory cytokine 1 ^{112, 113}, frizzled-related protein 5 ¹¹⁴ or the pseudogene Wdnm1-like ¹¹⁵. Adipokines may provide a molecular basis for the association between obesity and PCa, as well as with clinicopathological variables and aggressiveness.

1.5. ADIPOSE TISSUE, INFLAMMATION AND PROSTATE CANCER PROGRESSION

Obesity is considered a chronic subclinical inflammatory disease ¹¹⁶. In particular, adipose tissue produces high levels of circulating pro-inflammatory adipokines, such as

transforming growth factor beta 1 (TGFb), interleukin 1 (IL-1), IL-6, interleukin 8 (IL-8), MCP-1, TNFa or leptin ¹¹⁷. This is partially explained by the infiltration of macrophages. Although the circulating levels of many pro-inflammatory adipokines produced in adipose tissue with the enlargement of fat mass are expressed by adipocytes, activated macrophages also account for a significant production of pro-inflammatory cytokines ⁷⁴.

The increase in macrophage infiltration raises the hypothesis that obesity-associated increased macrophage infiltration contributes to tumorigenesis. Noteworthy, obesity was associated with increased macrophage infiltration in breast tumors ¹¹⁸. This ability for increased infiltrative capacity has been demonstrated in other tissues ^{119, 120}. Once inflammation has been initiated, there is an immunological mechanism that favors the activation and differentiation of T helper cells into Th1, a pro-inflammatory phenotype. In this context leptin may be a key modulator ¹²¹. Therefore, deregulation of inflammation in the tumor microenvironment is crucial for tumor development. This subclinical chronic inflammatory state has been recognized to be important in the pathophysiology of insulin resistance, type 2 diabetes and atherosclerosis ¹²², and possibly of cancer.

1.6. PHYSIOLOGICAL ROLE AND PATHOLOGICAL IMPLICATIONS OF PERIPROSTATIC ADIPOSE TISSUE

The mechanisms underlying a more aggressive biology of PCa in obese men remain undetermined, albeit the recognized endocrine and paracrine metabolic activity of adipose tissue ⁸⁸ may provide a link between this milieu and PCa development. It is now well established that the interactions between non-tumor cells in the microenvironment and the tumor cells are decisive of whether cancer cells progress towards metastasis or whether they remain dormant ¹²³.

In PCa, the extra-capsular extension of cancer cells into the periprostatic (PP) fat is a pathological factor related with worst prognosis ¹²⁴. Despite there is initially a capsular-like structure separating the PP fat from tumor cells, PCa cells generated within prostatic acini frequently infiltrate and even surpass the prostatic capsule, therefore interacting with the surrounding PP adipose tissue. Once extension beyond the capsule occurs, the PP adipose tissue-secreted factors, extracellular matrix components or direct cell-cell contact may influence the phenotypic behavior of malignant cells. Particularly in the prostate, the crosstalk between adipose tissue and prostate tumor, may impact the behavior of either PCa cells or adipose tissue cells. Indeed, recent findings focused on PP adipose tissue demonstrated that increased local production of IL-6 and PP fat thickness, were associated with PCa aggressiveness ¹²⁵⁻¹²⁷. Furthermore, fat depots from distinct

anatomical origin and obesity status have specific gene expression profiles ^{128, 129}, therefore it seems plausible that PP adipose tissue might have a depot-specific secretory profile.

1.7. THE IMPACT OF TUMOR-DERIVED FACTORS ON ADIPOSE TISSUE: WHAT KIND OF FEED-BACK?

Current practices to establish the association between obesity and PCa rarely can reflect underlying molecular mechanisms. In recent years, adipose tissue was postulated as a highly active and large source of endocrine and metabolic activity. Conclusions from several studies underline the essential role of adipose tissue in tumor progression, since it produces molecules with oncologic potential.

The effect of adipose tissue on cancer development has been previously investigated, albeit the direct effects of cancer cells on adipose tissue function have received little attention. Previous findings, from studies on breast cancer cell lines suggest that cancer-derived factors induce adipogenesis, modulate stromelysin-3 production by adipocytes and induce adipose tissue-derived stem cells to differentiate into carcinoma-associated fibroblast-like cells ¹³⁰⁻¹³². Coculture of adipocytes with breast cancer cells also modified adipocytes phenotype and increased the expression of proteases and proinflammatory cytokines ¹³³.

Previous studies demonstrated that anatomical localization of fat depots influence the adipokine expression profile ^{128, 129}. Moreover, recent findings showed that PP adipose tissue thickness and the associated local production of IL-6 are associated with PCa aggressiveness ¹²⁵⁻¹²⁷. However, it remains to be determined the functional roles of PP adipose tissue, which can be relevant for the obesity-PCa association because of the localization and eventual crosstalk in extra-prostatic disease, further contributing to a more aggressive tumor phenotype. Prostate tumor-stimulated adipose tissue, particularly the PP adipose tissue due to anatomical proximity, may modulate the production of adipokines with oncologic potential, further influencing cancer development and aggressiveness.

1.8. ADIPOSE TISSUE-DERIVED STEM CELLS TOWARDS THE PROSTATE TUMOR

Accumulated evidence over the last years indicates that mesenchymal stem cells (MSC) represent a source of progenitors with pathological relevance. Bone-marrow

mesenchymal stem cells (BM-MSC) and adipose tissue-derived mesenchymal stem (stromal) cells (ASCs) share similar stem molecular signature ¹³⁴, although they have a distinct immunophenotypic profile ¹³⁵. Adipose stem cells have been described as CD31⁻/CD34⁺/CD45⁻/CD146⁻ ¹³⁶, with high proliferative potential and ability for migration and capillary formation ¹³⁷. These proliferative progenitor cells are modulated by the degree of adiposity and microenvironment (adipokines, hypoxia, oxidative stress) ¹³⁸.

From the complex association between obesity and PCa (PCa), meta-analyses have demonstrated that obese men are more likely to have aggressive PCa ^{33, 35}. The mechanisms behind this association are not yet understood, albeit recent reports suggest that ASC may represent a link between obesity and cancer ⁶². Both *in vitro* and *in vivo* studies strongly support that ASC home to tumor sites and promote tumor growth, either directly or after differentiation to endothelial cells or myofibroblasts ¹³⁹⁻¹⁴³.

Experimental data obtained from *in vivo* murine PCa models demonstrated that the presence of ASCs on prostate tumors was mediated by stromal derived factor – 1/C-X-C chemokine receptor type 4 axis ^{140, 141, 144}. Taken together, these findings support a prominent role for ASC in the association between obesity and PCa, even though no studies in humans have been reported.

2. AIMS AND STUDY OUTLINE

Our overall hypothesis is that excess adiposity alters prostatic exposure to molecular and cellular factors implicated in prostate cancer (PCa) progression, and that these changes at the adipose tissue, blood and gland levels mediate the resulting effect of adiposity-cancer crosstalk on the presentation and course of the disease.

At the translational research level we conducted Molecular Epidemiology and pharmacogenomic studies to uncover the role of combined polymorphisms from genes of adipokine pathways (multilocus genetic risk score) in PCa risk, aggressiveness and evaluate their predictive and prognostic value, across several clinically-driven key questions that remain unanswered. In a subsequent study we sought to evaluate the utility of body fat percent as a measure of obesity in determining risk of PCa, and to validate while adjusting for body fat the previously determined genetic risk score.

Alongside, we developed an experimental protocol to work with and collect human adipose tissue samples, in order to serve as basis for basic experimental research, with the purpose of unveiling potential mechanisms behind the obesity-PCa association. Blood, matched visceral and periprostatic adipose tissue and prostate samples were collected from patients submitted to radical prostatectomy or nodular prostate hyperplasia surgery. Several questions were addressed using these samples, 1) the influence of adipose tissue in PCa cell lines and viceversa, 2) the obesity-associated immunoinflammatory upregulation and their impact in prostate tumor and adipose tissue pathophysiology, 3) the relevance of periprostatic adipose tissue for PCa through molecular characterization of this tissue, 4) understand the deposition pattern of adipose-derived stem cells in adipose depots, in blood and in the prostate. The specific aims of this study were:

2.1. To analyze body fatness and germline genetic variants in candidate genes of adipokine pathways as risk factors for prostate cancer

2.1.1. To address the contribution of polymorphisms to prostate cancer susceptibility/aggressiveness and to assess the clinical utility of an adipokine genetic risk score, we conducted a prospective 3-year study in a cohort of over 1000 men submitted to prostatic biopsy;

2.1.2. To evaluate the usefulness of body fat percent (measured by bioimpedance analysis) as a measure of obesity and to validate the genetic risk score, a sample of 380 men elective for prostatic biopsy was analyzed;

2.1.3. To determine the predictive and prognostic utility of these genetic polymorphism, a cohort of nearly 500 prostate cancer patients receiving androgen

deprivation therapy were studied (clinical outcomes: time to castration-resistance, overall survival after androgen deprivation therapy initiation and metastasis at any time in the course of disease).

2.2. To understand the mechanisms involved in the causally invoked association between obesity and prostate cancer

2.2.1. To establish primary cultures of periprostatic and visceral adipose tissue explants and stromal-vascular fraction, which allow subsequent *in vitro* studies to investigate the molecular mechanisms involved in the crosstalk with prostate tumor cells;

2.2.2. To study the repercussion of obesity-associated inflammation in prostate tissue and periprostatic fat, and use mediation model analysis to determine which physiologic variables account for the association of adiposity with tumor characteristics;

2.2.3. To uncover the transcriptomic profile of periprostatic adipose tissue, according to obesity and prostatic disease status;

2.2.4. To investigate human adipose tissue depots as reservoirs of adipose stem cells, their frequency in peripheral blood and their deposition in the prostate.

3. MATERIAL AND METHODS

3.1. BODY COMPOSITION AND FUNCTIONAL SINGLE NUCLEOTIDE POLYMORPHISMS IN CANDIDATE GENES FROM ADIPOKINE PATHWAYS IN PROSTATE CANCER

3.1.1. Genetic polymorphisms in prostate cancer susceptibility and aggressiveness, and assessment of the clinical utility of an adipokine genetic risk score

3.1.1.1. PATIENTS

Participants were enrolled between September 2007 and October 2010, after being referred to the urology departments of São João Hospital and Porto Military Hospital (Porto, Portugal) for prostatic transrectal ultrasound guided biopsy (8-13 cores), on the basis of abnormal digital rectal examinations (DRE) and/or single baseline prostate specific antigen (PSA) levels over 2.5 ng/mL. Our study population consists of 1099 consecutively-admitted Caucasian men who had histological evaluation and consented for genotyping. Approval was obtained from research ethics committees at each institution.

In our study, from the prospectively enrolled men undergoing prostate biopsy, patients with non-prostate cancer (BPH or chronic prostatitis) were deemed controls for several orders of reasons: diagnosis was contemporary with cancers; advanced age at diagnosis allowed matching with elderly cancer patients; all undergone digital rectal examination, PSA estimate and prostate needle biopsy, making the possibility of crossover remote; most men have BPH or chronic prostatitis by the 7th-8th decades of life, making it normal in men of that age to carry benign prostatic disease, and corresponding to the median age at diagnosis in our prostate cancer (PCa) group (age 69.0 years); men without prostatic disease would only be eligible from a much younger range of ages, which could introduce bias.

Prostate pathology and Gleason scores were determined via biopsy. In re-biopsed individuals only the last, most relevant pathological diagnosis was considered. Ninety-three men were excluded from the study due to a pathology report of high-grade prostatic intraepithelial neoplasia or a biopsy suspicious of cancer only. None of the participants had undergone PCa treatment (hormonal castration, surgery, chemotherapy, or radiotherapy). All remaining 1006 eligible patients were included for molecular analysis.

3.1.1.2. GENETIC VARIANTS AND GENOTYPING

Candidate single nucleotide polymorphisms (SNPs) were selected from the best evidence from published studies and through public databases that provide information on

the phenotypic risks. Candidate genes involved in adipokine pathways known to affect oncogenesis were selected. SNPs with minor allele frequencies <0.05 were excluded. A total of 29 literature-defined putative functional SNPs in 19 different genes were selected, corresponding to 9 adipokine pathways (Supplementary Table 1 in PAPER VI).

Genotyping for 22 SNPs (two in *ADIPOQ*, *IL6*, *IL6R*, *KDR*, three in *VEGF*, *LEP*, two in *LEPR*, *PPARG*, *PPARGC1A*, *PPARD*, *SPP1*, *IGF1R*, *IGFBP3*, *IRS1*, *FGF2*, *FGFR2*, *TNFA*, *TNFRSF1A*) was performed using TaqMan allelic discrimination (Applied Biosystems), whereas 7 SNPs were genotyped through polymerase chain reaction - restriction fragment length polymorphism analysis (*IL6*-597/-572/-174, *ADIPOQ*+45, *IL6ST* Gly148Arg, *LEPR* Gln223Arg and *TNFA*-863), using previously described protocols¹⁴⁵⁻¹⁵⁰. For quality control we used non-template controls in all runs and blind replicate genotype assessment in 5% of the samples. For the majority of SNPs, we observed almost complete concordance among duplicates.

3.1.1.3. STATISTICAL ANALYSIS

We used means and medians as descriptive statistics for continuous variables and the Kolmogorov-Smirnov test to assess their departure from normality. The Mann-Whitney test was used to compare means between PCa and non-cancer groups. The chi-square test was used to test for departures from Hardy-Weinberg equilibrium for each SNP based on the distribution among the non-prostate cancer group.

Unconditional logistic regression was used to estimate age-adjusted odds ratios (aORs) and 95% confidence intervals (95% CIs) for the associations between the polymorphisms and development of PCa based on both recessive and dominant models. We examined the association of genetic markers with overall PCa, restricted to high-grade PCa (combined Gleason score ≥ 7), and restricted to high-risk PCa for metastasis (PSA at diagnosis ≥ 20 ng/mL and/or combined Gleason score ≥ 8). Sensitivity analyses were conducted on the risk-associated SNPs that exhibited deviation from Hardy-Weinberg equilibrium. This was done by restricting the non-prostate cancer group to normal/benign prostate hyperplasia histology, and with serum PSA < 4 ng/mL and then retesting the risk associations and departure from Hardy-Weinberg equilibrium.

To assess whether risk-associated SNPs affected time to clinical onset of disease we constructed Kaplan-Meier plots of the cumulative probabilities for having PCa diagnosed at different ages according to each SNP. This analysis was conducted among PCa cases only.

Stepwise multivariate logistic regression with backward elimination (P-value for retention=0.15) was conducted in SNPs with $aOR \leq 0.7$ or $aOR \geq 1.3$ (30% decrease or increase in odds of the outcome) plus age and PSA as continuous variables.

Bootstrapping analyses were performed through MonteCarlo simulation (1000 replications).

We constructed an inclusive multi-locus genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyses. For each SNP, the risk genotypes were coded as 1 and the non-risk alleles as 0. The model was determined by multiplying the β coefficient by the SNPs, plus the γ coefficient by the PSA value and the α coefficient by the age (Inclusive Genetic Risk Score = $\sum \beta_i \times X_i + \gamma \times \text{PSA} + \alpha \times \text{Age}$; where X_i =SNPs scaled for risk, β_i =coefficient for SNPs, γ =coefficient for PSA, α =coefficient for Age). A similar risk score was calculated based on a model that included only PSA and age at diagnosis. These models were fitted independently using all PCa and restricted to high-grade PCa as outcomes. A likelihood-ratio test was used to assess the goodness of fit between the logistic regression models.

We then assessed the clinical value of the above two scores in correctly predicting disease status by receiver operating characteristic (ROC) curve analysis. We compared the areas under the ROC curves (AUC) constructed with both scores (with and without genetic information), both for all PCa and high-grade cancers, using a non-parametric algorithm, as suggested by DeLong et al.¹⁵¹.

We evaluated the improvement in model performance (PSA and age risk score) introduced by the inclusion of the SNPs risk information, using the net reclassification improvement (NRI) and the integrated discrimination improvement (IDI) tests^{152, 153}. Since the NRI measurement is heavily dependent on the threshold levels used, we used a threshold probability between 15% and 45%, similar to those previously reported in such clinical context¹⁵⁴.

In order to validate the inclusive genetic risk score findings we performed logistic regression with 10 000 permutation (MonteCarlo simulation) in the full dataset (n=1006), and in a subset (the last 300 patients to be included in the study) from this full dataset, using the genetic risk score as continuous variable. All statistical analyses were conducted in STATA version 10.0 (StataCorp, College Station, Texas). For NRI and IDI calculations, we used the nriidi-package for Stata¹⁵³.

3.1.2. Body adiposity, adipokine pathway-based genetic risk score and prostate cancer risk

3.1.2.1. PATIENTS

Men referred to the urology department of São João Hospital, on the basis of abnormal digital rectal examination and/or single baseline PSA levels over 2.5 ng/mL, starting October 2009 until October 2010, were scheduled for guided transrectal

ultrasound prostatic biopsy (8-13 cores). Patients with previous history of PCa or prostate surgery were not included. Three-hundred ninety six consecutively-admitted Caucasian men were enrolled after signing an informed consent. Approval was obtained from research ethics committees.

Pathological information on biopsies included the diagnosis of normal prostate, BPH, chronic prostatitis, prostate inflammatory atrophy, high grade intraepithelial neoplasia and PCa with Gleason score. In re-biopsied individuals only the last, most relevant pathological diagnosis was considered. Only the benign conditions and PCa were deemed for analysis. Therefore, 16 patients were excluded from the study with diagnosis of high-grade prostatic intraepithelial neoplasia (n=12) or a biopsy suspicious of cancer only (n=4). High grade disease was defined as a biopsy Gleason score ≥ 7 .

3.1.2.2. ANTHROPOMETRIC MEASURES AND GENETIC RISK SCORE

Patients were instructed to come for examination and biopsy in the morning after an overnight fast and to refrain from strenuous exercise on the day preceding biopsy. Before biopsy, patients underwent height measurement on a wall-mounted stadiometer, and weight (to 0.1 Kg) and body fat percent (BFP) using a bipolar bioimpedance analyzer (TBF-300, Body Composition Analyzer, Tanita). Percent body fat was calculated with the manufacturer's software.

Selection of SNPs, genotyping and calculations to obtain the genetic risk score were described in detail in a previous work (PAPER VI). This risk score includes 6 SNPs (*LEPR* Gln223Arg (G>A), *SPP1*-66 T>G, *IGF1R*+3174 G>A, *IGFBP3*-202 A>C, *FGF2*+223 C>T, *IL6*-597 G>A), total PSA and age at diagnosis. For each participant, the coefficients were summed for each of these variables.

3.1.2.3. STATISTICAL ANALYSIS

We used means and medians as descriptive statistics for continuous variables and the Shapiro-Francia test to assess their departure from normality. As appropriate, the Mann-Whitney test and Student t-test were used to compare means (age, anthropometric and hormonal variables) between prostatic disease groups. The Kruskal Wallis followed by Mann-Whitney two samples tests were used for analyses of non parametric variables. The chi-square was used to test for departures from Hardy-Weinberg equilibrium for each SNP based on the distribution among the non-prostate cancer group.

Unconditional logistic regression was used to estimate age-adjusted odds ratios (aORs) and 95% confidence intervals (95%CI) for the associations between anthropometric variables and circulating hormones levels with risk for developing PCa, high-grade PCa (combined Gleason score ≥ 7), and high-risk PCa for metastasis (PSA at

diagnosis ≥ 20 ng/mL and/or combined Gleason score ≥ 8). To estimate the strength of the linear association between continuous variables, we calculated the respective regression equations. Non-parametric Spearman's correlations were computed to assess the statistical dependence between variables.

As previously described (PAPER VI), we calculated the genetic risk score for each participant by summing the coefficients of relevant variables (genetic polymorphisms in the genes *LEPR* Gln223Arg (G>A), *SPP1*-66 T>G, *IGF1R*+3174 G>A, *IGFBP3*-202 A>C, *FGF2*+223 C>T, and *IL6*-597 G>A, plus total PSA and age at diagnosis). The genetic risk score was validated with adjustment to adiposity measures (BMI and BFP), age at diagnosis and testosterone levels. Bootstrapping using Monte Carlo 10000 permutations test was then performed as an internal validation measure. Stratified analyses by BFP-defined obesity (cutoff 25%) were equally made. The software STATA version 10.0 (StataCorp, College Station, Texas) was used for statistical analyses.

3.1.3. Predictive and/or prognostic value of candidate genes from adipokine pathways in prostate cancer patients receiving androgen deprivation therapy

3.1.3.1. PATIENTS

Patients with histopathologically confirmed PCa, treated with androgen deprivation therapy between 1990 and 2009, were included in this study (n=483), after signing informed consent. Patients were recruited from 4 Hospitals in Portugal: Portuguese Institute of Oncology – Porto Centre, Porto Military Hospital, Porto Hospital Centre and Central Lisbon Hospital Centre. The research protocol and consent form were approved by the participating Institution's Ethics Committees.

All patients were treated with androgen deprivation therapy (ADT) (orchiectomy or luteinizing hormone releasing hormone agonist, LHRHa, with or without anti-androgen), after diagnosis with advanced/metastatic PCa or after relapsing disease from definitive local therapy, and followed up prospectively. Patients with adjuvant hormonal therapy for local disease were not considered to take part in this study. Hormonal treatment continued until castration-resistance on the basis of the PSA concentration, imaging, and clinical findings. The primary endpoint was resistance to hormonal castration (CRPCa), defined as the time from ADT initiation to two consecutive rises of PSA (1 week apart) greater than the PSA nadir, despite at least two consecutive hormonal manipulations or progression of osseous lesions (new or size increase soft tissue metastasis, or at least 2 new metastatic spots in bone scintigraphy)^{155, 156}. The secondary end points included overall survival, defined as the time from ADT initiation to death from any cause (ACM), and appearance of distant metastasis at any time during the course of the disease

(identified by x-rays, computed tomography scans or bone scintigraphy.). Information concerning clinical endpoints was collected on the basis of standardized extractions from patient files. Samples of peripheral blood for genotyping were collected independent of treatment initiation.

3.1.3.2. GENETIC VARIANTS AND GENOTYPING

Candidate genes involved in adipokine pathways known to affect oncogenesis were selected. SNPs were preferred from the best evidence from published studies. A total of 27 literature-defined putative functional SNPs in 17 different genes were chosen, corresponding to 9 adipokine pathways (Supplementary table 1 in PAPER VIII). In agreement with literature, we performed combinations of SNPs by adipokine pathway according to their functional repercussion (Supplementary table 2 in PAPER VIII).

Allelic discrimination through Taqman genotyping (Applied Biosystems) was used for 20 SNPs (two in *ADIPOQ*, rs1501299 and rs16861194; *IL6*, rs10499563; *IL6R*, rs2228145; *KDR*, rs2071559; three in *VEGF*, rs2010963, rs833061 and rs3025039; *LEP*, rs7799039; two in *LEPR*, rs1137100 and rs8179183; *PPARG*, rs1801282; *PGC1A*, rs8192678; *PPARG*, rs2016520; *OPN*, rs28357094; *IGF1R*, rs2229765; *IRS1*, rs1801278; *FGFR2*, rs2981582; *TNFA*, rs1800629; *TNFRSF1A*, rs4149570), whereas the remaining (three in *IL6*, rs1800797, rs1800796 and rs1800795; *ADIPOQ*, rs2241766; *IL6ST*, rs3729960; *LEPR*, rs1137101; *TNFA*, rs1800630) were genotyped by polymerase chain reaction - restriction fragment length polymorphism. Quality control included non-template controls in all runs and genotype reliability assessment in 5% of the samples.

3.1.3.3. STATISTICAL ANALYSIS

Clinicopathologic characteristics of patients were summarized as number and percentage of patients for categorical variables, or mean and standard deviation for continuous variables; PSA at diagnosis was dichotomized at 20 ng/mL because of its association with micrometastasis ⁴.

Multivariate analyses were performed after selecting the relevant confounding variables by empirical evaluation for each of clinical and genetic models. For time-to-event analyses, Cox regression models were used to assess hazard ratios (HR) and 95% confidence intervals (95%CI) for estimating the association of clinicopathological characteristics with the time to CRPCa and time to ACM. Logistic regression analyses were conducted to evaluate risk for metastasis. Since the optimal genetic model in association studies is not warranted, we tested based on the minor allele the dominant (aa + Aa genotype versus AA genotype) and recessive (aa genotype versus Aa + AA genotype) models and functional SNP combinations to evaluate the individual association

of 27 SNPs with three outcome measurements using Cox regression and logistic regression analyses. The model with the highest likelihood ratio was presented as the best-fitting genetic model for each SNP. Likewise, the functional combinations of SNPs by pathway were dichotomized (low/intermediate versus high and low versus intermediate/high) and the one with highest likelihood ratio was used. For the subset of clinical factors, SNPs and functional combinations of SNPs selected on the basis of probability <0.15 , a multivariate Cox proportional hazards model was derived by stepwise selection (P-value for retention < 0.05) to identify the independent predictive risks for CRPCa and ACM. A multivariate logistic regression model was performed to evaluate clinical and genetic predictive factors for PCa metastasis. Statistical analyses were conducted in STATA version 10.0 (StataCorp, College Station, Texas).

3.2. MECHANISMS CAUSALLY INVOKED IN THE ASSOCIATION BETWEEN OBESITY AND PROSTATE CANCER

3.2.1. PATIENTS AND SAMPLE COLLECTION

Seventy-six patients diagnosed with PCa (n=54) or BPH (n=22) and elective for prostatic surgery at the Portuguese Institute of Oncology Porto, Porto Hospital Centre and D. Pedro V Military Hospital, participated in this study. Inclusion criteria were: 45-75 years of age, fasting normoglycaemia (<126 mg/dL) without glycaemia lowering drugs and absence of previous treatments. All samples were collected with informed consent by participants, and with approval by the ethical committees of the hospitals included in the project.

In the operating room before anesthetic induction blood samples were collected from an already installed catheter. During surgery, after anesthetic induction and initial dissection to reach the prostate, samples of pre-peritoneal visceral (VIS) adipose tissue and anterior-lateral periprostatic (PP) adipose tissue were collected by urologic surgeons. These samples were immediately cleaned from macroscopic blood and rinsed with saline solution 0.9%. Thereafter, one fragment was embedded in a RNA preservation solution (RNAlater, Ambion) for use in gene expression studies and the other fragments were processed accordingly (Figure 2). When the prostate or the prostatic nodular hyperplasia was removed, 1 fragment was cut from the malignant region indicated previously in the biopsy, and immediately snap-frozen in liquid nitrogen or included in RPMI 1640 (Gibco, Invitrogen) with 1% penicillin/streptomycin (PS) (Gibco, Invitrogen) for further processing in the laboratory. Mirror samples were included in a histological cassette for pathological

analysis. Both prostate and adipose tissue samples were maintained at 37°C for further processing within one hour in the laboratory.

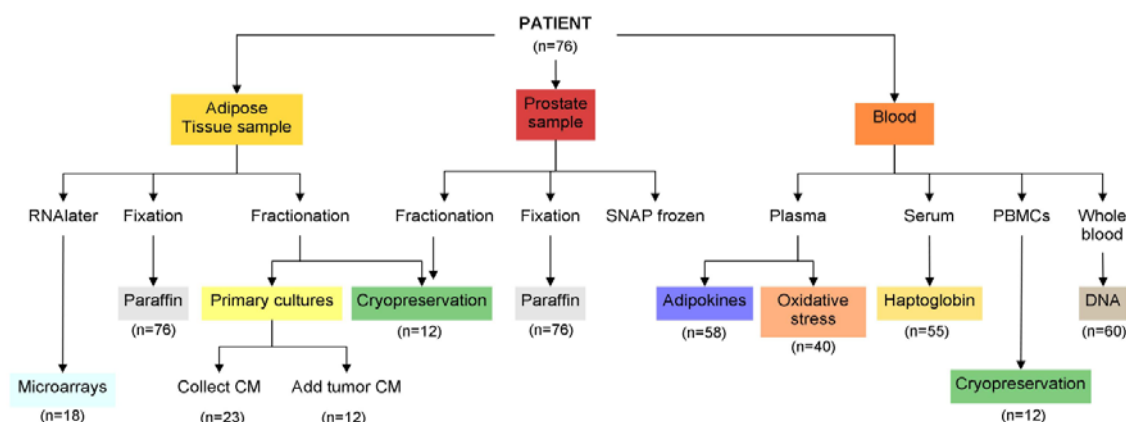


Figure 2. Flowchart resuming protocols for sample collection. In each patient, samples of periprostatic and visceral adipose tissue, of prostate and blood were assembled during surgery. The standard procedure for adipose tissue included separation into RNAlater for gene expression, fixation for paraffin embedding and fractionation. After fractionation cells were used for 3 experiments: primary cultures for collecting medium, primary cultures to stimulate with prostate cancer media and to freeze as cell suspension for flow cytometry experiments. The prostate sample was routinely used for snap frozen except when patients were included in the study of adipose stem cells, where the tissue was digested and the cell suspension stored for flow cytometry readings. Blood was always processed in the same way except for the samples of patients participating in the ASCs experiments or for measurements of oxidative stress markers in haemoglobin: 200 μ L of whole blood was used for DNA extraction, and the rest centrifuged to collect plasma and serum. In the ASCs study, blood was centrifuged with Ficoll and peripheral blood mononuclear cells collected. CM, conditioned medium.

3.2.2. ADIPOSE TISSUE PRIMARY CULTURES AND CONDITIONED MEDIUM EXPERIMENTS

For organotypic, whole adipose tissue (explants) culture, VIS and PP adipose tissue fragments were processed separately using a modified protocol from Thalmann et al.¹⁵⁷. Briefly, after incubation of explants (0.3 g/mL) for 16 hours in DMEM/F12 (Gibco, Invitrogen) supplemented with biotin 16 μ M (Sigma Aldrich), panthotenate 18 μ M (Sigma Aldrich), ascorbate 100 μ M (Sigma Aldrich) and PS 1% (Sigma Aldrich) (sDMEM/F12), fresh medium was added, which was referred to as time zero for time-course experiments. Explant cultures were maintained at 37°C and 5% CO₂. Following this time-point two different experiments were performed: 1) conditioned medium (CM) from tumoral PC-3 cell line was added to the adipose tissue explants (0% CM and 25% CM) and allowed to grow for 48h, or 2) the explants were allowed to grow alone during 48 hours to collect CM. At the end of adipose tissue cultures the undernatant was collected, centrifuged (20000g,

3 minutes), aliquoted and stored at -80°C as stimulated supernatant and explants CM, respectively.

In order to obtain the cells from the stromal-vascular fraction (SVF), other pieces of VIS and PP adipose tissue were incubated with collagenase (2 mg/mL) (Collagenase A, Roche) for 60 minutes at 37°C with agitation (120 rpm). After digestion, the solution was pipetted through a 100 µm cell strainer (BD Falcon) to remove undigested tissues. Following centrifugation the floating adipocyte layer was removed. Then the supernatant was discarded and the SVF cell pellet resuspended in sDMEM/F-12 with 10% Newborn Calf Serum (NCS) (Sigma Aldrich) and filtered through a 40 µm cell strainer (BD Falcon, BD Biosciences). Following erythrocyte lysis (Buffer EL, QIAGEN), SVFs were resuspended and seeded (500 µL of cell suspension) in wells coated with 0.2% gelatin (Sigma Aldrich) in sDMEM/F-12 medium with 10% NCS. Stromal-vascular fraction cells were maintained at 37°C and 5% CO₂. After 48 hours, fresh medium without NCS was added. This time was referred as time zero for time-course experiments. Forty-eight hours later media was removed for conditioned medium experiments, centrifuged and stored. Alternatively, cultures were stimulated with PC-3 CM (0% CM and 25% CM) for 48h. After this time, media was collected, centrifuged (20000g, 3 min), aliquoted and stored at -80°C. Figure 3 depicts the protocol used to process adipose tissue samples.

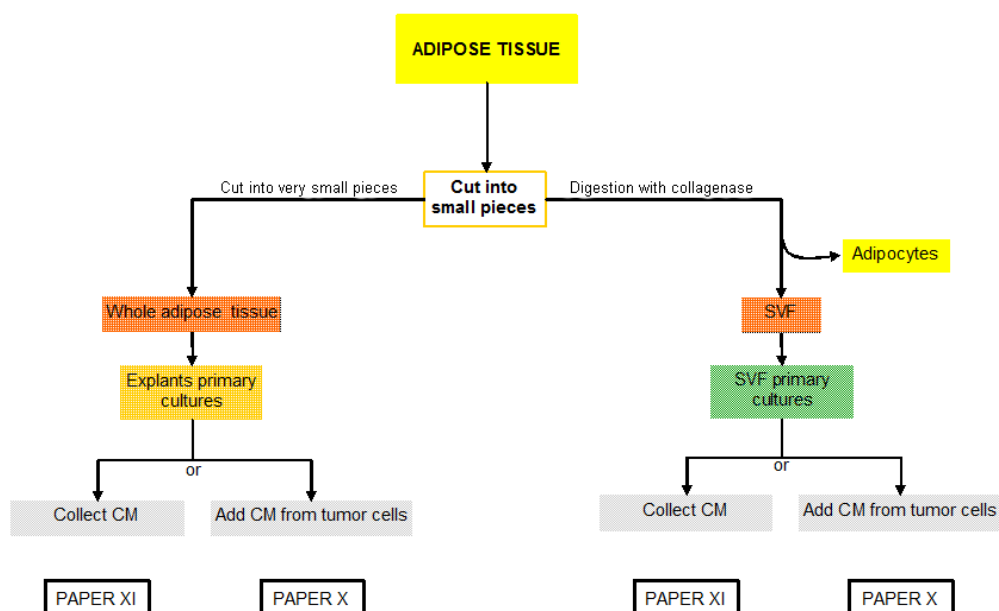


Figure 3. Flowchart of adipose tissue processing until experimentation. Detailed description in the text. SVF, stromal-vascular fraction

3.2.3. PROSTATE CANCER CELL LINES

PC-3 cell line was obtained directly from European Collection of Cell Cultures (ECCAC). RPMI 1640 supplemented with L-glutamine and Hepes (Gibco), 10% fetal

bovine serum (FBS) (Gibco) and 1% PS (Sigma Aldrich) was used as basal medium. Cells were incubated at 37°C with 5% CO₂. 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, when CM was collected, centrifuged at 20000g for 3 minutes (room temperature, RT), aliquoted and stored at –80°C.

LNCaP cell line was a kind gift from the General Pathology Laboratory at Faculty of Medicine, University of Coimbra (Portugal), although originally bought from American Type Culture Collection (ATCC). The basal medium used was RPMI 1640 supplemented with L-glutamine and Hepes (Gibco), 10% FBS (Gibco) and 1% PS (Sigma Aldrich). Cells were incubated at 37°C with 5% CO₂. For conditioned medium experiments, cells were seeded and allowed to grow until 80% confluence. Then, the medium was removed and replaced by RPMI 1640 plus PS (1%) without FBS for 24h, when supernatant was collected, centrifuged (20000g for 3 minutes, RT), aliquoted and stored at –80°C.

3.2.4. PROLIFERATION ASSAY

Cancer cells were seeded into 96-well plates (5x10³ and 10x10³ cells/well for PC-3 and LNCaP cells, respectively) and incubated for 24 hours in RPMI 1640 medium with 10% FBS. Next, supernatant was removed and new cell medium free from FBS, with (50% volume) or without (control) adipose tissue-derived conditioned medium was added to cancer cells. Media was removed after 24 hours, and cells were stored at –80°C. Then, the pellet was solubilized in a lysis buffer supplemented with a DNA-binding dye (CyQUANT cell proliferation assay, Invitrogen). DNA content was evaluated in each well by fluorimetry at 480/535 nm using a standard curve previously generated for each cell type, after plotting measured fluorescence values in samples versus cell number, as determined from cell suspensions using a hemocytometer. Samples were performed in duplicate and the mean value used for analyses.

3.2.5. CELL TRACKING AND ANALYSIS OF CELLULAR MOTILITY

For the time-lapse microscopy analysis (Zeiss Axiovert inverted-fluorescence microscope), exponentially growing cancer cells were seeded into 96-well plates at a density of 5x10³ and 10x10³ cells/well, for PC-3 and LNCaP, respectively. After 24 hours incubation in RPMI 1640 media supplemented with 10% FBS, supernatant was removed and new medium with (50% volume) or without (control, 0% CM) adipose tissue-derived conditioned medium, were added to cancer cells. At this time point the time-lapse experiment was started. A digital image of the field of interest was taken every 15 minutes for 24 hours, generating 85 frames that were arranged into sequences in .avi format (Zeiss Axiovert software). Two fields were selected in each well. The nucleus of each cell

was followed using manual tracking from the first to the last frame and results recorded (Zeiss LSM Image Browser version 3.2.0.70). We used mean speed (MS) and final relative distance to the origin (FRDO) as indicators to characterize cell trajectory and motility. Mean cell speed corresponds to the total distance covered during the experiment, divided by the duration of the experiment, which was considered to be representative of cell motility [17]. To assess the distance the cell migrated since its origin to the end of the observation, we analyzed the linear distance between the initial and final cell position that allows the identification of the statistical trend of cells that randomly explore a large area.

3.2.6. ZYMOGRAPHY

Matrix metalloproteinases 2 and 9 (MMP2 and MMP9) gelatinolytic activities were determined on substrate impregnated gels. Briefly, total protein from supernatants of adipose tissue primary cultures incubated or not with PC-3 CM (12 µg/well) were separated on 10% SDS-PAGE gels containing 0.1% gelatin (Sigma-Aldrich). Gels were washed with 2% Triton X-100 and incubated for 16-18h at 37°C in substrate buffer (50mM Tris-HCl, pH7.5, 10mM CaCl₂). 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).

3.2.7. GENOTYPING

Functional SNPs in genes of adipokine pathways were selected as previously described. In this study including 60 men submitted to prostatic surgery, 24 SNPs are included from 9 pathways. Taqman allelic discrimination (Applied Biosystems) was used in genotyping 19 SNPs (two in *ADIPOQ*, *IL6*, *IL6R*, *KDR*, two in *VEGF*, *LEP*, two in *LEPR*, *PPARG*, *PPARGC1A*, *PPARD*, *SPP1*, *IGFBP3*, *FGF2*, *FGFR2*, *TNFA*, *TNFRSF1A*), whereas 4 SNPs were genotyped through PCR-RFLP (*IL6*-174, *ADIPOQ*+45, *IL6ST* Gly148Arg, *LEPR* Gln223Arg), using previously described protocols (PAPER VI). The haptoglobin phenotype was determined in serum samples by polyacrylamide gel electrophoresis and peroxidase staining as previously described ¹⁵⁸. Genotyping quality control measures included non-template controls in all runs and replication in 5% of the samples.

3.2.8. MULTIPLEX MEASUREMENTS

Cell culture supernatants. Culture medium derived from PP adipose tissue-derived explants, matched SVFs and PC-3 CM were used for TNFa, MCP-1, IL-6 (Human cytokine/chemokine Milliplex MAP kit, 3-plex, Millipore, USA) and leptin, adiponectin

(Human adipocyte Milliplex MAP kit, 2-plex, Millipore, USA) analysis. Multiplex for TNF α , MCP-1 and IL-6 were performed for both explants and SVF supernatants and PC-3 CM, whereas multiplex for leptin and adiponectin was only run in explants samples, according to manufacturer's instructions. Sample concentrations were measured using Luminex 200 (Luminex, Arium, Portugal) and analyzed with Luminex 100 IS v2.2 software. A 5-parameter logistic method was used for calculating adipokine concentrations in samples. Median fluorescent intensity (MFI) of each sample was subtracted to the MFI of PC-3 CM. The intra- and inter-assay precisions were 8.1% and 11.6% for IL-6, 6.1% and 12.0% for MCP-1, 10.5% and 15.9% for TNF α , 4.2% and 21.4% for leptin and 6.6% and 11.0% for adiponectin, respectively. The minimum detectable concentration was 0.4 pg/mL for IL-6, 1.2 pg/mL for MCP-1, 0.2 pg/mL for TNF α , 27.4 pg/mL for leptin and 80.3 pg/mL for adiponectin.

Human plasma samples. The concentrations of some inflammatory markers were quantified in plasma samples (n=58) using a Luminex™ 200 platform (Luminex, Austin, TX, USA). The human immunoassay kit (Millipore Iberica, Madrid, Spain), with analyte-specific components for the measurement of IL1b, IL-6, IL-8, leptin, MCP-1, hepatocyte growth factor (HGF), insulin and nerve growth factor (NGF) was used in this study (HADCYMAG-61K). The standard curve was calculated using a five parameter-curve fitting and results were analyzed with the Luminex IS™ 2.3 software (Luminex). Calibrators, controls and samples were run in duplicate throughout the study.

3.2.9. ELISA

Osteopontin (OPN) and RANTES (CCL5) levels were determined in culture supernatants and PC-3 CM with commercially available human ELISA kits (R&D Systems), according to manufacturer's instructions. Absorbance of each sample was subtracted to the reading obtained from PC-3 CM. A 4-parameter logistic formula (GraphPad Prism 5 software) was used to generate standard curves. 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.

3.2.10. MITOCHONDRIAL DNA QUANTIFICATION

Samples were homogenized in Tripure reagent and DNA isolated and purified through Qlamp DNA blood mini kit (QIAGEN), according to the manufacturer instructions. To synthesize standard DNA, polymerase chain reaction (PCR) and cloning procedures were performed according to the protocol described by Song and Lewis¹⁵⁹. Standard DNA was quantified by spectrophotometry. The standard DNA stock was diluted from 15 to 150000 copies/ μ L for 16S and from 1 to 3000 copies/ μ L for *GAPDH*.

To determine the amount of mitochondrial DNA (mtDNA) relative to nuclear DNA, we used quantitative real-time PCR in the StepOne system (Applied Biosystems, USA) according to Song and Lewis ¹⁵⁹. Accurate amplification of the target amplicon was evaluated by melting curve analysis. Every reaction showed high PCR efficiencies (>90%) and good standard curve (correlation coefficient >0.99). Relative mtDNA copy number was calculated by the formula $[(16S \text{ copy number}) / (GAPDH \text{ copy number})]$.

3.2.11. HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Measurement of adipocyte size. Paraffin-embedded white adipose tissues obtained from pre-peritoneal VIS and PP fat depots were cut into 3 mm thick slides for haematoxylin-eosin and Mallory's Trichrome staining. Slides were observed and photographed at 200X (5 randomly selected different fields) in a Nikon Eclipse 50i microscope with a camera Nikon DS-Fi1. We estimated adipocyte area using the Image J software. Images were converted into a binary format with the following commands: Split Channels color, Gaussian Blur filter, auto Adjust brightness/contrast, Threshold. The binary black and white images were compared with the original images to ensure an accurate conversion. Minor adjustments, if needed, were made with the following commands: Draw tools – eraser and Color picker. The command ROI Manager was used to select cells (only those that are completely visualized in the field and that have a defined membrane). After setting the scale (1px:0.34 m) the total number and areas of adipocytes were calculated with the command Measure. Fifty to 100 adipocytes were measured for each sample.

Immunohistochemistry and scoring. Paraffin-embedded PP and VIS adipose tissue, and prostate tissues representative from tumor or adenoma areas were used for 3mm thick slides for immunohistochemistry. Briefly, slides were incubated with the primaries mouse anti-human CD68 antibody (dilution 1:100, 514H12 clone, Leica Biosystems) and CD31 (dilution 1:50, 1A10 clone, Leica Biosystems), followed by the respective secondary antibody and by the avidin-biotin complex reagents (Vectastain Elite ABC kit, DAKO). Negative controls omitting the primary antibody confirmed specificity. The reaction was developed with diaminobenzidine (Leica Biosystems), and sections were counterstained with hematoxylin.

Microvessel density was evaluated in tumor areas of slides from cancer patients and in whole tissue sections (separately for acinar and stromal regions) from benign disease patients. Any immunopositive (CD31⁺) single cell or cluster of cells clearly separated from adjacent clusters and background, with or without lumen, was considered an individual vessel. Areas of fibrosis, necrosis and inflammation, as well as vessels with a muscle wall, were excluded from the counting. Microvessels were counted in the three

most vascularized areas using a 200X field (0.74 mm²) by two observers simultaneously, and the average value was considered.

Macrophages were identified in the adipose parenchyma (CD68 within blood vessels were excluded) when cytoplasmic staining for CD68 was present along with an identifiable mononuclear nucleus. This was assessed after examination of all fields available per slide at high-power field magnification using light microscopy. Counting was performed by a pathologist blinded to sample data. In the nodular hyperplasia (in both epithelial and stromal areas) and prostate tumors macrophages were counted in the three densest areas in a 200X field.

3.2.12. FLOW CYTOMETRY ANALYSIS AND CELL SORTING

The following directly conjugated mouse anti-human antibodies were used (all from Biolegend): FITC-labeled anti-CD31, APC-labeled anti-CD34, PE-labeled anti-CD146, and PerCP-labeled anti-CD45. Antibodies non-reactive to human cells were used as control to determine the fluorescence background (all from Biolegend): PE-, FITC-, PerCP- and APC-conjugated isotypic antibodies. In short, cryopreserved cell suspension samples of adipose tissues and prostate were rapidly thawed and washed in PBS with 2% FBS (PBS-FBS). The cell pellet was resuspended, and samples or isotypic controls immunostained separately. Approximately 2×10^6 cells were stained with a cocktail of previously optimized concentrations of antibodies for 30 minutes at 4°C. For each sample, isotype matched controls were analyzed to acquire the appropriate settings. Data was collected in a BD FACScalibur cytometer (1 million gated events) and analyzed by FlowJo software (Ashland, OR). Cells expressing a specific marker combination were reported as percentage of the number of gated events. The rationale used for detection of the cell populations of interest is shown in Figure 1 of PAPER XII.

Prostatic cells identified as CD45⁻/CD34⁺/CD31⁻/CD146⁻ were purified by cell sorting in a BD FACS Aria cytometer (n=6). Briefly, thawed cell suspension samples were washed, labeled with antibodies and 1 µg/ml of propidium iodide at a final concentration of 2×10^6 cells/ml. Cell sorting was performed directly to cell lysing solution of RNeasy Micro Kit (Qiagen) and kept at -80°C until further processing.

3.2.13. RNA EXTRACTION AND REAL-TIME PCR

Fragments of adipose tissue (PP and VIS) were homogenized with a ULTRA-TURRAX T25 basic (IKA Werke GmbH, Germany) in QIAzol Reagent (Qiagen). Samples were purified with the RNeasy Lipid Tissue Mini Kit (Qiagen) according to manufacturer's instructions and treated with DNase I (RNase-free DNase set, Qiagen). An aliquot of prostate cell suspension from each sample was used for gene expression. RNA was

extracted from cells in tripure reagent (Roche), according to manufacturer directions, purified with the RNeasy Mini Kit (Qiagen) and treated with DNase I. For samples obtained after cell sorting, we used the RNeasy Micro Kit (Qiagen) according to manufacturer's instructions and DNase treatment. The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA) and quality was inspected for absence of degradation or genomic DNA contamination, using the Experion RNA StdSens Chips in the ExperionTM automated microfluidic electrophoresis system (BioRad, CA, USA). RNA was stored at -80°C until use. First strand cDNA was synthesized with 1 µg of total RNA from adipose tissues, 0.25 µg from prostate cell suspensions and 0.150 µg from sorted prostate cells using the AffinityScript QPCR cDNASynthesis Kit (Stratagene) according to manufacturer's instructions. The transcript levels of adipsin (*CFD*) and *CD133* genes (primers were respectively: *CFD*, forward: 5'-CCAAGCGCCTGTACGACGT-3', reverse: 5'-GGCCTTCTCCGACAGCTGT-3', and *CD133*, forward: 5'-GCTTTGCAATCTCCCTGTTG-3', reverse: 5'-TTGATCCGGGTTCTTACCTG-3') were quantified by qPCR using SYBR Green in an iCycler iQ5 Real-Time PCR (Bio-Rad). qPCR was performed in a 20 µl volume containing 5 µl of complementary cDNA, 10 µl of 2x Syber Green Supermix (BioRad, CA, USA), 2 µl of each primer (250 nM) and 1 µl H₂O PCR grade. After amplification, a threshold was set for each gene and cycle threshold-values (Ct-values) were calculated for all samples. The expression of the gene *PCA3* (assay ID Hs01371938_m1, Applied Biosystems) was evaluated through Taqman (StepOne, Applied Biosystems). The cDNA was amplified in a total volume of 20µL/well at the following conditions: 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, using the Taqman gene expression master mix (Applied Biosystems). Results were normalized, for adipose tissues and prostate, to the levels of the ribosomal *18S* (primers, forward: 5'-GGATCCATTGGAGGGCAAGT-3' and reverse: 5'-CCCAAGATCCAACCTACGAGCTT-3') and *28S* (primers, forward: 5'-AGCCGATCCATCATCCGCAATG and reverse: CAGCCAAGCTCAGCGCAAC), respectively; whereas for *PCA3* we used as reference the *18S* (assay ID Hs03003631_g1, Applied Biosystems) and *ACTB* (assay ID Hs01060665_g1, Applied Biosystems) genes. Relative quantification was calculated using the REST 2009 software, where relative mRNA expression was expressed as fold over the reference group. All samples were run in duplicate and average values used for calculations.

3.2.14. MICROARRAY HYBRIDIZATION, DATA PROCESSING AND ANALYSIS

For microarrays, total RNA was extracted from PP adipose tissue samples after homogenization with an ULTRA-TURRAX T25 basic (IKA Werke GmbH, Staufen,

Germany) in QIAzol reagent (Qiagen, Valencia, CA, USA) and purified through columns (RNeasy Lipid Tissue Mini kit, Qiagen) with DNase I treatment (RNase-free DNase set, Qiagen). Integrity and purity of RNA were assessed by on-chip electrophoresis using Experion (BioRad, USA).

From 1 µg total RNA, cDNA and biotin-labeled antisense cRNA were obtained and hybridized to a high-density oligonucleotide human genome array HG-U133 Plus 2.0 Affymetrix GeneChip Arrays (Affymetrix, Santa Clara, CA, USA). Background correction and normalization were done using robust multi-array average algorithm¹⁶⁰. Calculation of fold change values was performed using the NP and the non-cancer or localized PCa as reference. We used Linear Models for Microarray Data¹⁶¹ to identify differentially expressed genes. Briefly, it starts by fitting a linear model for each gene in the data; then an empirical Bayes method is used to moderate the standard errors for estimating the moderated t-statistics for each gene, which shrinks the standard errors towards a common value. This test is similar to a t-test method for each probe except that the residual standard deviations are moderated across genes to ensure more stable inference for each gene. The moderated standard deviations are a compromise between the individual genewise standard deviations and an overall pooled standard deviation. We used False Discovery Rate¹⁶² to evaluate the statistical significance of all genes.

We evaluated the gene function and network enrichments for selected genes (unadjusted $P < 0.01$) using Ingenuity Pathway Analysis software (Ingenuity Systems, CA, USA). LoPCa group of patients was not included in this analysis. Genes with known HUGO gene symbols and their corresponding fold change were uploaded into the software. Networks of these genes were algorithmically generated based on their connectivity and assigned a score. The score takes into account the number of focus genes in the network and the size of the network to approximate its relevance to the original list of focus genes. The identified networks are presented as a figure indicating molecular relationships between genes/gene products. Canonical pathway analysis identified the pathways which were more significant to the input data set.

To validate the microarray data, a number of representative genes and microRNAs were selected to be studied by Real-Time PCR. For gene expression analysis, cDNA was generated using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA), whereas for microRNAs expression we used the Taqman MicroRNA RT kit (Applied Biosystems), according to the manufacturer's instructions. Transcript levels of the selected genes (*CFH*, assay ID Hs00962366_m1; *FADS1*, assay ID Hs00203685_m1; *ANGPT1*, assay ID Hs00375822_m1; *CLDN10*, assay ID Hs01075312_m1; *LEP*, assay ID Hs00174877_m1; *CFH*, assay ID Hs00962366, all from Applied Biosystems) and microRNAs (*mir-let-7-a2*, assay ID 121214_mat) were quantified by Real-Time PCR

(StepOne, Foster City, CA, Applied Biosystems). The cDNA was amplified using the following conditions, both for genes (Taqman Gene Expression Master Mix, Applied Biosystems) and microRNA (Taqman Universal Master Mix, Applied Biosystems): 95 °C for 10 min, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Results were normalized to the levels of 18S gene (assay ID Hs03003631_g1, Applied Biosystems) and *mir-103* for microRNAs (assay ID 000439, Applied Biosystems), according to previous reports with adipose tissue^{163, 164}. Gene and microRNA expression was calculated using the REST 2009 software, where relative expression was expressed as fold over the reference group. The products of the *PCA3* gene amplification (assay ID Hs01371938_m1, Applied Biosystems) were verified by 1.5% agarose gel electrophoresis, and acquired using the GelDoc XR system (BioRad) and Quantity One software (BioRad).

3.2.15. STATISTICAL ANALYSIS

Data is overall presented as mean±S.E.M. Adequate adjustment of measurements of adipose tissue mtDNA copy number, protein, adipose tissue gelatinase activity, and PCa cell count and motility (final relative distance to origin), were log₁₀-transformed to become normally distributed, whether adjusted or not to adipose tissue sample weight, before statistical analysis. Adequate adjustment of results per gram of adipose tissue was performed when comparing between the fractions and depots of adipose tissue.

Departure from normality was always tested using Kolomogorov-Smirnov. Unpaired t-test or Mann-Whitney U tests were used for statistical comparison. One-way ANOVA with between groups' post-hoc Scheffe test or post-hoc Dunnett test, and the independent samples *t*-test, were used as appropriate. Whenever means for different groups wanted to be compared and normality conditions were not satisfied we used the Kruskal-Wallis test followed by Mann Whitney test once a significant P was obtained or only Mann Whitney test.

Cell populations evaluated by flow cytometry are expressed as percent of CD45-/CD34+ cells adjusted by gram of tissue or only the percent of cells in blood samples analysis. Values for percent of adipose stem cells per gram were log₁₀-transformed to become normally distributed. Unpaired t-test was used for statistical comparison between means. The REST 2009 software is used to determine gene expression ratios. We used 10000 iterations to evaluate whether significant differences existed between samples and controls, while taking into account reaction efficiency and reference gene normalization. Independent measures ANOVA with post-hoc Tukey or Kruskal-Wallis tests were used for comparisons of clinicopathologic variables between groups by stage of disease in the microarray study. Unpaired t-test was used for statistical comparison between

obese/overweight versus normoponderal group. Analyzes were performed using SPSS 17.0 and $p < 0.05$ was considered significant.

4. RESULTS AND DISCUSSION

The results are presented and fully discussed in the section Appendixes, as papers or submitted manuscripts. Here we present a shorter version of results and discussion, highlighting the most relevant findings. Whenever necessary, the reader is remitted to the appropriate table or figure in APPENDIXES section.

4.1. BODY COMPOSITION AND FUNCTIONAL SINGLE NUCLEOTIDE POLYMORPHISMS IN CANDIDATE GENES FROM ADIPOKINE PATHWAYS IN PROSTATE CANCER

4.1.1. Performance of an adipokine pathway-based multilocus genetic risk score for prostate cancer risk prediction (PAPER VI)

A total of 449 histologically confirmed prostate cancer (PCa) and 557 non-prostate cancer patients were included in the analyses. Descriptive clinicopathologic characteristics are depicted in Table 1 of PAPER VI. We evaluated the associations between each individual single nucleotide polymorphism (SNP) on PCa susceptibility (Supplementary table 2 in PAPER VI). From this analysis 7 polymorphisms, *LEPR* Gln223Arg, *FGF2*+223 C>T, *IL6R* Asp358Ala, *IGF1R*+3174 G>A, *IL6*-597 G>A, *IGFBP3*-202 A>C and *SPP1*-66 T>C, were found to be associated with PCa (overall, high-grade or high-risk for metastasis PCa). Age-stratification on the aforementioned SNPs indicated that effects were mostly restricted to subjects below the median age (Supplementary table 3 in PAPER VI). Kaplan-Meier analysis with Log-rank tests showed a shorter waiting time-to-onset of PCa in *IL6R* Asp358Ala C-allele carriers (P=0.026) and in *IGF1R*+3174 AA homozygous (P=0.002) (Figure 1 in PAPER VI).

To test our hypothesis that genetic variability in SNPs from adipokine pathways may contribute a combined effect for PCa risk and/or aggressiveness, we estimated the overall mutually-adjusted effects by stepwise multivariate logistic regression. The SNPs in *LEPR* Gln223Arg, *SPP1*-66 T>G, *IGF1R*+3174 G>A, *IGFBP3*-202 A>C, *FGF2*+223 C>T and *IL6*-597 G>A, plus age and prostate specific antigen (PSA) remained independently associated with risk for overall, and for high-grade PCa (Table 1). In the PCa group with high risk for metastasis, only the *LEPR* Gln223Arg, *SPP1*-66 T>G and *FGF2*+223 C>T genetic variants, age and PSA persisted (Table 1). Within all groups, bootstrap analysis (1000 replications) confirmed results (Table 1).

Table 1. Stepwise multivariate logistic regression and Bootstrap analyses

	All PCa				Restricted to high-grade PCa				Restricted to high-risk PCa for Metastasis			
	Multivariate model		Bootstrap		Multivariate model		Bootstrap		Multivariate model		Bootstrap	
	Genotype	OR (95%CI) ^a	OR (95%CI) ^b	OR (95%CI) ^b	OR (95%CI) ^a	OR (95%CI) ^b	OR (95%CI) ^b	OR (95%CI) ^b	OR (95%CI) ^a	OR (95%CI) ^b	OR (95%CI) ^b	OR (95%CI) ^b
Age at diagnosis		1.03 (1.01-1.05)	1.02 (1.00-1.04)	1.03 (1.01-1.05)	1.03 (1.01-1.05)	1.03 (1.01-1.06)	1.03 (1.01-1.06)	1.03 (1.01-1.06)	1.07 (1.03-1.11)	1.07 (1.03-1.11)	1.07 (1.03-1.11)	1.07 (1.03-1.11)
PSA at diagnosis		1.07 (1.04-1.09)	1.06 (1.04-1.09)	1.07 (1.05-1.10)	1.07 (1.05-1.10)	1.07 (1.04-1.11)	1.07 (1.04-1.11)	1.07 (1.04-1.11)	1.07 (1.04-1.09)	1.07 (1.04-1.09)	1.14 (1.09-1.19)	1.14 (1.09-1.19)
<i>LEPR</i> Gln223Arg (A>G)	G carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent
	AA	1.52 (1.14-2.02)	1.53 (1.13-2.07)	1.56 (1.15-2.12)	1.56 (1.15-2.12)	1.57 (1.14-2.14)	1.57 (1.14-2.14)	1.57 (1.14-2.14)	1.50 (0.91-2.45)	1.50 (0.91-2.45)	1.55 (0.93-2.58)	1.55 (0.93-2.58)
<i>SPP1</i> -66 T>G	T carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent
	GG	1.86 (1.07-3.23)	1.77 (1.00-3.13)	1.97 (1.10-3.52)	1.97 (1.10-3.52)	1.89 (1.03-3.49)	1.89 (1.03-3.49)	1.89 (1.03-3.49)	2.64 (1.16-6.01)	2.64 (1.16-6.01)	2.52 (1.12-5.64)	2.52 (1.12-5.64)
<i>IGF1R</i> +3174 G>A	G carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	---	---	---	---
	AA	1.33 (0.93-1.89)	1.34 (0.94-1.93)	1.40 (0.96-2.05)	1.40 (0.96-2.05)	1.39 (0.93-2.09)	1.39 (0.93-2.09)	1.39 (0.93-2.09)	---	---	---	---
<i>IGFBP3</i> -202 A>C	A carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	---	---	---	---
	CC	1.40 (1.02-1.92)	1.38 (1.01-1.88)	1.40 (1.00-1.95)	1.40 (1.00-1.95)	1.39 (1.00-1.93)	1.39 (1.00-1.93)	1.39 (1.00-1.93)	---	---	---	---
<i>FGF2</i> +223 C>T	T carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent
	CC	1.45 (0.98-2.14)	1.45 (0.98-2.16)	1.55 (1.00-2.38)	1.55 (1.00-2.38)	1.54 (1.00-2.38)	1.54 (1.00-2.38)	1.54 (1.00-2.38)	2.20 (1.01-4.78)	2.20 (1.01-4.78)	2.22 (1.02-4.85)	2.22 (1.02-4.85)
<i>IL6</i> -597 G>A	AA	Referent	Referent	Referent	Referent	Referent	Referent	Referent	---	---	---	---
	G carriers	1.42 (0.92-2.19)	1.37 (0.88-2.13)	1.61 (0.99-2.62)	1.61 (0.99-2.62)	1.58 (0.97-2.56)	1.58 (0.97-2.56)	1.58 (0.97-2.56)	---	---	---	---

Age and PSA analyzed as continuous variables. PCa, prostate cancer; PSA, prostate specific antigen. ^a Stepwise multivariate logistic regression; ^b MonteCarlo simulation (1000 replications). Empirical confounding variables were independently analyzed in each model (overall PCa and both restricted groups).

Adipose tissue deregulation has been proposed as a relevant mechanism underlying obesity-related cancer, due to inappropriate release of biologically active adipokines. Thus, functional SNPs in genes coding for molecules involved in adipokine pathways may modulate the expression, transport, or signaling of adipokines, thereby influencing PCa risk and biology. Our findings show that SNPs in genes from adipokine pathways (leptin, interleukin-6, fibroblast growth factor 2, osteopontin, and insulin growth factor) may influence the development of PCa and aggressive disease.

We tested each SNP for association with two clinically-relevant definitions of unfavorable outcomes: high-grade (combined Gleason score ≥ 7) and high-metastasis risk (combined Gleason score ≥ 8 and/or PSA ≥ 20 ng/mL) PCa. Combined Gleason score is a powerful predictor of disease progression and mortality¹⁶⁵, whereas Gleason score ≥ 8 is associated with aggressive biological behavior and increased risk of occult disseminated disease¹⁶⁶. We found functional variants in genes from leptin, osteopontin, insulin growth factor, fibroblast growth factor 2 and interleukin 6 pathways to be related with high-grade PCa, while SNPs in the leptin, osteopontin and fibroblast growth factor 2 axis associate with high-metastasis risk PCa. These pathways are known to be involved in aggressive PCa, lending support for these SNPs as clinical markers of aggressive disease.

Age-adjusted logistic regression showed increased risk for PCa (aOR=2.5, 95%CI: 2.0-3.2) and high grade PCa (aOR=2.8, 95%CI: 2.2-3.5) using the inclusive risk score as continuous variable (Table 2). The logistic regression models (inclusive versus PSA and age) were significantly different, for all PCa ($P=0.0002$) and high-grade PCa ($P=0.0001$), after likelihood ratio test.

Table 2. Validation of the genetic risk score

	Full dataset (n=1006)	Full dataset (n=1006)	Subset (n=300)
Odds ratios			
Non-PCa vs. PCa	2.52 (1.99-3.19) *	2.53 (1.98-3.22) **	2.24 (1.50-3.34) **
NonPCa vs. HGPCa	2.77 (2.17-3.54) *	2.97 (2.27-3.89) **	2.73 (1.68-4.46) **
AUC			
Non-PCa vs. PCa	---	0.68 (0.65-0.71)	0.70 (0.64-0.76)
Non-PCa vs. HGPCa	---	0.71 (0.68-0.75)	0.75 (0.69-0.81)

* Logistic regression analysis to compute age-adjusted Odds Ratios (ORs) and respective 95% confidence intervals (95% CIs) for PCa and high grade disease, using age and GRS as continuous variables. ** Odds ratios were calculated by MonteCarlo bootstrapping (10 000 replications), using inclusive genetic risk score as a continuous variable. PCa, prostate cancer; HGPCa, high grade PCa; AUC, Area under the curve.

The inclusive (age and PSA added to the multi-locus genetic set) linear risk score computed on the basis of the above logistic regression model was validated as overall risk predictor by MonteCarlo simulation in the original (n=1006) and subsample (n=300) datasets. As shown in Table 2, after 10000 permutations there was good reliability in odds ratios and confidence intervals for PCa and high-grade PCa.

Figure 4 shows the receiver operator characteristic (ROC) curves for the all-inclusive genetic risk score and for the age and PSA-based risk score. The AUC estimates for both outcomes (all PCa and high-grade PCa) were significantly higher for the all-inclusive score than with the age plus PSA predictor, $P=0.0099$ and $P=0.0196$, respectively (Figure 4). The statistically superior predictive value of the all-inclusive score was confirmed via the net reclassification improvement (NRI) (all PCa: 9.5%, $P<0.0001$, high-grade PCa 13.3%, $P<0.0001$) and integrated discrimination improvement (IDI) (all PCa: 0.021, $P<0.0001$, high-grade PCa: 0.024, $P<0.0001$) comparisons.

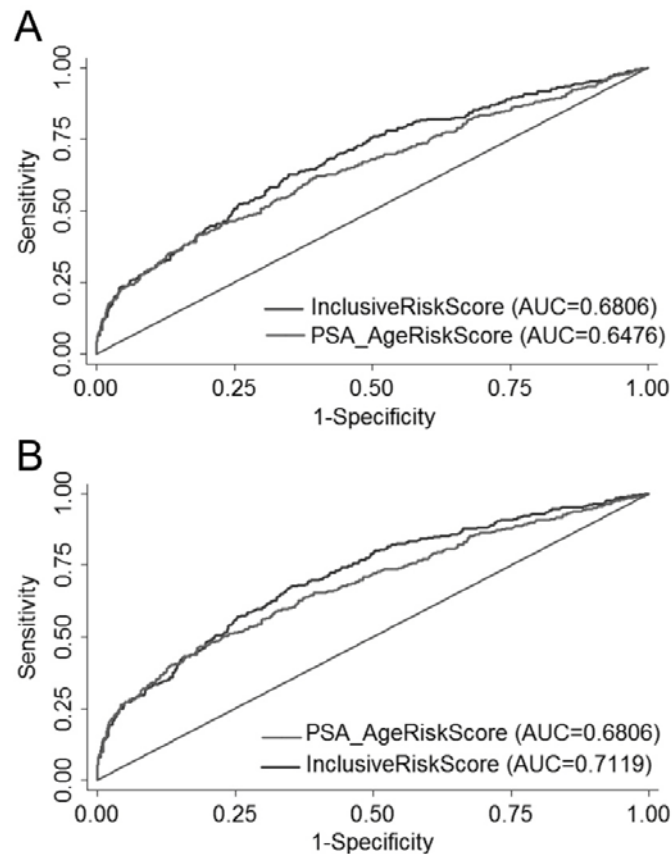


Figure 4. ROC curves and AUC for the inclusive risk score and PSA plus age alone, for all prostate cancer (A) and restricted to high-grade prostate cancer (B). The Likelihood ratio test was used to estimate the difference between risk models for all prostate cancer and high-grade prostate cancer ($P=0.0002$ and $P=0.0001$, respectively).

Although a wealth of evidence demonstrates the effects of individual adipokines on prostate carcinogenesis, it is unlikely that the overall pathophysiological impact is due to the influence of a single adipokine *in vivo*. We showed that consideration of the cumulative susceptibility contributed by SNPs from adipokine pathways helps in risk stratification. Our analyses indicate that the inclusive (age and PSA added to the multi-locus genetic set) risk score provides improvements in discrimination and prediction of all PCa, and high-grade PCa. We suggest that risk genotypes in the inclusive model may cooperate to influence the endocrine and paracrine activity of adipokine pathways that leads to tumor development and progression.

Genotype distributions in four SNPs deviated from Hardy-Weinberg equilibrium (Supplementary table 1 in PAPER VI). In sensitivity analysis of three relevant SNPs, equilibrium was achieved after restricting the control group to constrained conditions, whereas the trend towards increased risk remained stable, regardless of control group used (restricted or unrestricted) (Supplementary table 4 in PAPER VI). Three of these four deviated SNPs ended up in the all-inclusive risk score. Therefore, as an additional step to clarify the relative importance of these SNPs we tested a four SNPs risk score (excluding the 3 SNPs that were not in equilibrium). The risk for PCa and high-grade PCa remained significantly associated with the four SNPs risk score (OR=2.6, 95%CI: 2.0-3.4 and OR=2.4, 95%CI: 1.5-4.0, respectively), still after bootstrap validation using 10000 permutations (OR=2.6, 95%CI: 2.0-3.4 and OR=3.1, 95%CI: 2.3-4.1, respectively).

The logistic regression models (all-inclusive and PSA plus age) also persisted significantly different with the 4 SNPs risk score, for all PCa ($P=0.0107$) and high-grade PCa ($P=0.0057$), after likelihood ratio test. Despite AUC estimates for the all-inclusive score compared to the age plus PSA didn't reach statistical significance (all PCa, $P=0.0683$ and high-grade PCa, $P=0.0723$), the predictive value of the all-inclusive score remained higher in the NRI (all PCa: 7.3%, $P=0.0002$, high-grade PCa: 6.7%, $P=0.0004$) and IDI (all PCa: 0.011, $P=0.0012$, high-grade PCa: 0.013, $P=0.0008$) comparisons. These findings show that the predictive and discriminative ability of the genetic score based on 4 SNPs decreased but remained significant. Therefore, we used the all inclusive score.

In this cohort of men subjected to prostate biopsy due to abnormal clinical and/or PSA findings where an extensive biopsy scheme was used, we showed that by adding a genetic score based on 7 SNPs significantly improved the discriminative ability of an established parsimonious model with only PSA and age. The AUC increased significantly from 0.65 to 0.68 for all PCa and from 0.68 to 0.71 in high grade PCa, when the genetic variants were added to the model. Furthermore, the improved discriminative ability and PCa risk persisted with a four SNPs risk score (excluding SNPs deviated from Hardy-

Weinberg equilibrium). The inclusion of further functional SNPs in a susceptibility model for PCa is warranted, in order to determine a multi-locus model to accurately predict PCa and disease aggressiveness. The use of improved risk models, such as the one described here, may impact public health if shown to have clinical utility when combined with individualized screening and risk reduction strategies.

4.1.2. Body adiposity, adipokine pathway-based genetic risk score and prostate cancer risk (PAPER VII)

This study included 380 men submitted to prostatic biopsy, comprising 154 diagnosed with PCa and 226 without histological evidence of cancer. Age, anthropometric and hormonal characteristics are shown in PAPER VII (Table 1). Mean age at diagnosis, body mass index (BMI), body fat percent (BFP) and total PSA were significantly higher in PCa compared with non-PCa ($P<0.0001$, $P=0.011$, $P=0.008$ and $P<0.0001$, respectively), whereas mean height and free/total PSA ratio were lower in cancer patients ($P=0.045$ and $P<0.0001$, respectively).

We found a higher percent of subjects with PCa, compared with non-PCa, in the obese category according to BFP (53% versus 43%), when the population was globally analyzed. Table 3 presents the ORs and respective 95%CI according to normoponderal, overweight and obesity categories of BFP and BMI.

Table 2 in PAPER VII shows the classification of subjects as normal, overweight or obese according to BMI or BFP. Eighth percent of the men with BMI defined as normal were actually obese given their BFP, while only 2% considered obese by BMI were in reality normal according to BFP. Additionally, 65% of the BMI overweight-classified subjects were after all obese considering their BFP. Moreover, in normal men (according to BMI) the mean BFP was significantly higher for PCa compared with non-PCa group ($P=0.001$) (Figure 5). Concordantly, within the BMI-defined normal group, we found that men with a BFP corresponding to overweight and obesity are at increased risk for developing PCa (OR, 2.5, 95%CI, 1.1-5.5 and OR, 8.2, 95%CI, 2.0-33.0, respectively) and high-grade PCa (OR, 2.6, 95%CI, 1.0-6.7 and OR, 8.2, 95%CI, 1.7-40.9, respectively), compared with BFP-defined normal.

Studies in distinct populations reported similar findings^{51-53, 167}. The limited diagnostic performance of BMI in intermediate ranges of body weight can be explained by the inability of BMI to discriminate between BFP and lean mass⁵¹, since the majority of human body weight comes from lean mass, particularly in men. Present findings show the existence of risk for PCa already in subjects with a BMI $< 30 \text{ Kg/m}^2$ but with highly

Table 3. Age-adjusted odds ratios and 95% CI of prostate cancer according to different categories of anthropometric measures

Cutoffs	Non-PCa	All Prostate Cancers		Restricted to HGPCa ^a		Restricted to HRPcCaMtx ^b	
		Cases	aOR (95%CI)	Cases	aOR (95%CI)	Cases	aOR (95%CI)
Height tertiles, cm							
< 164.9	60	57	Referent	45	Referent	20	Referent
165-170.29	91	54	0.6 (0.4-1.1)	39	0.6 (0.3-1.0)	18	0.7 (0.3-1.4)
≥ 170.3	75	43	1.1 (0.4-1.1)	35	0.7 (0.4-1.3)	10	0.6 (0.2-1.4)
Body fat percentage							
Normal (< 20)	58	17	Referent	13	Referent	7	Referent
Overweight (20 - 25)	71	55	2.4 (1.2-4.7)	43	2.4 (1.1-5.0)	13	1.3 (0.5-3.7)
Obese (≥ 25)	97	82	2.4 (1.3-4.6)	63	2.4 (1.1-4.7)	28	1.8 (0.7-4.6)
Body mass index, Kg/m ²							
Normal (< 25)	96	51	Referent	37	Referent	12	Referent
Overweight (25 – 30)	106	70	1.1 (0.7-1.8)	57	1.2 (0.7-2.0)	25	1.4 (0.6-3.0)
Obese (≥ 30)	24	33	2.4 (1.2-4.5)	25	2.4 (1.2-5.1)	11	3.1 (1.1-8.6)

^a HGPCa, High-grade PCa, Gleason score ≥ 7; ^b HRPcMtx, High Risk PCa for metastasis (PSA at diagnosis ≥20 ng/mL and/or Gleason score ≥ 8); aOR, age-adjusted odds ratio and 95%CI, 95% confidence interval; Adjusted ORs using age as a continuous variable

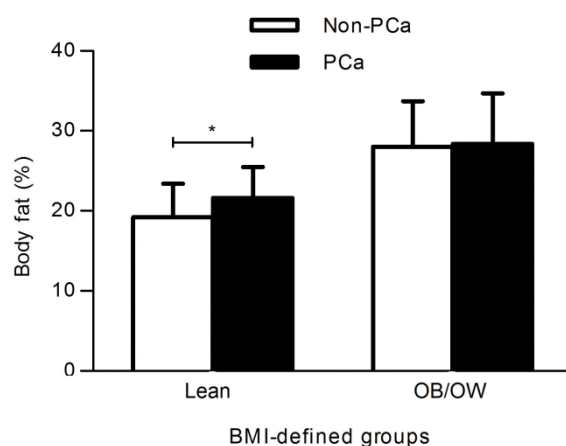


Figure 5. Differences in BFP between PCa and non-PCa groups in BMI-defined non-obese men. Mean±S.E.M.; PCa, prostate cancer; BMI, body mass index; OB/OW, obese and overweight group; *P=0.001 (Mann Whitney test).

increased adiposity. Even though BMI is widely used as a proxy measure of body fat, it does not inform on body composition as evidenced herein.

Considering the current understanding of adipose tissue effects on cancer^{57, 74, 168-170}, it would be expected that the association obesity-PCa would be more consistent. In our results, subjects with normal and mildly elevated BMI had a great variety of BFP, which are likely to explain the inconsistent relationship between BMI and PCa. Noteworthy, it was shown that BMI has its worst diagnostic performance to detect obesity in the elderly⁵¹, precisely the age group when most men with PCa are diagnosed.

Interestingly, an inverse significant association was found between both body fat percent and BMI with testosterone levels ($r=-0.210$, $P<0.0001$ and $r=-0.204$, $P<0.0001$, respectively) (Figure 6). In agreement, men in the top tertile of serum testosterone levels had decreased risk for having high-risk PCa for metastasis compared to the bottom tertile, while serum insulin-like growth factor 1 did not impact the risk for PCa (Supplementary table 4 in PAPER VII). One way excess adipose tissue may be linked to PCa risk and poorer disease pathology, is through its effect in hormone levels. The increased rate of peripheral conversion of testosterone to estradiol by aromatase in fat cells, results in decreased bioavailability of testosterone⁴⁸, which has been associated with advanced and poorly differentiated PCa¹⁷¹⁻¹⁷⁴. Indeed, in our study men in the top tertile of serum testosterone levels were at lower risk for having a cancer of the prostate at high-risk for developing metastasis. In agreement with previous findings^{175, 176}, the testosterone levels in our population were also inversely correlated with BMI and BFP.

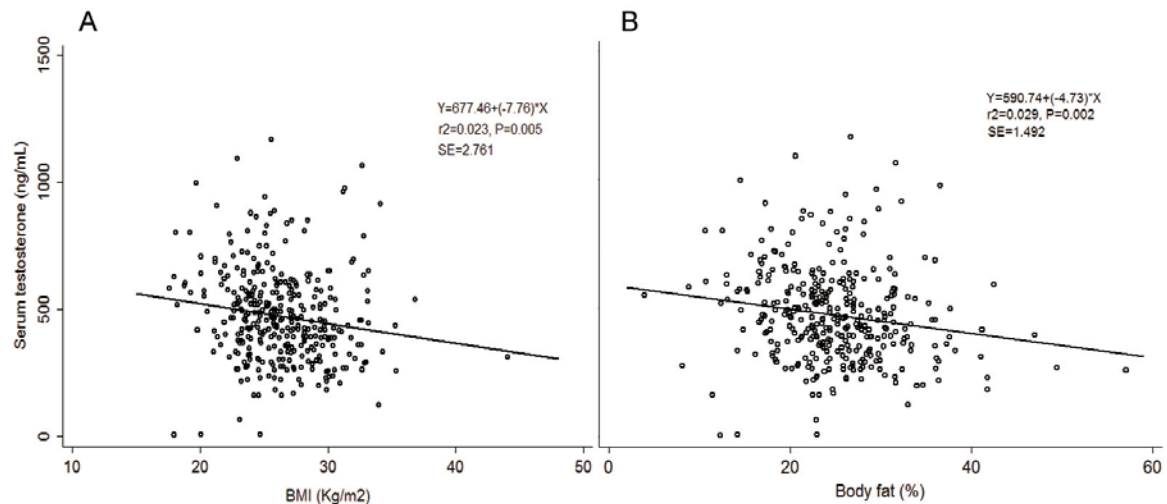


Figure 6. Linear regression analyses and equations to estimate the strength of association between BMI (A) and BFP (B) with circulating testosterone levels. PCa, prostate cancer; Non-PCa, non prostate cancer; BMI, body mass index.

Our findings challenge the use of BMI to detect excess adiposity, particularly for men in the intermediate range of BMI, where it fall short to distinguish between excess in body fat or preserved lean mass. Therefore, in Portugal and other European countries, with a high percentage of overweight men (BMI 25-30 Kg/m²)^{177, 178}, it is desirable to include BFP measurements in the standard assessment of patients in the urological outpatient clinic both for diagnosis and therapeutic management, in order to implement obesity-related measures.

The genetic risk score adjusted for BMI, BFP, testosterone, prolactin and age remained significantly associated with increased risk for all PCa and high-grade PCa risk (Table 4). Stratification according to BFP-defined obesity cut-off (25%), showed that the GRS risk for overall and high-grade PCa was increased in the obese group (Table 4), but not in BMI-determined obese. Moreover, in the misclassified group as non-obese by BMI (< 30 Kg/m²) but BFP-defined as obese (BFP > 25%), the GRS was associated with risk for PCa and high-grade PCa on multivariate analysis (OR, 3.8, 95%CI, 1.8-8.1 and OR, 4.0, 95%CI, 1.7-9.5, respectively).

The gene-environment interaction that likely explains the obesity-PCa association should involve several genes and pathways from different cells (adipose tissue cells, tumor cells, immune cells) in specific environmental contexts. In a previous study, we developed an adipokine pathways-based genetic risk score and showed it was a better predictor of PCa than a parsimonious PSA plus age risk model. However, in that study, anthropometric or body composition data was not available, remaining untested the genetic risk score (GRS)-associated risk after adjustment for BMI and BFP.

Table 4. Multivariate analysis of Genetic Risk Score and risk for prostate cancer, overall and in obese or non obese men according to body fat percent-defined obesity cutoff

	All PCa		High grade PCa ^a	
	Multivariate	Bootstrap ^b	Multivariate	Bootstrap ^b
GRS	aOR (95%CI)	aOR (95%CI)	aOR (95%CI)	aOR (95%CI)
All subjects	2.1 (1.5-3.1)	2.1 (1.4-3.3)	2.4 (1.6-3.6)	2.4 (1.4-4.0)
Restricted to BFP				
< 25%	1.5 (1.0-2.3)	1.5 (0.9-2.7)	1.8 (1.1-2.9)	1.8 (0.9-3.6)
≥ 25%	3.2 (1.7-6.1)	3.2 (1.8-6.0)	3.5 (1.7-7.2)	3.5 (1.7-7.5)

^a High-grade PCa (Gleason score ≥ 7); aOR, age-, BMI-, testosterone- and BFP-adjusted odds ratio and 95%CI (95% confidence interval); GRS, age, BMI and testosterone analyzed as continuous variables; PCa, prostate cancer. ^b Monte Carlo simulation with 10000 permutations.

We found that the GRS was significantly associated with increased risk for developing overall and high-grade PCa, after adjustment for adiposity measures, age and testosterone levels. Furthermore, stratification according to BFP-defined obesity (cutoff 25%) we observed that GRS risk was increased only in obese subjects (for overall and high-grade PCa). Thus, the use of GRS could be more useful to predict PCa in the obese group of men (BFP ≥ 25%). Mechanistically, these genotypes of functional SNPs might impact the endocrine and paracrine activity of adipokine pathways that leads to tumor development and progression. This effect is stronger in men with excess fatness, revealing eventual synergies between adipokine SNPs and obesity status in association with overall and high-grade PCa.

Implementing direct and simple measures of body fatness together with measures of body fat distribution may be helpful in stratifying men at risk for PCa and aggressive disease; and by adding other predictive markers, such as DNA genetic variants, will certainly enhance the accuracy to predict PCa. In light of the rising incidence of obesity worldwide, identifying obesity as a risk factor for prostate malignancy is important, since it may be one of the few modifiable risk factors for PCa.

4.1.3. Combined analysis of functional polymorphisms in genes of adipokine pathways as predictive and prognostic markers in patients receiving androgen deprivation therapy (PAPER VIII)

Four hundred and eighty three patients treated with androgen deprivation therapy (ADT) in the course of their disease were analyzed in this study. Empirical analyses using univariate Cox or logistic regression identified clinical factors and individual single

nucleotide polymorphisms (SNPs) that predicted castration-resistant prostate cancer (CRPCa) and all-cause mortality (ACM), and that were associated with risk for metastasis (Table 1, supplementary tables 3 and 4, and Figures 1 and 2 in PAPER VIII). Then, the predictive effects of SNPs with $P < 0.15$ (univariate analysis) on time to CRPCa and ACM following ADT and risk for metastasis were evaluated together with significant clinicopathologic predictors from empirical examination. This analysis showed the effect of *ADIPOQ* +45 and *ADIPOQ* +276 variants, and tumoral necrosis factor alpha (TNFa) activation profile on CRPCa was robust after adjustment for clinical factors in a multivariate analysis (Table 5). Analysis of the secondary endpoint, ACM, after adjusting for other predictors, showed that *ADIPOQ* +45 T>G and *IL6R* Asp358Ala remained significant predictors for time to ACM (Table 5), and that *PPARD* CC carriers also were independently associated with increased overall survival after ADT (HR=0.25, 95%CI: 0.08-0.81). On multivariate analysis, carrying the *LEPR* 223 G-allele remained associated with greater risk of developing distant metastatic disease (Table 4 in PAPER VIII).

In this study aiming to understand if germline polymorphisms in adipokine pathways are determinants of the response to ADT, the time to CRPCa was influenced by two SNPs in *ADIPOQ* and by combined SNPs in TNFa pathway activation. The predictive ability of *ADIPOQ* +45 extended towards the secondary end point ACM, together with *PPARD* and *IL6R* genetic polymorphisms. Additionally, results also suggest an association of *LEPR* Gln223Arg polymorphism with development of metastasis.

Factors that influence the response to ADT includes clinical stage, Gleason score, PSA and metastasis¹⁷⁹, although SNPs may also be useful for prognosticating the response to ADT. By analyzing 27 SNPs of 17 genes related with 9 adipokine pathways, we have identified two SNPs, *ADIPOQ* +45 and *ADIPOQ* +276 and a functional *TNFA/TNFRSF1A* combination that are associated with CRPCa after controlling for clinical prognostic factors. The mechanisms responsible for CRPCa development are not definitely established. Despite increasing interest in androgen receptor-dependent pathway, other independent pathways have been described^{22, 180}, in which CRPCa cells use alternative survival pathways to overcome the growth inhibition imposed by ADT^{22, 181}. ADT is known to induce changes in adiposity and adipokine levels in circulation^{182, 183}. Adipokine pathways, have been implicated in intracellular signals such as those activated in hormonal castration resistance¹⁸⁴. Furthermore, mitogenic and anti-apoptotic effects of some adipokines (e.g. leptin, IL-6, IGF-1) seem to be limited to hormone-refractory PCa cells^{100, 185, 186}.

Table 5. Association between adipokine pathway SNPs and time-to-CRPCa and time-to-ACM following ADT

	Time-to-CRPCa		Time-to-ACM	
	aHR (95%CI)	P	aHR (95%CI)	P
Age at diagnosis, years	--	--	1.06 (1.03-1.10)	< 0.0001
Metastasis at ADT initiation				
No	Referent		Referent	
Yes	2.61 (1.80-3.79)	< 0.0001	1.86 (1.23-2.80)	0.003
Clinical stage				
Localized	Referent		Referent	
Advanced	2.40 (1.52-3.78)	< 0.0001	4.70 (2.80-7.90)	< 0.0001
Gleason score				
≤ 7 (3+4)	--		Referent	
≥ 7 (4+3)	--	--	1.82 (1.20-2.78)	0.005
CRPCa				
No	--		Referent	
Yes	--	--	4.70 (2.80-7.90)	< 0.0001
<i>ADIPOQ</i> + 45				
T carrier	Referent		Referent	
GG	4.14 (1.63-10.56)	0.003	5.20(1.82-14.86)	0.002
<i>ADIPOQ</i> +276				
T carrier	Referent		--	
GG	0.70 (0.50-0.97)	0.034	--	--
TNFA activation				
Low/intermediate	Referent		--	
High	0.62 (0.41-0.93)	0.022	--	--
<i>PPARD</i>				
T carrier	--		Referent	
CC	--	--	0.25 (0.08-0.81)	0.021
<i>IL6R</i>				
CC	--		Referent	
A carrier	--	--	0.61 (0.37-0.99)	0.047

ADT, androgen deprivation therapy; ACM, all-cause mortality; CRPCa, castration-resistant prostate cancer; SNP, single nucleotide polymorphism; TNFA, tumor necrosis factor alpha; *ADIPOQ*, adiponectin gene; *PPARD*, peroxisome-proliferator activated receptor delta; *IL6R*, interleukin 6 receptor; aHR(95%CI), adjusted hazard ratio and 95% confidence interval. Covariates included in multivariate model for CRPCa: PSA at diagnosis (<20 vs. ≥20 ng/mL); clinical stage (localized vs. advanced); Gleason (<3+4 vs. ≥4+3); definitive therapy (RP/RT vs. No); metastasis at ADT initiation (Yes vs. No); *VEGF*+405; *VEGF*-460; *IL6*-572; *IL6R*; *IL6ST*; *IL6* activation LI_H; *ADIPOQ* +45; *ADIPOQ* +276; *ADIPOQ* haplotype; *LEPR*223; *LEP* activation; *TNFA*-863; *TNFA* activation LI_H; *TNFA* expression L_IH. Covariates included in multivariate model for ACM: Age at diagnosis (continuous); PSA at diagnosis (<20 vs. ≥20 ng/mL); clinical stage (localized vs. advanced); Gleason (<3+4 vs. ≥4+3); treatment modality (aLHRH vs. combined ADT); metastasis at ADT initiation (Yes vs. No); resistance to hormonal castration (Yes vs. No); *VEGF*+405/-460 activation LI_H; *IL6*-572; *IL6R*; *ADIPOQ* +45; *ADIPOQ* +276; *LEPR*223; *PPARD*.

In our study, *ADIPOQ* +45 G homozygous and *ADIPOQ* +276 T carriers had a shorter time to CRPCa. Although *ADIPOQ* polymorphisms have been inconsistently associated with PCa risk^{67, 187, 188}, our study is the first to evaluate *ADIPOQ* SNPs in association with CRPCa. When we combined SNPs in TNFa pathway [*TNFA* -863 C>A

(rs1800630), *TNFA* -308 G>A (rs1800629) and *TNFRSF1A* -329 G>T (rs4149570)] according to functional characteristics, the carriers of low/intermediate TNFa activity profile had shorter time to CRPCa, notwithstanding case-control studies showed lack of association between *TNFA* variants at locus -863 or -308 and PCa^{189, 190}. TNFa-mediated activation of tumoral necrosis factor receptor 1 (TNFR1) signaling is critical for activating tumor-reactive T cells and arresting multistage carcinogenesis^{191, 192}. The very low levels of testosterone as result of ADT, together with increased inflammatory markers¹⁹³, create an inflammatory environment for tumor under ADT. We hypothesize that this scenario may be altered by polymorphisms in *ADIPOQ* and by low/intermediate TNFa activation, which likely result in decreased inflammatory magnitude, further depressing immunosurveillance.

The few studies that examined the role of germline polymorphisms in association with PCa mortality yielded inconsistent results¹⁹⁴⁻¹⁹⁷. In our cohort, where all patients received ADT for treating PCa while taking into consideration previous treatment modalities, *ADIPOQ* +45 T>G, *PPARD* -87 T>C and *IL6R* Asp358Ala, remained independently associated with decreased survival. The common genetic variant of *IL6R* that results in Asp-by-Ala substitution at codon 385 (A > C nucleotide substitution) is associated with increased soluble IL6R and IL-6 serum levels and with altered IL6R membrane binding^{198, 199}. We observed after multivariate analysis that *IL6R* C homozygous remained significantly associated with reduced overall survival following ADT, which might correspond to higher activation of trans-signaling pathway associated with C-allele.

We found that *LEPR* 223 G carriers were at increased risk for developing metastatic disease. Although this SNP yielded mixed results in case-control studies^{67, 69}, G carriers have a stronger leptin-binding affinity²⁰⁰. In addition, this polymorphism is associated with plasma soluble LEPR concentration and may influence receptor recycling and degradation^{201, 202}, thereby influencing free leptin levels and receptor availability at cell surface. There has been particular interest in the *PPARD* -87 T>C polymorphism that alters the binding affinity of the SP1 transcription factor, with carriers of the minor C-allele having higher transcriptional activity²⁰³. We observed in a cohort of PCa patients treated with ADT that CC carriers had increased survival.

In a time where alternative therapeutic opportunities arise in advanced PCa^{23, 24}, poor prognostic germline polymorphisms may be used along with known clinical factors to foster the capacity to prognosticate clinical course after ADT initiation, thereby helping clinical reasoning. Present findings also encourage the need for clarifying the effectiveness of personalized therapies targeted towards adiponectin, tumoral necrosis factor and leptin pathways. If confirmed, our findings might help targeting patients with

precocious ADT failure and mortality, and at risk for metastasis development in the course of disease.

4.2. MECHANISMS CAUSALLY INVOKED IN THE ASSOCIATION BETWEEN OBESITY AND PCA

4.2.1. A two-way street: reciprocal regulation of human adipose tissue and prostate cancer (PAPER IX and X)

In these experiments we tried to reproduce *in vitro* the interaction between adipose tissue-produced molecules and prostate tumor cells. Our purpose was to understand the impact of periprostatic (PP) and visceral (VIS)-derived factors and its fractions in key mechanisms that are hallmarks of malignant aggressiveness phenotype in PCa cells and viceversa.

The production of adipokines in adipose tissue has been widely studied in subcutaneous and VIS adipose tissue ²⁰⁴. 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 ^{157, 205}. Thus, conditioned medium obtained after primary culture of adipose tissue and stromal-vascular fraction (SVF), may reflect the adipokines usually produced *in vivo* ²⁰⁶. It is a heterogeneous organ that consists of multiple cell types: adipocyte fraction, which contains lipid-loaded adipocytes, and SVF fraction, which includes preadipocytes, endothelial cells, fibroblasts, stem cells, macrophages and other immune cells ²⁰⁷. The fractions of adipose tissue differ in that while explants reflect an organotypic cell culture system of whole adipose tissue, the major characteristic of SVF fraction culture is the depletion of adipocytes and absence of extracellular matrix. In order to investigate which fraction influenced tumor cells, we cultured paired explants and SVF fraction cells. To allow comparison between depots and adipose tissue fractions, the cell count was adjusted per gram of adipose tissue.

These *in vitro* experiments showed that the PP depot has the potential to modulate extra-prostatic tumor cells' microenvironment through increased matrix metalloproteinases (MMPs) activity and by promoting PCa cell survival and migration. On the other hand, we report here on reactive PP adipose tissue, where tumor factors can regulate the expression of adipokines with oncologic potential, MMP activity and mitochondrial DNA. Adipocytes are likely to be relevant players in these mechanisms.

In vitro experiments with adipose tissue-derived CM in prostate cancer cell lines

The contribution of PP adipose tissue, which is often infiltrated by malignant cells, to cancer progression is largely unknown. Thus, this study aimed to determine if PP adipose tissue is linked with aggressive tumor biology in PCa. In order to investigate the proteolytic profile of PP adipose tissue, we evaluated gelatinase activity in conditioned medium from culture of PP adipose tissue explants, according to age at diagnosis, BMI, pathologic status and Gleason grade of donors (Table 1 in PAPER X). MMP9 was significantly elevated in obese/overweight compared to normoponderal subjects ($P=0.036$). To understand which fraction of PP adipose tissue contributes to enhanced gelatinase activity, we analyzed paired explant and SVF fraction cultures from PP adipose tissue (Figure 7). Our results indicate that the proteolytic activity of both MMP2 and MMP9 is higher in cultures of adipose tissue explants than in the correspondent SVF fractions. A similar proteolytic pattern is present between explants and SVF fractions of VIS adipose tissue. Additionally, we observed that PP adipose tissues present higher MMP2 but not MMP9 activity, as compared with adipose tissue from a distinct anatomical fat depot (median pre-peritoneal VIS region) (Figure 7).

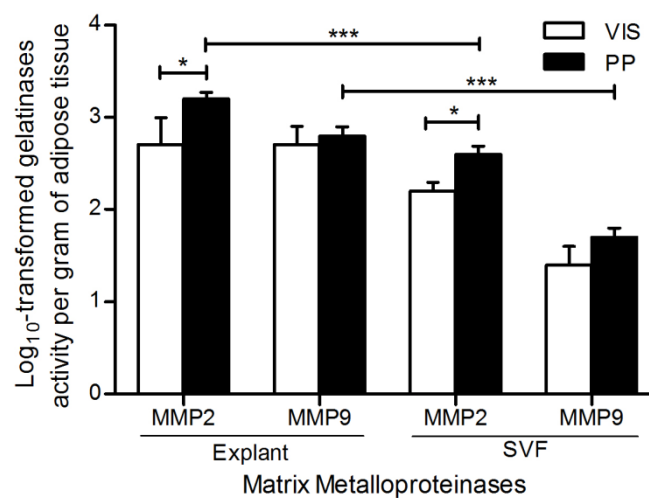


Figure 7. Gelatinolytic activity of periprostatic adipose tissue and comparison with visceral pre-peritoneal fat depot. Analyses were performed in explants and stromalvascular fraction primary culture of 21 samples of PP adipose tissue and 10 samples of VIS adipose tissue. Independent samples *t*-test was used. *** $P<0.0001$ between explants and SVF fraction; * $P<0.05$ in the comparison among fat depots. MMP, matrix metalloproteinase; VIS, visceral; PP, periprostatic; SVF, stromal-vascular fraction.

In tumors, cancer cells are not the only source of MMPs. In our study, MMP9 activity was significantly elevated in the PP adipose tissue of overweight/obese men ($BMI \geq 25 \text{ Kg/m}^2$), implying excess body fat and the PP fat depot in the modulation of extra-capsular cancer cells' microenvironment. Concordantly, other studies found MMP9 to be

positively correlated with BMI ¹⁰⁶. Their expression in prostate tumors is related with disease progression and metastasis ¹⁰², whereas MMP9 was shown to increase growth factors bioavailability and to elicit epithelial-to-mesenchymal transition in tumor cells ^{103, 104}, therefore promoting an aggressive phenotype. A recent report indicated that oesophageal tumors from obese patients express more MMP9 and that co-culture of VIS adipose tissue explants with tumor cells up-regulated MMP2 and MMP9 ¹⁰⁵. Noteworthy, cancer-derived factors stimulate other surrounding cells, including adipose tissue cells, to synthesize MMPs ¹⁷⁰. Interestingly, our findings showed that media from explants and PP adipose tissue depot presented the higher gelatinolytic activity per gram of adipose tissue, compared with SVF cultures- and VIS adipose tissue-derived media. Although the amount of MMP9 has been described to be higher in SVF fraction of adipose tissue compared with adipocytes ¹⁰⁶, the latter have greater plasticity to increase MMPs expression when interacting with other cells in adipose tissue ^{106, 208}. The increased activity of metalloproteinases in CM from adipose tissue explants in culture compared with SVF, likely reflect the additive effect or interaction between cells of the SVF fraction plus adipocytes. We found that MMP2 activity was increased in PP versus VIS adipose tissue supernatants. Although there is no evidence of MMP2 role in adipose tissue/cancer cells crosstalk, recent findings suggest MMP2 is upregulated in tumor cells co-cultured with adipose tissue explants and that its expression and activation is modulated by several adipokines (e.g. Wdm1-like and visfatin) ^{105, 109, 115}. Additionally, other MMPs, notably MMP11, have been shown to be correlated with breast cancer-induced adipocyte's activated state ^{131, 133}. If confirmed, our findings may reveal a novel specific proteinase expression and activity pattern in PP adipose tissue favorable to PCa progression.

In order to examine whether soluble factors secreted by PP adipose tissue alter tumor cell behavior, its proliferative potential on an aggressive hormone-refractory PCa cell line was investigated. We observed that factors secreted from explants of both PP and VIS adipose tissue increase proliferation of hormone-refractory PCa cells, whereas only VIS SVF culture-derived factors stimulated proliferation (Figure 8A). The log10-transformed PC-3 cell count per gram of adipose tissue, was significantly higher after stimulation with explants culture-derived CM compared with SVF, independently of the adipose tissue depot ($P<0.0001$) (Figure 7B). Interestingly, the SVF-derived CM of PP adipose tissue had a stronger proliferative effect than SVFs of VIS origin ($P=0.007$) (Figure 8B).

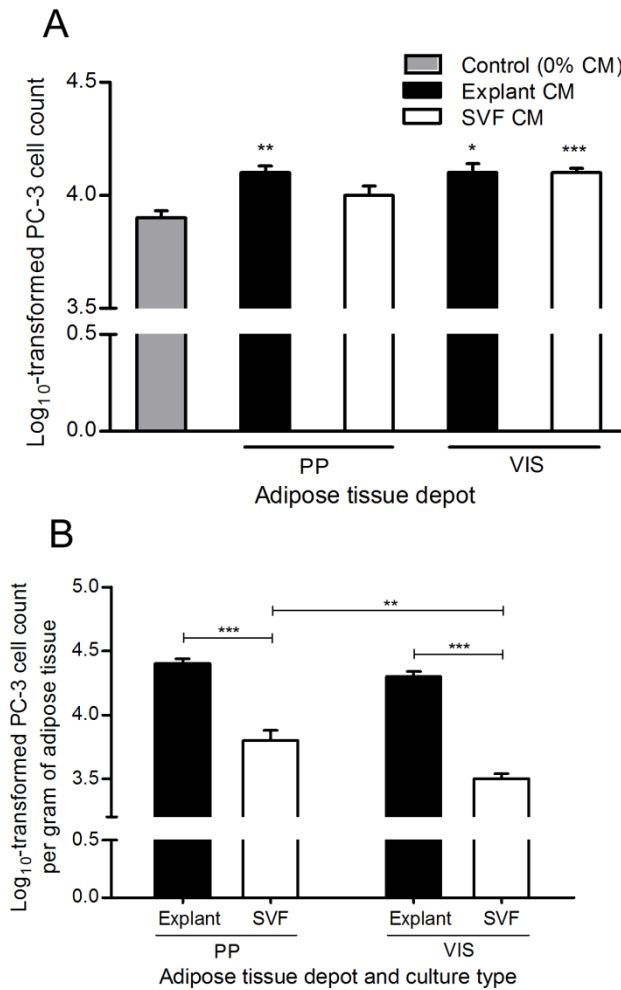


Figure 8. Influence of conditioned medium from distinct adipose tissue origins in the proliferation of PC-3 cells. Analyses were performed using conditioned medium of 21 samples of periprostatic (PP) and 10 samples of visceral (VIS) adipose tissue, after explants and stromal-vascular fraction primary cultures. A. Effect of adipose tissue-derived CM on PC-3 cell proliferation, in comparison with control (0% CM) (** $P < 0.01$ in relation with 0% CM, one-way ANOVA with two-sided post-hoc Dunnett test). B. PC-3 cell proliferation was normalized per gram of adipose tissue and compared according to fat depot and adipose tissue fraction (** $P < 0.01$ and *** $P < 0.0001$ between groups, independent samples t -test). CM, conditioned medium; PP, periprostatic; SVF, stromal-vascular fraction; VIS, visceral.

The influence of PP adipose tissue secreted factors for cell proliferation of another less aggressive hormone-sensitive PCa cell line was subsequently examined. Interestingly, while these cells also respond to the proliferative stimulus of CM from SVF fraction ($P < 0.0001$), an inhibitory effect in LNCaP cells was observed with explants CM ($P < 0.05$), independently of fat depot (Figure 9A). Comparisons between adipose tissue fractions, explants versus SVF-derived CM, in LNCaP cell proliferation were conducted using the logarithmically-transformed cell count per gram of adipose tissue (Figure 9B). For VIS but not PP adipose tissue, there was an increased influence of explants

compared to SVF CM in LNCaP cell proliferation ($P<0.0001$). Furthermore, when compared with visceral SVF CM, the SVF CM from PP adipose tissue increased LNCaP cell proliferation (Figure 9B).

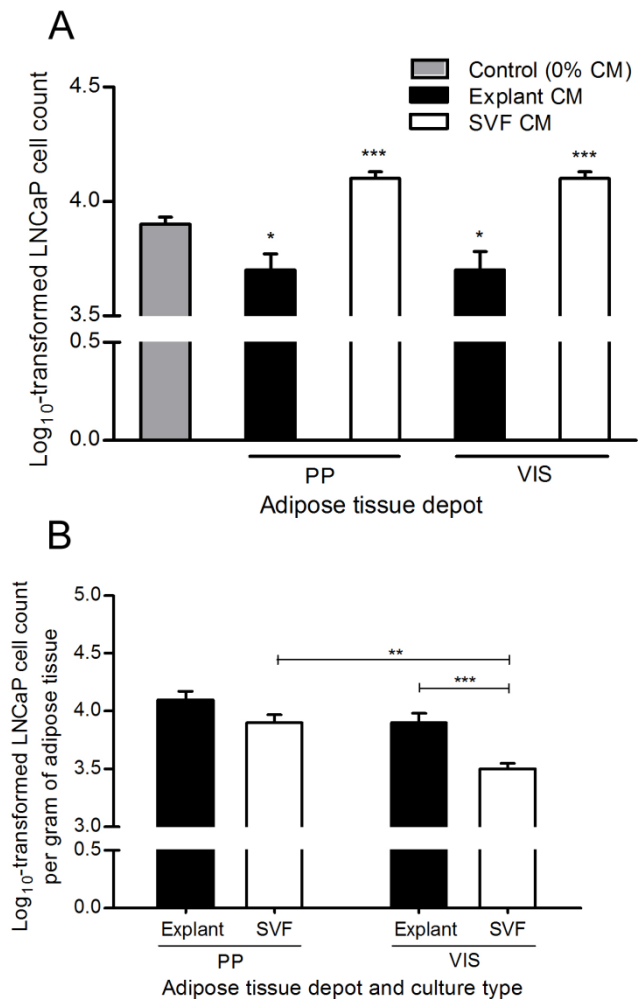


Figure 9. Influence of conditioned medium from adipose tissue in the proliferation of LNCaP cells. Analyses were conducted using conditioned medium of periprostatic (PP) and visceral (VIS) adipose tissue from 10 subjects after explants and stromal-vascular fraction primary cultures. A. Influence of adipose tissue-derived CM in LNCaP cell proliferation, in comparison with control (0% CM) (* $P<0.05$ and ** $P<0.01$, relative to control, two-sided post-hoc Dunnett test). B. Comparison of the effect of CM from distinct adipose tissue depot and fractions in LNCaP proliferation after tissue weight normalization (** $P<0.01$ and *** $P<0.0001$ between groups, independent samples t -test). CM, conditioned medium. SVF, stromal-vascular fraction. PP, periprostatic; VIS, visceral.

It is well-established that adipose tissue secretes a wide array of molecules²⁰⁹. These adipokines, exclusively or partially secreted by adipocytes or SVF fraction cells, are likely to have a role in modulating the risk of cancer progression^{57, 74, 168}. Few studies examined the effect of adipocytes in PCa cells growth^{100, 210}. While a proliferative effect was observed in hormone-refractory PC-3 cells, these findings didn't replicate in LNCaP

cells²¹⁰. In fact, the mitogenic and anti-apoptotic effects of several adipokines, alone and combined, in PCa cell growth (e.g. leptin, IL-6, insulin-like growth factor 1, IGF-1), seems to be limited to hormone-refractory PCa cells^{100, 185, 186, 211, 212}. Previous studies also report on the suppression of LNCaP cell growth as response to adipokines (e.g. TNF α , decreased expression of vascular endothelial growth factor, VEGF), not observed in hormone-refractory cells^{210, 213-215}. Contrary to explants, CM from SVF cultures induces cancer cell proliferation, independently of cell line, except for the SVF from PP adipose tissue in PC-3 cells. Cells that constitute the SVF fraction of adipose tissue, where macrophages have a modulatory role, are known to secrete several angiogenic and antiapoptotic factors^{90, 216, 217}, which ultimately can impact PCa cells growth. The lack of proliferative effect observed for the SVF fraction from PP adipose tissue may partially be due to the reported low number of macrophages in PP fat depot¹²⁷, diminishing the proliferative stimulus in PCa cells.

In this study, proliferation was increased with CM from PP and VIS explants versus SVF CM in PC-3 cells, whereas LNCaP cells only proliferated significantly more with VIS explants compared to VIS SVF. As the highest proliferation was seen following stimulation with CM from explants we speculate adipocytes may be the main effectors. Other studies also found a proliferative effect of adipocytes in PCa cells^{100, 210}. Adipocytes add significantly to the proliferative effect in hormone-refractory PCa cells, even though the adipokines responsible by these results have yet to be determined. Alternatively, since explants culture preserve the paracrine signals by maintaining the existing crosstalk among the different cell types²⁰⁶, we hypothesize that the higher proliferative stimulus conferred by explants CM likely reflects a co-stimulatory and/or additive effect of adipokines produced by adipocytes and by the stromal vascular fraction cells.

The enhanced proteolytic activity of PP and VIS adipose tissues led us to investigate their putative effect on PCa cell motility. Therefore, the motile behavior of the PC-3 hormone-refractory and of the LNCaP hormone-sensitive PCa cell lines were analyzed using adipose tissue samples from 4 additional subjects. In the first subject by subject analysis we observed that CM from any adipose tissue fraction or depot elicited, in comparison to untreated cells (control) increased motility, independently of donor's clinicopathological characteristics (data not shown). Figure 10 shows motile parameters of PCa cells in response to adipose tissue CM. Comparing with control, LNCaP cells stimulated with CM from any fraction or depot always resulted in higher mean speed and final relative distance to origin (FRDO) (Figure 10A). In PC-3 cells, while mean speed was higher for any CM condition compared with control, the FRDO was only increased after stimulation with CM from explants, both from PP and VIS depot (Figure 10B).

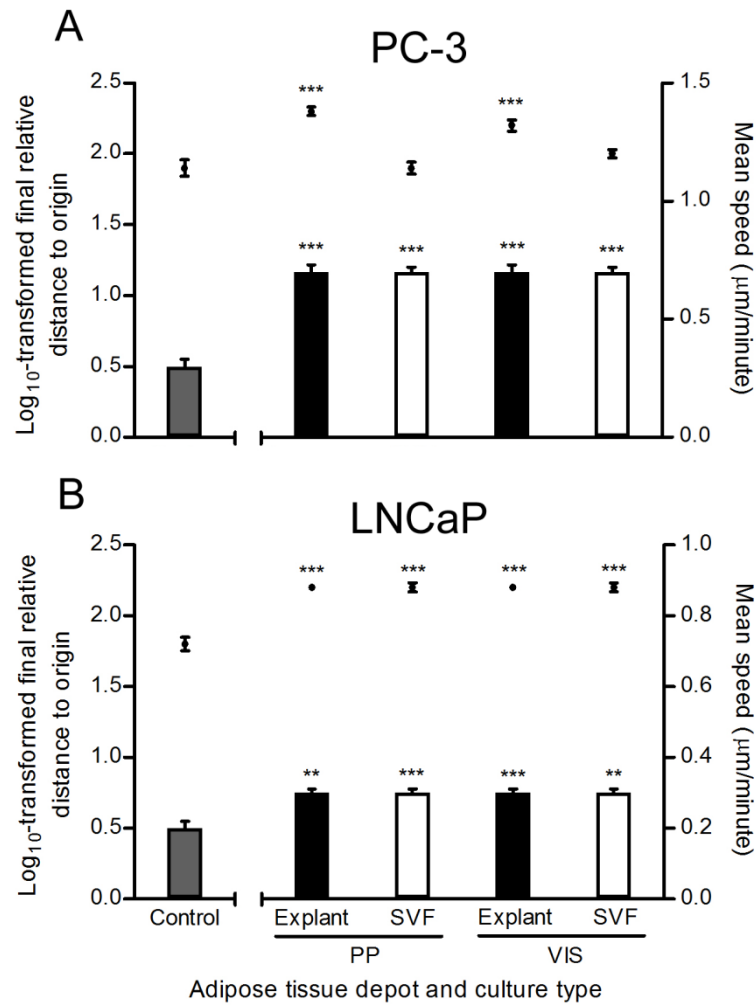


Figure 10. Motility of PC-3 and LNCaP cells upon stimulation of adipose tissue-derived CM from explants and SVF. Influence of adipose tissue fractions in cell motility parameters. Data represent mean±S.E.M. of at least 20 representative cell trajectories per each tested condition, with conditioned medium of primary adipose tissue cultures from four distinct subjects. Bars represent mean speed and plots the logarithmically transformed final relative distance to origin (FRDO). A. FRDO and MS of PC-3 cells (**P<0.0001 relative to control). B. FRDO and MS of LNCaP cells (**P<0.01 and ***P<0.0001 relative to control). In the log-transformed FRDO we used one-way ANOVA with post-hoc Dunnett test (two-sided), whereas the mean speed was analyzed using Kruskal Wallis followed by Mann Whitney test. SVF, stromal-vascular fraction; PP, periprostatic; VIS, visceral.

After adjustment of motility parameters to adipose tissue weight, in order to compare different culture types and depots, only the LNCaP cells mean speed was not statistically different between PP and VIS depot. Otherwise, motile parameters were higher after stimulation with CM from PP depot (Figure 11). For both PC-3 (Figure 11A) and LNCaP (Figure 11B) cells stimulated with explant-derived CM from PP and VIS adipose tissue, the mean speed and FRDO were significantly higher in comparison to SVF (P<0.0001).

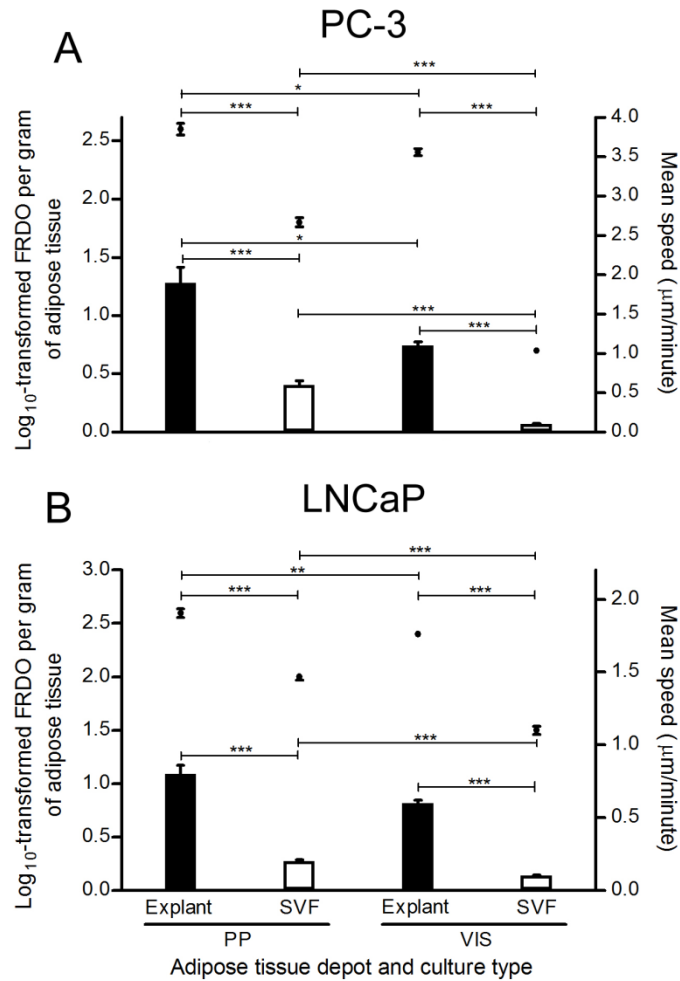


Figure 11. Motility of PC-3 and LNCaP cells upon stimulation of adipose tissue-derived CM from explants and SVF. Data represent mean±S.E.M. of at least 20 representative cell trajectories per each tested condition, from four distinct subjects. Bars represent mean speed per gram of adipose tissue and plots the logarithmically transformed final relative distance to origin (FRDO) per gram of adipose tissue. A. FRDO and MS of PC-3 cells (*P<0.05 and ***P<0.0001 between treatment conditions). B. FRDO and MS of LNCaP cells (**P<0.01 and ***P<0.0001 between conditions). Analyses on MS were performed with Mann Whitney test, whereas FRDO was analyzed using independent samples *t*-test. SVF, stromal-vascular fraction; PP, periprostatic; VIS, visceral.

The mechanisms involved in tumor cell movement are far from fully elucidated, although various biophysical processes are considered to be involved²¹⁸. These mechanisms are modulated by the activation of several signaling pathways, such as PI3K, ERK/MAPK and c-Src tyrosine kinase²¹⁸, which are known downstream signals of adipokines¹⁸⁴. In fact, many adipokines [e.g. IGF-1, osteopontin (OPN), leptin, adiponectin, VEGF, thrombospondin, interleukin -8 (IL-8) and interleukin 6 (IL-6)] have been shown to modulate different steps of cell motile behavior^{94, 219-230}. Several key

pathways and molecules involved in this process can be induced by factors secreted by adipose tissue, hence supporting the increased motility we found in stimulated PCa cells.

Explants-derived CM, whether from VIS or PP origin exerted consistently, also across cell lines, an increased effect in migration speed and final relative distance to origin, when compared with SVF fraction. It is possible that explants CM, which reveal the secretory profile of adipocytes plus SVF cells, produce more motile factors and exclusive secretion of others (e.g. leptin and adiponectin), thereby resulting in increased total distance/mean speed and final relative distance to origin of PCa cells.

The anatomical origin of adipose tissue accounts for increased gelatinolytic activity and different proliferative and migratory stimulus. CM from PP results in higher log10-transformed PC-3 and LNCaP cell count per gram of adipose tissue, only when SVF CM was used. Furthermore, adipose tissue from PP origin exerted the stronger motile effect (of both analyzed parameters) in PC-3 cells compared to VIS depot, independently of the culture type. In LNCaP cells only the PP explants-derived CM didn't impact the mean speed more than CM from VIS explants. These findings suggest that VIS and PP fat pads may have distinct relative cellular composition or are differently programmed to secrete molecules involved in the regulation of cell proliferation and motility. We recently found increased amount of adipose stem cells (CD34+/CD45-/CD31-/CD146-) in PP compared with VIS adipose tissue (Ribeiro R, unpublished observations, PAPER XII).

Tumor cell progression depends on itself as well as on the surrounding microenvironment, which is able to influence proliferation, migration and metastatic behavior of tumor cells by modulating the extracellular matrix and growth factor production²³¹. If the tissues where tumor cells exist provide the missing extrinsic signals, then cells will proliferate and acquire an invasive phenotype, which may lead to metastasis. Whole PP fat, not only SVFcells, seems to warrant the necessary factors to induce a specific microenvironment for PCa tumor cells, which ultimately may result, as we found, in tumor cell survival, increased motility and availability of extracellular proteases. During cell migration, pericellular proteolysis of extracellular matrix is important for cell protrusion. The increased production of MMPs found in PP adipose tissue can fuel invasive and metastatic behavior of PP fat-infiltrating PCa cells.

In vitro experiments with prostate cancer cell-derived CM in periprostatic adipose tissue

We were interested in studying if tumor cell-derived factors could influence PP adipose tissue behavior. After 48h of stimulation with CM from the PC-3 cell line, PP adipose tissue-derived explants increased protein expression of TNFa 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 40% (p=0.045) (Figure 12). The effects on monocyte

chemoattractant protein 1 (MCP-1), leptin and RANTES (CCL5) expression 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 analysis concerning obesity and stage of disease, showed that obesity did not influence the production of adipokines, despite the increased expression of TNF α , IL-6 and OPN in PP adipose tissue of overweight/obese group (data not shown). Interestingly, following treatment with PC-3 CM, the expression of TNF α , IL-6 and OPN protein was higher for PP adipose tissue-derived explants from patients with locally advanced disease ($p=0.016$, $p=0.004$ and $p=0.030$, respectively).

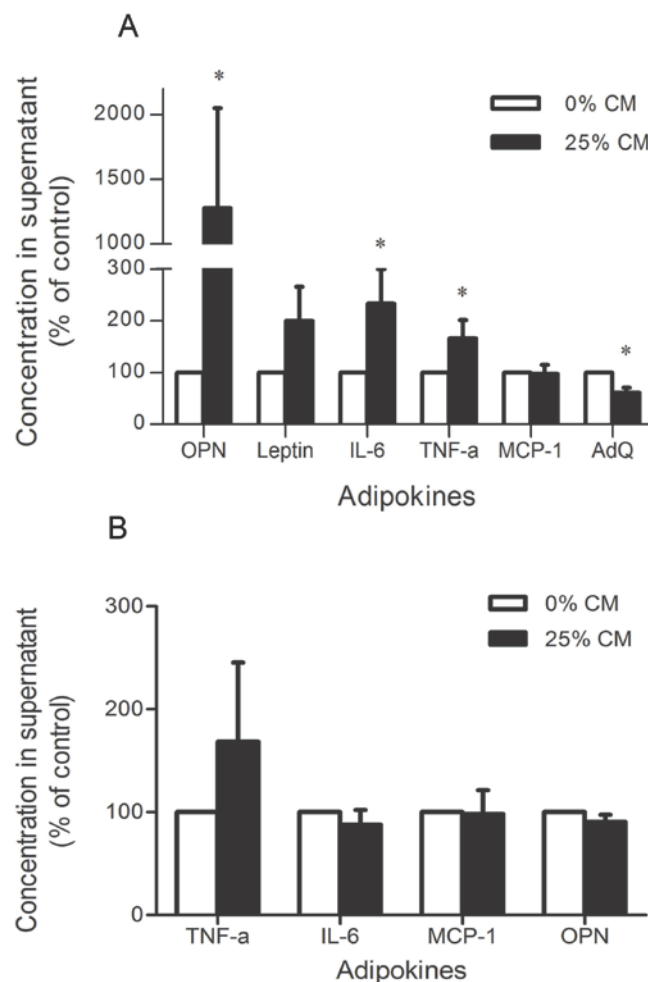


Figure 12. Percent increase in adipokines concentration measured in supernatants of adipose tissue explants (A) and stromal vascular fraction (SVF) (B), after stimulation with conditioned medium (CM) from PC-3 prostate tumor cells (mean $\pm$ S.E.M.). OPN, osteopontin; IL-6, interleukin-6; TNF-a, tumoral necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; AdQ, adiponectin; CCL5, RANTES. * $p<0.05$, compared with control (0% CM).

One of the mechanisms that causally invoke obesity with cancer progression includes deregulation of adipokine production ⁷⁴. The only study on PP adipose tissue, evidenced increased IL-6 local concentration, and its correlation with tumor aggressiveness ¹²⁷. Studies of IL-6 circulating levels suggest that IL-6 is involved in PCa progression and has prognostic significance ^{232, 233}. Interestingly, in this study we demonstrated that after exposure to PCa cell CM, the PP adipose tissue explants increased their production of IL-6. Likewise, adipocytes cocultured with breast cancer cells overexpressed IL-6 ¹³³. The increased IL-6 availability may induce neuroendocrine differentiation, intra-prostatic androgen production and activation of androgen receptor ^{234, 235}. The adipokine OPN is an instigator of tumor growth and progression, contributing to disease aggressiveness ^{236, 237}. It has been implicated in tumor cells malignancy *in vivo*, in tumor cell dormancy, and as a plasma marker for aggressive disease ^{108, 236}, even though its role as a PP adipose tissue-derived molecule with paracrine effect in locally advanced PCa has not been evaluated so far. In the present study, we observed a near 13-fold increased production of OPN by PP adipose tissue in response to PCa cell CM, suggesting a significant contribution to OPN local production, further eliciting tumor progression. Another adipose tissue secretory product is TNF α , a mediator of cancer-related inflammation ²³⁸, which has a direct effect causing genetic damage to cells, but also enhances malignant cell survival and induces epithelial-to-mesenchymal transition ^{239, 240}. Its circulating levels correlate with the extent of disease in patients with PCa ²³³. We found increased TNF α expression by stimulated PP adipose tissue 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 ²⁴¹. Adiponectin modulates the AMP-activated protein kinase (AMPK) pathway, acting as a direct endogenous inhibitor of inflammation, angiogenesis and cancer invasiveness ⁵⁷. Additionally, a 25-year prospective study demonstrated a protective effect of adiponectin serum levels for developing high-grade PCa and dying from the cancer ²⁴². Thus, the observed reduction of adiponectin production by PP adipose tissue explants following stimulation agrees with a favorable tumor microenvironment orchestrated by tumor cell-derived factors.

Our results suggest that the PP adipose tissue has plasticity to be metabolically more active (not only by adipocyte hypertrophy), after induction by unknown specific factors produced by PC-3 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.

Next, we found that the *in vitro* MMP9 activity of PP adipose tissue was higher in stimulated explants ($p=0.043$), whereas a trend for higher activity of MMP2 was observed

in SVF cultures ($p=0.088$) (Figure 13). Noteworthy, we observed an increased MMP2 activity following PC-3 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) copies number in explants but not in SVF ($p=0.033$ and $p=0.687$, respectively) (Figure 14).

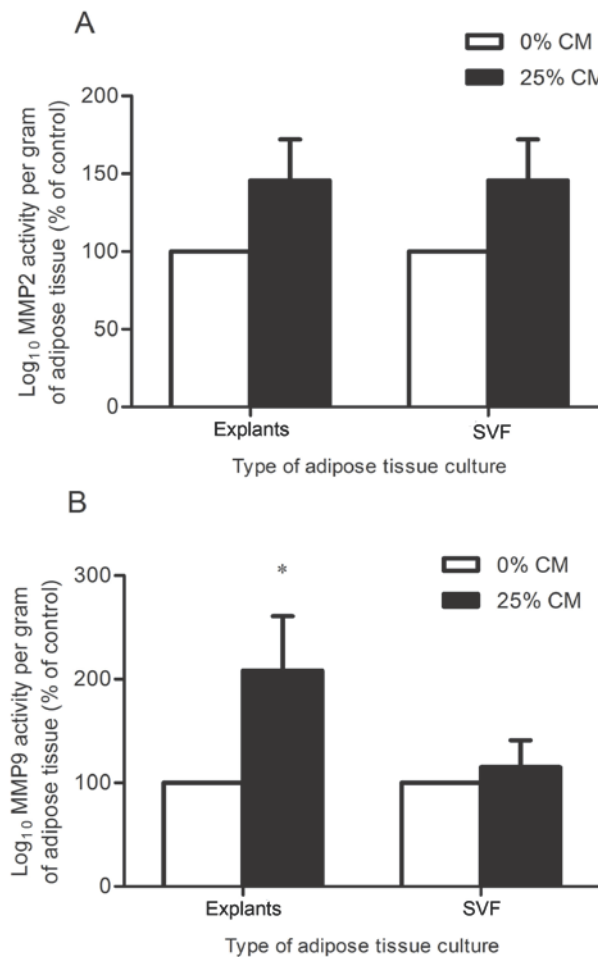


Figure 13. Log-transformed (Log₁₀) matrix metalloproteinase 2 (MMP2) (A) and matrix metalloproteinase 9 (MMP9) (B) activity per gram of tissue in adipose tissue explants and in stromal vascular fraction (SVF) after stimulation with PC-3 CM (mean±S.E.M.). * $p<0.05$, compared with control (0% CM).

In this study, we found that upon stimulation of PP adipose tissue with PCa cell CM, explants start to present higher MMP9 activity. Since inflammatory stimuli induces MMP expression²⁴³, the inflammatory mediators produced by tumor cells, such as IL-6, may explain MMP activity. Cumulatively, the observation that MMP9 expression in adipocytes increases over 100-fold when cocultured with macrophages¹⁰⁶, suggests that adipocytes have a great plasticity for increasing MMP9, possibly explaining our findings on explants.

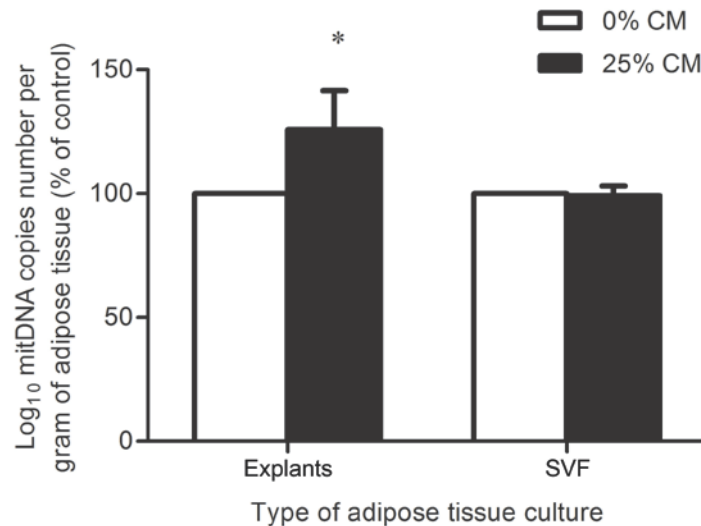


Figure 14. Log-transformed mitochondrial DNA (Log_{10} mitDNA) copies number adjusted per gram of adipose tissue in adipose tissue explants and stromal vascular fraction (SVF) after stimulation with conditioned medium (CM) from PC-3 prostate cancer cell line (mean $\pm$ S.E.M.). * $p < 0.05$, compared with control (0% CM).

Interestingly, the level of MMP9 activity was not modulated by obesity, whereas MMP2 was increased in obese patients. Catalan et al.²⁴⁴ also showed higher MMP2 activity in the VIS 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 function²⁴⁵, and that increasing numbers of mtDNA copies are associated with increased number of mitochondria per cell and with mitochondrial function^{246, 247}. 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)²⁴⁵, thus affecting overall adipocyte physiology and influencing the synthesis and release of adipokines. Since we observed increased metabolic activity of PP adipose tissue 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 factors¹³⁰, since adipocyte differentiation has been associated with increased mitochondrial content in adipocytes^{248, 249}.

Our data suggest that in the context of extra-prostatic disease, upon tumor cell stimulation, PP adipose tissue may acquire a phenotype favorable to aggressive PCa.

4.2.2. Obesity and prostate cancer: gene expression signature of human periprostatic adipose tissue (PAPER XI)

In this study we used, for the first time, a global gene expression profiling in PP adipose tissue to unravel genes and regulatory pathways associated with obese and overweight (OB/OW) and with PCa. Subjects presented similar characteristics between prostatic disease groups (Table 1 in PAPER XI). As expected by selecting the groups, OB/OW evidenced higher mean BMI (29.1 ± 1.8 vs. 23.1 ± 1.2 kg/m², $P < 0.0001$) and serum leptin levels (8.6 ± 1.3 vs. 1.9 ± 0.7 µg/mL, $P = 0.001$) than normoponderal (NP) subjects. For each group of prostate disease [benign prostatic hyperplasia (BPH), localized PCa (LoPCa) and locally advanced PCa (LAPCa)], significantly higher BMI was observed in OB/OW subjects compared with NP ($P = 0.016$, $P < 0.0001$ and $P = 0.013$, respectively).

Because prostate tumor cells infiltrate the PP fat and before advancing with microarray experiments, we confirmed whether PP adipose tissue samples were free from PCa cells by examining the expression of the *PCA3* gene. Lack of expression of this gene in the PP adipose tissue of cancer patients was demonstrated (Figure 1 in PAPER XI).

In the present study, forty-six percent of the well-characterized genes included in the array were expressed at detection level in PP adipose tissue, which is comparable to omental adipose tissue¹²⁹. The comparison of PP adipose tissue gene expression of non-diabetic OB/OW to NP men with prostatic disease identified for the first time 34 differently expressed genes (Table 2 in PAPER XI). When comparing between prostatic diseases (PCa versus BPH) the PP adipose tissue of age- and BMI-matched nondiabetic men, 119 altered genes were found (Table 3 in PAPER XI).

Two relevant but opponent pathways, lipolysis and adipo/lipogenesis, have a significant role in energy balance. In our study, consistent with other reports on VIS adipose tissue^{129, 250}, altered expression of genes involved in lipolysis were found in association with obesity and excess adiposity. The combined functional dysregulation of these genes suggests that obese PP adipose tissue has anti-lipolytic and adipo/lipogenic gene expression profile. Furthermore, our data suggests increased cell growth and anti-apoptosis, extensive to endothelial, progenitor or adult lineages in the PP adipose tissue of OB/OW. Taken together, these anti-lipolytic, adipo/lipogenic, proliferative and anti-apoptotic effects in the PP adipose tissue of OB/OW likely result in fat mass expansion, conferring increased capacity of enlarged adipocytes to express adipokines and increase fatty acids supplies, which might impact the local energy and growth factors availability, thereby casting the local environment to allow cancer progression^{168, 251}. This environment in the PP adipose tissue of OB/OW may at least partially explain the described association of obesity and excess adiposity with the progression of PCa^{38, 252}.

We also found altered genes in PP adipose tissue of OB/OW that are involved in immunity and inflammation. Combined, the altered genes likely contribute towards an enhanced local mild immunoinflammatory environment, observed in PP adipose of OB/OW might further influence tissue remodeling and contribute towards tumor progression [44, 45]. In addition, the altered expression of *LEP* and *ANGPT1* genes, which we found in PP adipose tissue, may have roles beyond adipose tissue itself. Prostate cancers express the leptin receptor ²⁵³ and leptin staining is significantly increased in malignant prostates and poorly differentiated tumors ²⁵³. Also angiopoietin 1 and its receptor Tie-2 were found in both prostate tumor cells and capillaries ²⁵⁴, where they can induce sprouting angiogenesis ²⁵⁵. Herein, we showed that the PP adipose tissue gene expression profile of OB/OW subjects may contribute to increased local adiposity, mild immunoinflammatory environment and production of molecules with oncogenic potential (Figure 15).

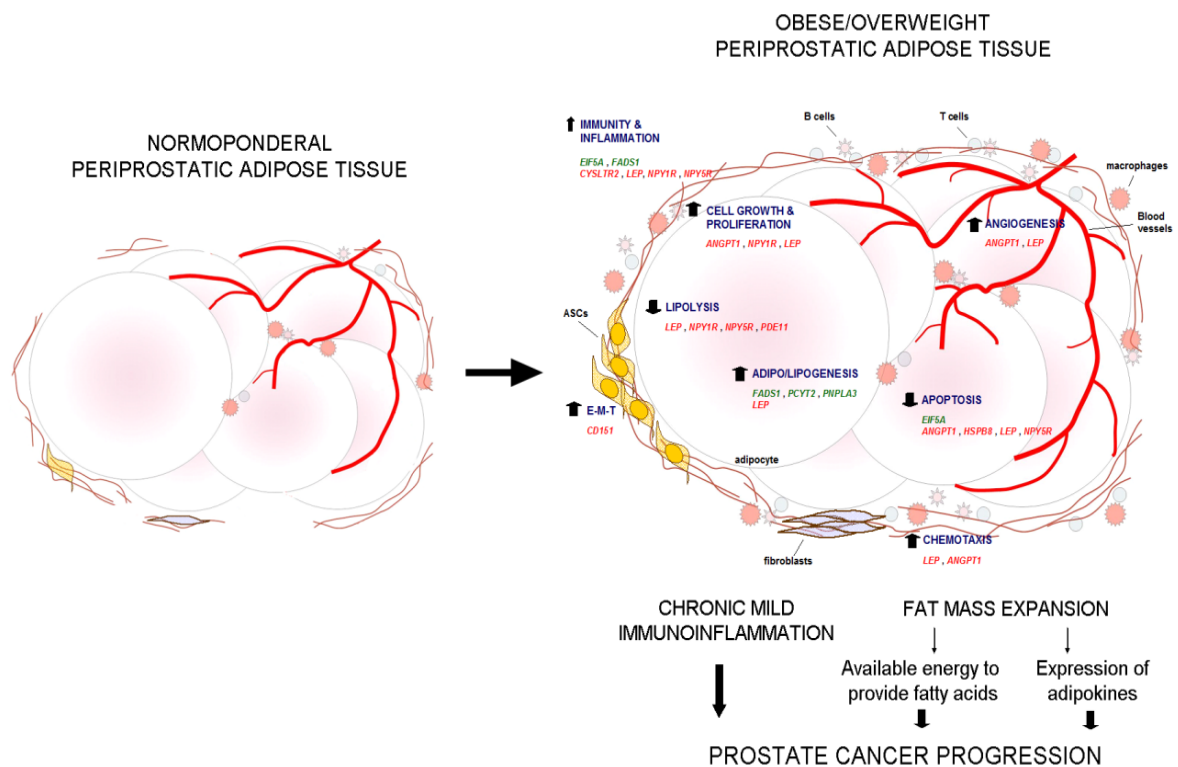


Figure 15. Resume of PP adipose tissue gene expression profile in obese/overweight. Genes with altered expression are associated with the regulation of functional biological processes. Altered function likely results in chronic mild immunoinflammatory response and fat mass expansion, which ultimately impact prostate cancer progression. ASCs, adipose-derived stem cells; E-M-T, epithelial-to-mesenchymal transition. Down-regulated genes are in green color and up-regulated are red.

The PP adipose tissue gene expression signature of men with PCa was determined, in order to find if functional alterations associate with PCa besides the previously reported PP thickness and protein measurements [9-12]. Our findings revealed that altered gene networks pertained to cell cycle and proliferation regulation. These

findings support a positive cell cycle regulation and permissive stimulus for growth and proliferation of PP adipose tissue cells. Cumulatively, pro-apoptotic genes were down-regulated, with canonical analysis showing involvement of the p53 pathway in adipose tissue of cancer subjects. Here, we also show altered expression of genes that associate with cellular differentiation of overall, adipocyte and endothelial lineages²⁵⁶⁻²⁵⁹. Thus, particularly the adipocyte and vascular biology of PP adipose tissue seem to be committed towards the differentiated state in men with PCa. Consistent with this, the microRNA *mir-let-7-a2* was overexpressed in the PP adipose tissue of PCa patients. The involvement of the *let-7* micro RNA in adipocyte differentiation has been described²⁶⁰. Taken together, the impact of these gene expression results on cell cycle and proliferation, in apoptosis and differentiation of PP adipose tissue cellular components supports fat mass accrual, which agrees with findings showing increased PP fat pad thickness in PCa patients^{125, 261}. Furthermore, bulky adipocytes predispose to increased adipokine secretion and availability of fatty acids, which may influence PCa progression^{168, 251}.

The complement system is important in immunosurveillance against tumors, albeit malignant cells are usually resistant to complement-mediated lysis^{262, 263}. Our finding of *CFH* and *CFHR1* up-regulation in PP adipose tissue of subjects with PCa suggests an eventual increased inhibitory modulation of the complement activity in prostate tumor cells and evasion to attack. The altered genes in PP adipose tissue with a role in inflammation and immunity might contribute to an environment with immunological cellular dormancy and reduced immunosurveillance, which may facilitate PCa progression (Figure 16).

The gene expression signature of PP adipose tissue from PCa patients seems to evidence altered gene expression across distinct cell types, with repercussion in stimulus for cell cycle regulation, cell proliferation and differentiation, and anti-apoptosis. Additionally, we found altered genes involved in immunological cell dormancy and reduced immunosurveillance, namely complement-related genes. Our findings suggest that PP adipose tissue gene expression profile of both OB/OW and PCa subjects is likely to cause a local environment favorable to PCa progression.

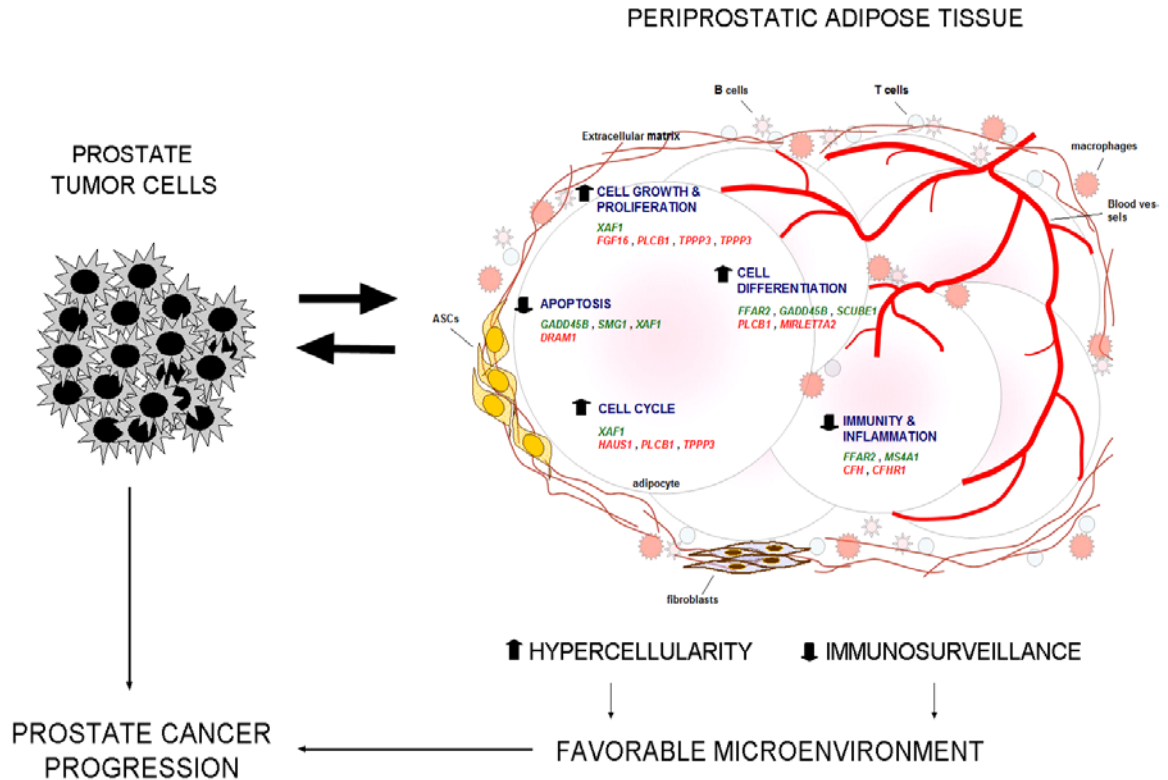


Figure 16. Resume of gene expression profile in periprostatic adipose tissue of patients with prostate cancer. Periprostatic adipose tissue of patients bearing prostate cancer likely reveals the interactions between tumor cells and adipose tissue cells. The functional repercussions of altered genes in patients with prostate cancer prompt periprostatic adipose tissue to increased hypercellularity and decreased immunosurveillance. The resulting influence of this favorable microenvironment is to foster prostate cancer progression. ASCs, adipose-derived stem cells; down-regulated genes are in green color whereas up-regulated are red.

4.2.3. Human periprostatic adipose tissue as a potential source of progenitor stem cells to prostate cancer (PAPER XII)

Recently, adipose tissue was found to contain also progenitor $CD45^{-}/CD34^{+}/CD31^{-}/CD146^{-}$ cells (ASC), which are highly represented in adipose tissue stromal vascular fraction (SVF), and display multipotency and proliferative capacity^{62, 136, 264}. One recently published study demonstrated that the neighboring adipose tissue considerably contributes with ASC to tumor vascular and fibrovascular stroma²⁶⁵, while another work found that different fat depots possess unique capacities to promote tumor growth²⁶⁶. Hence, we sought to investigate the accumulation of ASC in human PP adipose tissue, and to evaluate if these cells were found in the prostate of cancer or BPH patients.

First, we evaluated *PCA3* mRNA transcripts in adipose tissue samples, since it informs about the presence of prostate tumor cells. The tumor samples used for ASC

quantification from PCa patients overexpressed *PCA3* (659-fold, $P=0.011$), thus confirming tumor samples were representative of malignancy areas.

When comparing the amount of ASC between adipose tissue from distinct anatomic origins, we found it was significantly increased in PP versus VIS depot ($P<0.0001$), even after stratified analyses by prostatic disease (BPH, $P=0.001$; PCa, $P=0.012$) (Figure 17A). Interestingly, in PP adipose tissue the quantity of ASC was significantly lower for PCa compared with BPH patients ($P=0.023$), whereas the inverse was observed in VIS adipose tissue ($P=0.012$) (Figure 17B). To strengthen these findings, we evaluated adipsin (*CFD*) mRNA transcripts abundance in adipose tissue, whose expression was shown to be highly up-regulated in ASC. In agreement with figure 17A, *CFD* was overexpressed by 2-fold in PP compared to VIS adipose tissue ($P=0.008$) (Figure 17C). Figure 17D schematically represents the relative ASC frequencies found in adipose depots, according to prostatic disease.

These findings suggest unique progenitor cell population amounts residing in PP adipose tissue and differences between anatomic adipose depots, which has been previously appreciated for other anatomic origins^{266, 267}.

We hypothesize that the hypoxia, inflammation and oxidative stress conditions arising over the course of prostatic gland cancerous or hyperplasic development^{268, 269}, may induce recruitment of ASC to the involving PP adipose tissue from blood and eventually other fat depots or stimulate their local proliferation. The most accessible option for tumors that necessitate overcoming growth demands is to exploit resources in close proximity to the site of tumor development²⁶⁵. In the case of PCa, the enveloping PP adipose tissue may be such a reservoir of ASC. However, local recruitment of these cells towards the tumor may result, as we observed, in reduced amount of ASC in the PP fat depot compared with BPH. Whether ASC migrate from PP adipose tissue into the prostate through the visceral pelvis fascia, which is conceivable in light of their previously described capacity to move and ability to produce matrix proteinases²⁷⁰ or through the blood, is an interesting issue that remains to be established.

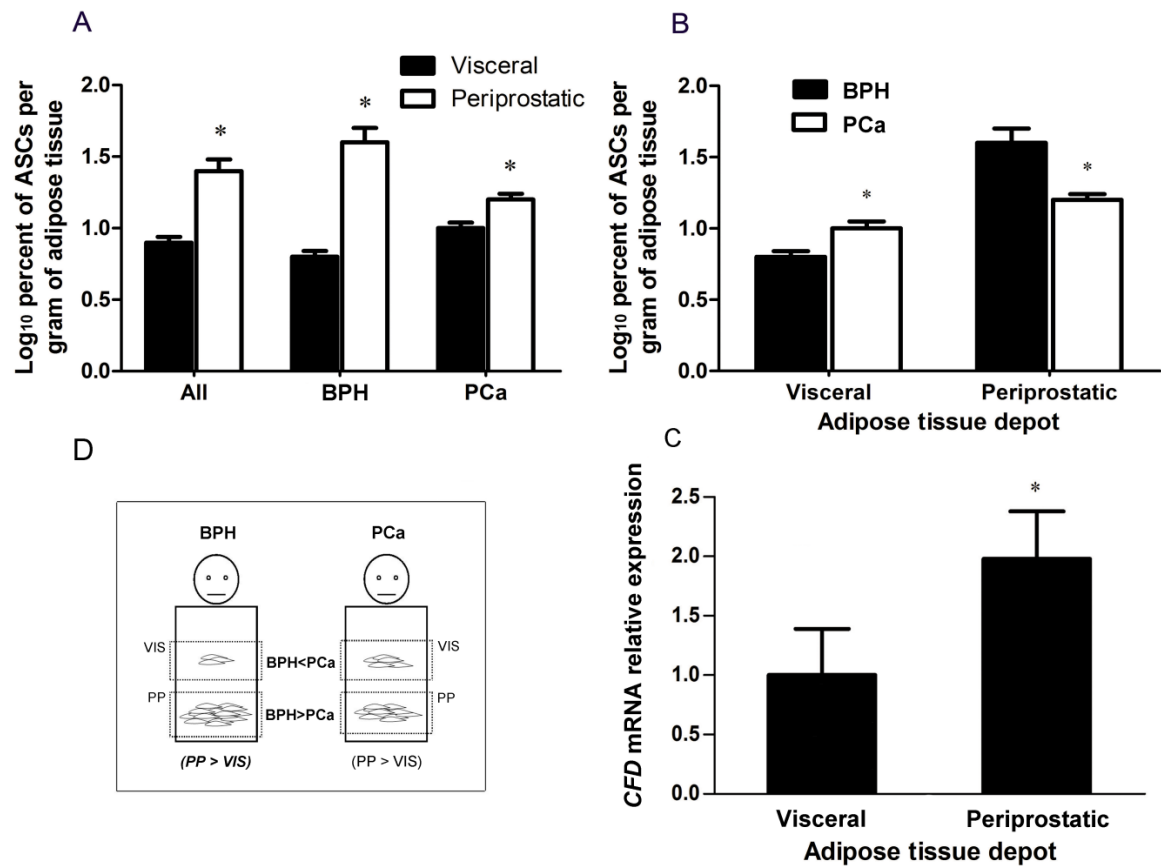


Figure 17. Adipose stem cells quantity and *CFD* gene expression in periprostatic and visceral adipose tissue. A. Comparison of log₁₀ percent of adipose stem cells per gram of tissue between visceral and PP fat depots, overall and for BPH and PCa groups (* $P < 0.05$); B. Differences in the log₁₀ percent of adipose stem cells per gram of adipose tissue between BPH and PCa patients within each fat depot (* $P < 0.05$); C. Comparison of adipisin (*CFD*) gene expression between PP and VIS depots (* $P = 0.008$); D. Scheme depicting the relative quantities of adipose stem cells among adipose depots and according to prostatic disease. Bars represent mean $\pm$ S.E.M. from 12 men (BPH, $n = 6$ and PCa, $n = 6$). PCa, prostate cancer; BPH, benign prostatic hyperplasia; VIS, visceral adipose tissue; PP, periprostatic adipose tissue.

In the prostate, the amount of ASC cells was increased in cancers compared with BPH ($P = 0.043$) (Figure 18A). Furthermore, *CFD* expression was 10-fold increased in prostate tumors from cancer patients ($P = 0.186$), whereas in isolated ASC (sorted cells) from the prostate it was also increased in tumors compared to prostatic tissue of BPH ($P = 0.192$) (Figure 18B). Moreover, the normalized *CFD* expression was well correlated with the amount of ASC cells in the prostatic tissue (Spearman coefficient, $r = -0.71$, $P = 0.047$). CD133 is a specific stem and progenitor cell marker for a variety of cells, albeit it is not expressed in ASC. Interestingly, *CD133* expression was up-regulated in prostatic tumors of cancer patients by 3.6-fold ($P = 0.025$) (Figure 18C), but not in isolated ASC (sorted cells) (Figure 18D).

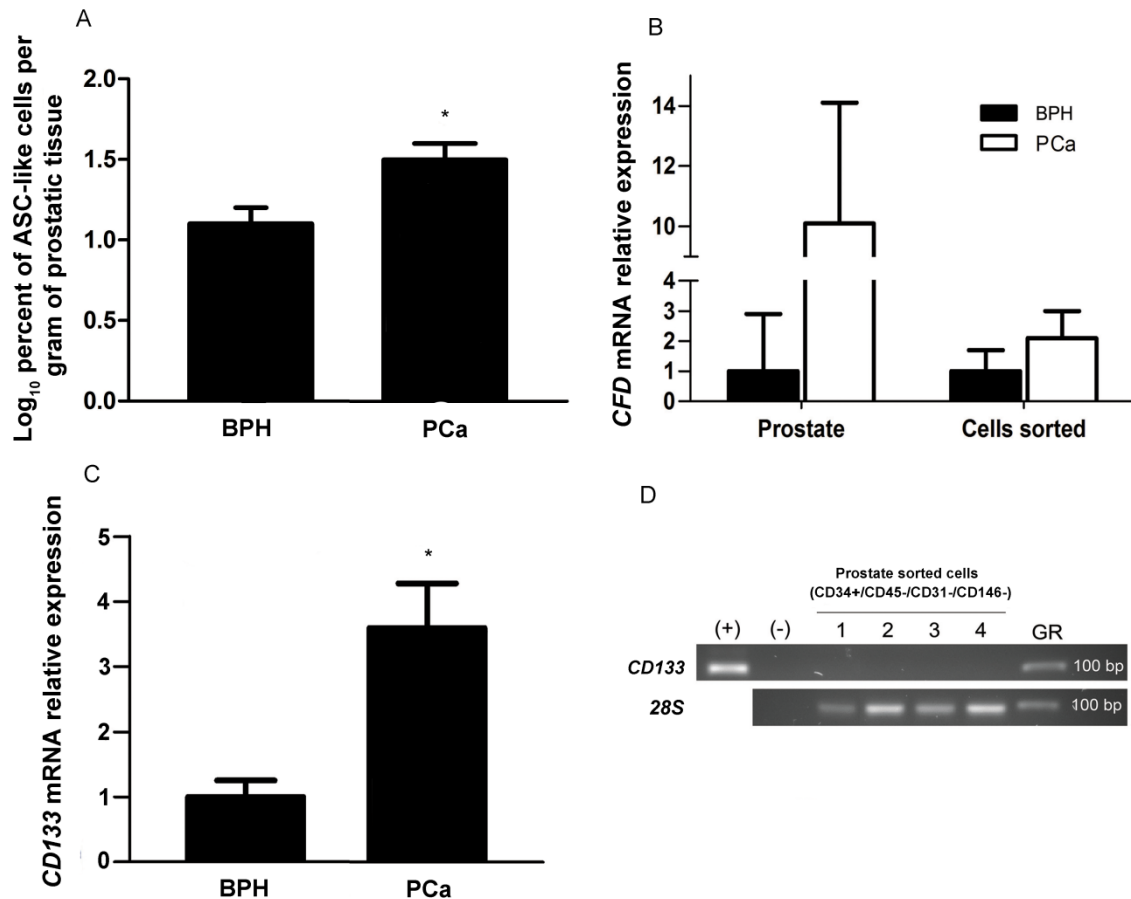


Figure 18. Quantification of adipose stem cells in prostatic tissue and gene expression in prostate tissue and $CD45^+/CD34^+/CD31^+/CD146^-$ sorted cells. A. Log₁₀-transformed amount of adipose stem cells per gram of prostatic tissue in BPH and PCa patients (* $P=0.021$); B. Adipsin gene (*CFD*) expression in prostatic tissue and $CD45^+/CD34^+/CD31^+/CD146^-$ cells sorted from prostate samples, according to prostatic disease (BPH, $P=0.186$ and PCa, $P=0.192$). C. Comparison of *CD133* gene expression in prostatic tissue between BPH and PCa (* $P<0.025$). Bars represent mean $\pm$ S.E.M. from 12 men (BPH, $n=6$ and prostate cancer, $n=6$). D. Representative gel electrophoresis of target *CD133* and reference *28S* qPCR products from $CD45^+/CD34^+/CD31^+/CD146^-$ cells sorted from prostate cell suspensions (HBP, 1-2 and PCa, 3-4). (+) positive control, using RNA from prostate tumor sample; (-) negative control; GR, gene ruler. PCa, prostate cancer; BPH, benign prostatic hyperplasia.

In the peripheral blood, although the ASC cell population was barely detectable, we found a trend for increased ASC-like cells in cancer compared to BPH patients (0.05 ± 0.01 and 0.03 ± 0.005 , respectively, $P=0.068$).

In the prostatic tissue, we found higher amounts of $CD45^+/CD34^+/CD31^+/CD146^-$ progenitor cells in tumors, whereas a trend for increased number in circulation was observed for cancer patients compared with BPH subjects. Our findings in the human prostate are concordant with previous results in murine models, which demonstrated that ASC from adipose tissue home to tumors, through the modulation of the stromal derived factor – 1/C-X-C chemokine receptor type 4 axis^{140, 141, 271}. This axis was shown to be

highly activated both in PCa and benign hyperplasia ^{272, 273}, driving the recruitment of ASC.

In order to analyze the simultaneous influence of being overweight/obesity and bearing cancer in ASC frequencies, we compared subjects with these characteristics (OB/OW + PCa) to the other patients combined into one group (Others) (Figure 19).

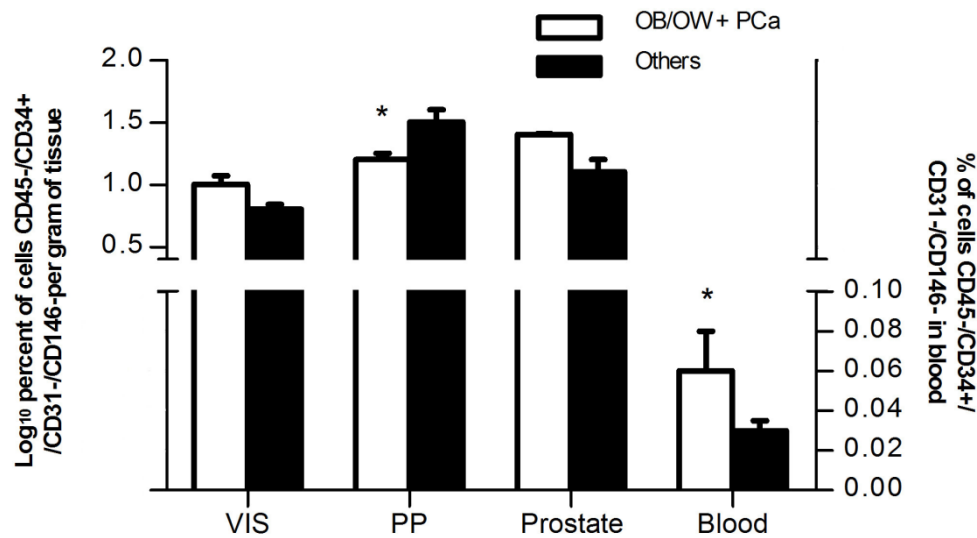


Figure 19. CD45⁺/CD34⁺/CD31⁻/CD146⁻ progenitor cells in adipose tissue, prostate and peripheral blood and prostate cancer-obesity phenotype. OB/OW + PCa, includes obese/overweight (OB/OW) patients with prostate cancer (PCa) (n=4); Others, includes OB/OW (n=5) and lean controls (n=1), and lean patients with cancer (n=2). PP, periprostatic adipose tissue; VIS, visceral adipose tissue. *P<0.05 in comparison with Others.

As initially suggested ⁶², a recent study confirmed increased frequency of progenitor CD34⁺ cells in the circulation of obese (BM $\geq$ 30 kg/m²) patients with colorectal cancer ²⁷⁴. A similar analysis was conducted in our group of patients, although the low number of obese patients prompted us to use a distinct cutoff (overweight and obese subjects with BM $\geq$ 25 kg/m²), which has been reported before now ^{37, 275}. We found a significantly higher frequency of CD45⁺/CD34⁺/CD31⁻/CD146⁻ progenitors in the circulation of OB/OW patients with PCa, than in lean patients with PCa and OB/OW controls. Since we also observed that ASC frequency is decreased in PP adipose tissue and increased in the prostate of OB/OW men with PCa, their increase in blood may account for one possible route for ASC migration. Some reports indicate that ASC play a role in obesity and are regulated by diet and obesity ^{267, 276}.

To our knowledge, no prior reports evaluated the ASC pool in human prostate and PP adipose tissue. If confirmed by further studies, the source of tumor stroma from adipose tissue depots that lie in close proximity to cancer foci, besides revealing a

mechanistic link between obesity and cancer progression may identify surgical and medical interventions to disrupt tumor-stroma interactions.

5. CONCLUSIONS

5.1. OBESITY, BODY COMPOSITION AND GERMLINE GENETIC VARIANTS OF ADIPOKINE PATHWAYS IN PROSTATE CANCER RISK AND PROGNOSIS

In a relatively large sample of over 1000 subjects, we identified SNPs in genes of adipokine pathways that are associated with prostate cancer (PCa) development and with a more aggressive phenotype. Further, the inclusion of single nucleotide polymorphisms in an innovative risk score model significantly improved the performance of PSA and age to predict overall PCa and high-grade PCa risk in biopsed men.

In another study, we validated the inclusive genetic risk score and confirmed that the adipokine-based genetic risk score performs well independently of body mass index (BMI) and body fat percent (BFP), although only BFP-determined obese men had genetic risk score-associated risk. Noteworthy, in this study we uncovered for the first time, in a population of elderly men submitted to prostate biopsy, that BMI has limited diagnostic performance to appropriately identify subjects with excess body fatness, particularly in intermediate ranges of body weight. Actually, individuals classified by BFP as overweight or obese are at increased risk for developing PCa overall and high-grade PCa, even within the group of subjects misclassified as normal through BMI. Direct and simple measures of body fatness and measures of body fat distribution may be helpful in stratifying men at risk for PCa and aggressive disease; and the eventual combination of these measurements with other predictive and prognostic tools, such as genetic markers, may enhance the capacity to accurately predict PCa and provide preventive and therapeutic guidance.

Among men treated with androgen deprivation therapy (ADT), our report was the first to examine genetic polymorphisms in adipokine pathways and to reveal it might influence outcome prediction. Poor prognostic germline polymorphisms may be used along with known clinical factors to foster the capacity to prognosticate clinical course after ADT initiation, thereby helping clinical reasoning. Present findings also encourage the need for clarifying the effectiveness of personalized therapies targeted to adiponectin, tumoral necrosis factor and leptin pathways in CRPCa. If confirmed, our findings might help targeting patients with precocious ADT failure and mortality, and at risk for metastasis development in the course of disease.

Although these are single reports, they represent a proof of principle that individual germline variation in adipokine pathways has impact not only on the risk of developing PCa but also on the subsequent response to therapy. Furthermore, in light of the rising

incidence of obesity worldwide, identifying obesity as a risk factor for PCa and aggressive disease is important, since it may be one of the few modifiable risk factors for PCa, therefore, with impact in the implementation of new adjuvant therapies, reinforcing public health guidelines and the definition of chemoprevention strategies.

5.2. MECHANISMS CAUSALLY INVOKED IN THE ASSOCIATION BETWEEN OBESITY AND PROSTATE CANCER

If the tissues where tumor cells exist provide the missing extrinsic signals, then cells will proliferate and acquire an invasive phenotype, which may lead to metastasis. Whole periprostatic (PP) fat, seems to warrant the necessary factors to induce a specific microenvironment for PCa tumor cells, which ultimately may result, as we found, in tumor cell survival, increased motility and availability of extracellular proteases. Besides the observed effect of adipose tissue derived factors in tumor proliferation, we report that tumor factors can regulate expression of adipokines with oncologic potential (osteopontin, tumoral necrosis factor alpha, interleukin 6 and adiponectin), matrix metalloproteinase (MMP) activity and mitochondrial DNA in PP adipose tissue. Thus, in the context of extra-prostatic disease, the adipose tissue is in close contact with tumor cells and may acquire a phenotype favorable to aggressive PCa. These findings support the existence of crosstalk between PP adipose tissue cells and prostate tumor cells. Furthermore, the increased production of MMPs by PP adipose tissue can fuel invasive and metastatic behavior of PP fat-infiltrating PCa cells.

In another study, first in human PP adipose tissue, obesity/overweight- and PCa-associated gene expression changes were analyzed by microarrays. We found that obesity and excess adiposity modifies the expression of PP adipose tissue genes to ultimately foster fat mass growth. In patients with PCa the expression profile of PP adipose tissue may result in a tumor microenvironment with hypercellularity and reduced immunosurveillance. Interestingly, this data suggests that environmental factors such as body fatness may regulate the gene expression pattern even among small fat depots and that there is a distinct PP adipose tissue expression profile in cancer patients. Both findings are liable to promote a favorable environment for PCa progression.

We reported for the first time in humans, that adipose tissue, particularly the PP fat depot, may represent a source of progenitor cells with potential to migrate to prostate tumor stroma. The present study further supports adipose-derived stem cells migration through the blood in obese men with PCa, although contiguous paths should be considered. If confirmed and extended by following studies, these findings may impact PCa therapeutic interventions aimed at disrupting tumor-stroma crosstalk.

These previously unrecognized findings suggest a role for PP adipose tissue in PCa progression, and as a candidate explanatory mechanism to the causally invoked association between obesity and aggressive PCa. After confirmation by larger studies, these findings may impact PCa therapeutic targets and reveal new opportunities for the implementation or reinforcement of chemoprevention strategies and healthy lifestyles in obesity-associated aggressive PCa.

6. FUTURE STUDIES

Future studies are warranted, to confirm and further explore the relevance of body composition and body fat distribution in prostate cancer etiology and progression, and to add information to allow the comprehension of adipose tissue biological mechanisms involved in carcinogenesis and aggressiveness. In the course of our work many questions were raised to be addressed in future studies:

- Verify in surgical specimens whether prostate tumors in contact with periprostatic adipose tissue have distinct molecules expressed, known to be relevant for prostate cancer; and evaluate retrospectively if those patients with invading tumors to the surrounding adipose tissue have worst prognosis;

- In light of recent findings concerning the role of adipocyte-driven factors in triggering mesenchymal transition in cancer cells, it would be challenging to evaluate the expression of epithelial-to-mesenchymal markers in prostate cancer cells within the prostate and extra-prostatic where they contact with adipose tissue. This line of research could shed light and establish the role of periprostatic adipose tissue in prostate cancer aggressiveness and metastasis;

- The majority of patients with prostate cancer do not die from the primary tumor, but from complications following skeletal metastasis. Once tumors metastasize to the bone, the disease is largely incurable, and current treatments are mainly palliative. Obesity, bone marrow adiposity and adipokines can regulate cancer-induced bone disease, and could be key mediators in disease pathogenesis. A molecular and cellular study of bone marrow-derived adipocytes would reveal interesting clues in a puzzling mechanism such as bone metastasis;

- Follow the clues from the microarrays study and search for the mechanistic role of some altered genes. Immunohistochemistry in surgical specimens and gene expression experiments in *in vitro* coculture systems;

- Study in a more comprehensive qPCR target-gene expression in periprostatic adipose tissue target genes encoding new adipokines, that may add information to the eventual local microenvironment surrounding the prostate;

- After this work, the obesity-cancer association remains an open area of research to explore, using the methods and know how presented here and others, in other cancer models. Particularly interesting, although still without other than epidemiological evidence, would be renal cancer.

- Macrophages adopt an M2 phenotype after exposure to prostate cancer conditioned medium. It would be interesting to observe the outcome of adding adipose tissue medium together with macrophages medium to tumor cells. Alternatively, a coculture system including all three cell types (adipocytes, macrophages and prostate tumor cells) would certainly mimic the microenvironment conditions found in periprostatic adipose tissue.

7. REFERENCES

1. Boyle P, Ferlay J. Cancer incidence and mortality in Europe, 2004. *Ann Oncol* 2005;16:481-8.
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71-96.
3. Quinn M, Babb P. Patterns and trends in prostate cancer incidence, survival, prevalence and mortality. Part I: international comparisons. *BJU Int* 2002;90:162-73.
4. Heidenreich A, Bellmunt J, Bolla M, et al. EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. *Eur Urol* 2011;59:61-71.
5. Andriole GL, Crawford ED, Grubb RL, 3rd, et al. Mortality results from a randomized prostate-cancer screening trial. *N Engl J Med* 2009;360:1310-9.
6. Schroder FH, Hugosson J, Roobol MJ, et al. Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med* 2009;360:1320-8.
7. Damber JE, Aus G. Prostate cancer. *Lancet* 2008;371:1710-21.
8. Vickers AJ, Roobol MJ, Lilja H. Screening for Prostate Cancer: Early Detection or Overdetection? *Annu Rev Med* 2011.
9. Etzioni R, Penson DF, Legler JM, et al. Overdiagnosis due to prostate-specific antigen screening: lessons from U.S. prostate cancer incidence trends. *J Natl Cancer Inst* 2002;94:981-90.
10. Carter HB. Assessing risk: does this patient have prostate cancer? *J Natl Cancer Inst* 2006;98:506-7.
11. Draisma G, Boer R, Otto SJ, et al. Lead times and overdetection due to prostate-specific antigen screening: estimates from the European Randomized Study of Screening for Prostate Cancer. *J Natl Cancer Inst* 2003;95:868-78.
12. Roddam AW, Duffy MJ, Hamdy FC, et al. Use of prostate-specific antigen (PSA) isoforms for the detection of prostate cancer in men with a PSA level of 2-10 ng/ml: systematic review and meta-analysis. *Eur Urol* 2005;48:386-99; discussion 98-9.
13. Vickers AJ, Gupta A, Savage CJ, et al. A panel of kallikrein marker predicts prostate cancer in a large, population-based cohort followed for 15 years without screening. *Cancer Epidemiol Biomarkers Prev* 2011;20:255-61.
14. Roobol MJ, Schroder FH, van Leeuwen P, et al. Performance of the prostate cancer antigen 3 (PCA3) gene and prostate-specific antigen in prescreened men: exploring the value of PCA3 for a first-line diagnostic test. *Eur Urol* 2010;58:475-81.
15. Salami SS, Schmidt F, Laxman B, et al. Combining urinary detection of TMPRSS2:ERG and PCA3 with serum PSA to predict diagnosis of prostate cancer. *Urol Oncol* 2011.
16. Stenman UH, Abrahamsson PA, Aus G, et al. Prognostic value of serum markers for prostate cancer. *Scand J Urol Nephrol Suppl* 2005:64-81.
17. Vickers AJ, Cronin AM, Bjork T, et al. Prostate specific antigen concentration at age 60 and death or metastasis from prostate cancer: case-control study. *BMJ* 2010;341:c4521.
18. Scardino PT, Weaver R, Hudson MA. Early detection of prostate cancer. *Hum Pathol* 1992;23:211-22.
19. Kabalin JN, McNeal JE, Price HM, Freiha FS, Stamey TA. Unsuspected adenocarcinoma of the prostate in patients undergoing cystoprostatectomy for other causes: incidence, histology and morphometric observations. *J Urol* 1989;141:1091-4; discussion 3-4.
20. De Marzo AM, Platz EA, Sutcliffe S, et al. Inflammation in prostate carcinogenesis. *Nat Rev Cancer* 2007;7:256-69.
21. Chung LW, Baseman A, Assikis V, Zhau HE. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J Urol* 2005;173:10-20.
22. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer* 2001;1:34-45.
23. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010;363:411-22.

24. de Bono JS, Logothetis CJ, Molina A, et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011;364:1995-2005.
25. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003;348:1625-38.
26. Finucane MM, Stevens GA, Cowan MJ, et al. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. *Lancet* 2011;377:557-67.
27. Fesinmeyer MD, Gulati R, Zeliadt S, Weiss N, Kristal AR, Etzioni R. Effect of population trends in body mass index on prostate cancer incidence and mortality in the United States. *Cancer Epidemiol Biomarkers Prev* 2009;18:808-15.
28. Quinn M, Babb P. Patterns and trends in prostate cancer incidence, survival, prevalence and mortality. Part II: individual countries. *BJU Int* 2002;90:174-84.
29. Kuczmarski RJ, Flegal KM, Campbell SM, Johnson CL. Increasing prevalence of overweight among US adults. The National Health and Nutrition Examination Surveys, 1960 to 1991. *JAMA* 1994;272:205-11.
30. Popkin BM. The nutrition transition in low-income countries: an emerging crisis. *Nutr Rev* 1994;52:285-98.
31. Popkin BM. Comment: obesity patterns and the nutrition transition in China. *Arch Intern Med* 1994;154:2249, 53.
32. Freedland SJ, Platz EA. Obesity and prostate cancer: making sense out of apparently conflicting data. *Epidemiol Rev* 2007;29:88-97.
33. Hsing AW, Sakoda LC, Chua S, Jr. Obesity, metabolic syndrome, and prostate cancer. *Am J Clin Nutr* 2007;86:s843-57.
34. MacInnis RJ, English DR. Body size and composition and prostate cancer risk: systematic review and meta-regression analysis. *Cancer Causes Control* 2006;17:989-1003.
35. Cao Y, Ma J. Body mass index, prostate cancer-specific mortality, and biochemical recurrence: a systematic review and meta-analysis. *Cancer Prev Res (Phila)* 2011;4:486-501.
36. Ploussard G, de la Taille A, Bayoud Y, et al. The risk of upstaged disease increases with body mass index in low-risk prostate cancer patients eligible for active surveillance. *Eur Urol* 2012;61:356-62.
37. Capitanio U, Suardi N, Briganti A, et al. Influence of obesity on tumour volume in patients with prostate cancer. *BJU Int* 2011.
38. Freedland SJ, Banez LL, Sun LL, Fitzsimons NJ, Moul JW. Obese men have higher-grade and larger tumors: an analysis of the duke prostate center database. *Prostate Cancer Prostatic Dis* 2009;12:259-63.
39. Chu DI, De Nunzio C, Gerber L, et al. Predictive value of digital rectal examination for prostate cancer detection is modified by obesity. *Prostate Cancer Prostatic Dis* 2011;14:346-53.
40. Banez LL, Hamilton RJ, Partin AW, et al. Obesity-related plasma hemodilution and PSA concentration among men with prostate cancer. *JAMA* 2007;298:2275-80.
41. Baillargeon J, Pollock BH, Kristal AR, et al. The association of body mass index and prostate-specific antigen in a population-based study. *Cancer* 2005;103:1092-5.
42. Fowke JH, Matthews CE. PSA and body composition by dual X-ray absorptiometry (DXA) in NHANES. *Prostate* 2010;70:120-5.
43. Freedland SJ, Platz EA, Presti JC, Jr., et al. Obesity, serum prostate specific antigen and prostate size: implications for prostate cancer detection. *J Urol* 2006;175:500-4; discussion 4.
44. Fowke JH, Motley SS, Wills M, et al. Prostate volume modifies the association between obesity and prostate cancer or high-grade prostatic intraepithelial neoplasia. *Cancer Causes Control* 2007;18:375-84.
45. Fontaine KR, Heo M, Allison DB. Obesity and prostate cancer screening in the USA. *Public Health* 2005;119:694-8.
46. Scales CD, Jr., Curtis LH, Norris RD, Schulman KA, Dahm P, Moul JW. Relationship between body mass index and prostate cancer screening in the United States. *J Urol* 2007;177:493-8.

47. Parekh N, Lin Y, Dipaola RS, Marcella S, Lu-Yao G. Obesity and prostate cancer detection: insights from three national surveys. *Am J Med* 2010;123:829-35.
48. Rohrmann S, Shiels MS, Lopez DS, et al. Body fatness and sex steroid hormone concentrations in US men: results from NHANES III. *Cancer Causes Control* 2011;22:1141-51.
49. Gross M, Ramirez C, Luthringer D, et al. Expression of androgen and estrogen related proteins in normal weight and obese prostate cancer patients. *Prostate* 2009;69:520-7.
50. Romero-Corral A, Lopez-Jimenez F, Sierra-Johnson J, Somers VK. Differentiating between body fat and lean mass-how should we measure obesity? *Nat Clin Pract Endocrinol Metab* 2008;4:322-3.
51. Romero-Corral A, Somers VK, Sierra-Johnson J, et al. Accuracy of body mass index in diagnosing obesity in the adult general population. *Int J Obes (Lond)* 2008;32:959-66.
52. Gomez-Ambrosi J, Silva C, Galofre JC, et al. Body adiposity and type 2 diabetes: increased risk with a high body fat percentage even having a normal BMI. *Obesity (Silver Spring)* 2011;19:1439-44.
53. Gomez-Ambrosi J, Silva C, Galofre JC, et al. Body mass index classification misses subjects with increased cardiometabolic risk factors related to elevated adiposity. *Int J Obes (Lond)* 2011.
54. MacInnis RJ, English DR, Gertig DM, Hopper JL, Giles GG. Body size and composition and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev* 2003;12:1417-21.
55. Wallstrom P, Bjartell A, Gullberg B, Olsson H, Wirfalt E. A prospective Swedish study on body size, body composition, diabetes, and prostate cancer risk. *Br J Cancer* 2009;100:1799-805.
56. von Hafe P, Pina F, Perez A, Tavares M, Barros H. Visceral fat accumulation as a risk factor for prostate cancer. *Obes Res* 2004;12:1930-5.
57. Mistry T, Digby JE, Desai KM, Randeva HS. Obesity and prostate cancer: a role for adipokines. *Eur Urol* 2007;52:46-53.
58. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008;8:915-28.
59. Hsing AW, Reichardt JK, Stanczyk FZ. Hormones and prostate cancer: current perspectives and future directions. *Prostate* 2002;52:213-35.
60. Renehan AG, Roberts DL, Dive C. Obesity and cancer: pathophysiological and biological mechanisms. *Arch Physiol Biochem* 2008;114:71-83.
61. de Ferranti S, Mozaffarian D. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clin Chem* 2008;54:945-55.
62. Zhang Y, Bellows CF, Kolonin MG. Adipose tissue-derived progenitor cells and cancer. *World J Stem Cells* 2010;2:103-13.
63. Langeberg WJ, Isaacs WB, Stanford JL. Genetic etiology of hereditary prostate cancer. *Front Biosci* 2007;12:4101-10.
64. Lindstrom S, Schumacher F, Siddiq A, et al. Characterizing associations and SNP-environment interactions for GWAS-identified prostate cancer risk markers--results from BPC3. *PLoS One* 2011;6:e17142.
65. Febbo PG. Genomic approaches to outcome prediction in prostate cancer. *Cancer* 2009;115:3046-57.
66. Aly M, Wiklund F, Xu J, et al. Polygenic risk score improves prostate cancer risk prediction: results from the stockholm-1 cohort study. *Eur Urol* 2011;60:21-8.
67. Moore SC, Leitzmann MF, Albanes D, et al. Adipokine genes and prostate cancer risk. *Int J Cancer* 2009;124:869-76.
68. Ribeiro R, Vasconcelos A, Costa S, et al. Overexpressing leptin genetic polymorphism (-2548 G/A) is associated with susceptibility to prostate cancer and risk of advanced disease. *Prostate* 2004;59:268-74.
69. Kote-Jarai Z, Singh R, Durocher F, et al. Association between leptin receptor gene polymorphisms and early-onset prostate cancer. *BJU Int* 2003;92:109-12.
70. Wang MH, Helzlsouer KJ, Smith MW, et al. Association of IL10 and other immune response- and obesity-related genes with prostate cancer in CLUE II. *Prostate* 2009;69:874-85.

71. Silha JV, Krsek M, Sucharda P, Murphy LJ. Angiogenic factors are elevated in overweight and obese individuals. *Int J Obes (Lond)* 2005;29:1308-14.
72. Gomez-Ambrosi J, Catalan V, Ramirez B, et al. Plasma osteopontin levels and expression in adipose tissue are increased in obesity. *J Clin Endocrinol Metab* 2007;92:3719-27.
73. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 2001;280:E745-51.
74. Park J, Euhus DM, Scherer PE. Paracrine and Endocrine Effects of Adipose Tissue on Cancer Development and Progression. *Endocr Rev* 2011.
75. Freedland SJ, Eastham J, Shore N. Androgen deprivation therapy and estrogen deficiency induced adverse effects in the treatment of prostate cancer. *Prostate Cancer Prostatic Dis* 2009;12:333-8.
76. Calabro F, Sternberg CN. Current indications for chemotherapy in prostate cancer patients. *Eur Urol* 2007;51:17-26.
77. Attar RM, Takimoto CH, Gottardis MM. Castration-resistant prostate cancer: locking up the molecular escape routes. *Clin Cancer Res* 2009;15:3251-5.
78. Ross RW, Oh WK, Xie W, et al. Inherited variation in the androgen pathway is associated with the efficacy of androgen-deprivation therapy in men with prostate cancer. *J Clin Oncol* 2008;26:842-7.
79. Hamada A, Sissung T, Price DK, et al. Effect of SLCO1B3 haplotype on testosterone transport and clinical outcome in caucasian patients with androgen-independent prostatic cancer. *Clin Cancer Res* 2008;14:3312-8.
80. Yang M, Xie W, Mostaghel E, et al. SLCO2B1 and SLCO1B3 may determine time to progression for patients receiving androgen deprivation therapy for prostate cancer. *J Clin Oncol* 2011;29:2565-73.
81. Lindstrom S, Adami HO, Balter KA, et al. Inherited variation in hormone-regulating genes and prostate cancer survival. *Clin Cancer Res* 2007;13:5156-61.
82. Bao BY, Pao JB, Huang CN, et al. Significant associations of prostate cancer susceptibility variants with survival in patients treated with androgen-deprivation therapy. *Int J Cancer* 2012;130:876-84.
83. Tsuchiya N, Wang L, Suzuki H, et al. Impact of IGF-I and CYP19 gene polymorphisms on the survival of patients with metastatic prostate cancer. *J Clin Oncol* 2006;24:1982-9.
84. Teixeira AL, Ribeiro R, Cardoso D, et al. Genetic polymorphism in EGF is associated with prostate cancer aggressiveness and progression-free interval in androgen blockade-treated patients. *Clin Cancer Res* 2008;14:3367-71.
85. Teixeira AL, Ribeiro R, Morais A, et al. Combined analysis of EGF+61G>A and TGFB1+869T>C functional polymorphisms in the time to androgen independence and prostate cancer susceptibility. *Pharmacogenomics J* 2009;9:341-6.
86. Ribeiro R, Lopes C, Medeiros R. Leptin and prostate: implications for cancer prevention--overview of genetics and molecular interactions. *Eur J Cancer Prev* 2004;13:359-68.
87. Wegiel B, Evans S, Hellsten R, Otterbein LE, Bjartell A, Persson JL. Molecular pathways in the progression of hormone-independent and metastatic prostate cancer. *Curr Cancer Drug Targets* 2010;10:392-401.
88. Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, Burrell MA. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 2001;280:E827-47.
89. Matsuzawa Y, Funahashi T, Nakamura T. Molecular mechanism of metabolic syndrome X: contribution of adipocytokines adipocyte-derived bioactive substances. *Ann N Y Acad Sci* 1999;892:146-54.
90. Rehman J, Traktuev D, Li J, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004;109:1292-8.

91. Gabrielsson BG, Johansson JM, Jennische E, et al. Depot-specific expression of fibroblast growth factors in human adipose tissue. *Obes Res* 2002;10:608-16.
92. Baillargeon J, Rose DP. Obesity, adipokines, and prostate cancer (review). *Int J Oncol* 2006;28:737-45.
93. Kim HJ, Lee YS, Won EH, et al. Expression of resistin in the prostate and its stimulatory effect on prostate cancer cell proliferation. *BJU Int* 2011;108:E77-83.
94. Huang CY, Yu HS, Lai TY, et al. Leptin increases motility and integrin up-regulation in human prostate cancer cells. *J Cell Physiol* 2011;226:1274-82.
95. Gruen ML, Hao M, Piston DW, Hasty AH. Leptin requires canonical migratory signaling pathways for induction of monocyte and macrophage chemotaxis. *Am J Physiol Cell Physiol* 2007;293:C1481-8.
96. Zheng Q, Dunlap SM, Zhu J, et al. Leptin deficiency suppresses MMTV-Wnt-1 mammary tumor growth in obese mice and abrogates tumor initiating cell survival. *Endocr Relat Cancer* 2011;18:491-503.
97. Iyengar P, Combs TP, Shah SJ, et al. Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. *Oncogene* 2003;22:6408-23.
98. Mistry T, Digby JE, Desai KM, Randeva HS. Leptin and adiponectin interact in the regulation of prostate cancer cell growth via modulation of p53 and bcl-2 expression. *BJU Int* 2008;101:1317-22.
99. Lamarre NS, Ruggieri MR, Sr., Braverman AS, Gerstein MI, Mydlo JH. Effect of obese and lean Zucker rat sera on human and rat prostate cancer cells: implications in obesity-related prostate tumor biology. *Urology* 2007;69:191-5.
100. Onuma M, Bub JD, Rummel TL, Iwamoto Y. Prostate cancer cell-adipocyte interaction: leptin mediates androgen-independent prostate cancer cell proliferation through c-Jun NH2-terminal kinase. *J Biol Chem* 2003;278:42660-7.
101. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2:161-74.
102. Lichtinghagen R, Musholt PB, Stephan C, et al. mRNA expression profile of matrix metalloproteinases and their tissue inhibitors in malignant and non-malignant prostatic tissue. *Anticancer Res* 2003;23:2617-24.
103. Chakrabarti S, Patel KD. Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. *Exp Lung Res* 2005;31:599-621.
104. Lin CY, Tsai PH, Kandaswami CC, et al. Matrix metalloproteinase-9 cooperates with transcription factor Snail to induce epithelial-mesenchymal transition. *Cancer Sci* 2011;102:815-27.
105. Allott EH, Lysaght J, Cathcart MC, et al. MMP9 expression in oesophageal adenocarcinoma is upregulated with visceral obesity and is associated with poor tumour differentiation. *Mol Carcinog* 2011.
106. Unal R, Yao-Borengasser A, Varma V, et al. Matrix metalloproteinase-9 is increased in obese subjects and decreases in response to pioglitazone. *J Clin Endocrinol Metab* 2010;95:2993-3001.
107. Bouloumie A, Sengenès C, Portolan G, Galitzky J, Lafontan M. Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. *Diabetes* 2001;50:2080-6.
108. McAllister SS, Gifford AM, Greiner AL, et al. Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell* 2008;133:994-1005.
109. Patel ST, Mistry T, Brown JE, et al. A novel role for the adipokine visfatin/pre-B cell colony-enhancing factor 1 in prostate carcinogenesis. *Peptides* 2010;31:51-7.
110. Adya R, Tan BK, Punna A, Chen J, Randeva HS. Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis. *Cardiovasc Res* 2008;78:356-65.

111. Varma V, Yao-Borengasser A, Bodles AM, et al. Thrombospondin-1 is an adipokine associated with obesity, adipose inflammation, and insulin resistance. *Diabetes* 2008;57:432-9.
112. Brown DA, Lindmark F, Stattin P, et al. Macrophage inhibitory cytokine 1: a new prognostic marker in prostate cancer. *Clin Cancer Res* 2009;15:6658-64.
113. Ding Q, Mracek T, Gonzalez-Muniesa P, et al. Identification of macrophage inhibitory cytokine-1 in adipose tissue and its secretion as an adipokine by human adipocytes. *Endocrinology* 2009;150:1688-96.
114. Ouchi N, Higuchi A, Ohashi K, et al. Sfrp5 is an anti-inflammatory adipokine that modulates metabolic dysfunction in obesity. *Science* 2010;329:454-7.
115. Wu Y, Smas CM. Wdnm1-like, a new adipokine with a role in MMP-2 activation. *Am J Physiol Endocrinol Metab* 2008;295:E205-15.
116. Scherer PE. Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes* 2006;55:1537-45.
117. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol* 2006;6:772-83.
118. Morris PG, Hudis CA, Giri D, et al. Inflammation and increased aromatase expression occur in the breast tissue of obese women with breast cancer. *Cancer Prev Res (Phila)* 2011;4:1021-9.
119. Ehses JA, Perren A, Eppler E, et al. Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes* 2007;56:2356-70.
120. Varma V, Yao-Borengasser A, Rasouli N, et al. Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action. *Am J Physiol Endocrinol Metab* 2009;296:E1300-10.
121. Ribeiro R, Araujo A, Lopes C, Medeiros R. Immunoinflammatory mechanisms in lung cancer development: is leptin a mediator? *J Thorac Oncol* 2007;2:105-8.
122. de Luca C, Olefsky JM. Inflammation and insulin resistance. *FEBS Lett* 2008;582:97-105.
123. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell* 2011;147:275-92.
124. Cheng L, Darson MF, Bergstralh EJ, Slezak J, Myers RP, Bostwick DG. Correlation of margin status and extraprostatic extension with progression of prostate carcinoma. *Cancer* 1999;86:1775-82.
125. van Roermund JG, Hinnen KA, Tolman CJ, et al. Periprostatic fat correlates with tumour aggressiveness in prostate cancer patients. *BJU Int* 2011;107:1775-9.
126. Sumitomo M, Asakuma J, Yoshii H, et al. Anterior perirectal fat tissue thickness is a strong predictor of recurrence after high-intensity focused ultrasound for prostate cancer. *Int J Urol* 2010;17:776-82.
127. Finley DS, Calvert VS, Inokuchi J, et al. Periprostatic adipose tissue as a modulator of prostate cancer aggressiveness. *J Urol* 2009;182:1621-7.
128. Dolinkova M, Dostalova I, Lacinova Z, et al. The endocrine profile of subcutaneous and visceral adipose tissue of obese patients. *Mol Cell Endocrinol* 2008;291:63-70.
129. Gomez-Ambrosi J, Catalan V, Diez-Caballero A, et al. Gene expression profile of omental adipose tissue in human obesity. *FASEB J* 2004;18:215-7.
130. Hirano T, Morii H, Nakazawa K, et al. Enhancement of adipogenesis induction by conditioned media obtained from cancer cells. *Cancer Lett* 2008;268:286-94.
131. Andarawewa KL, Motrescu ER, Chenard MP, et al. Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. *Cancer Res* 2005;65:10862-71.
132. Jotzu C, Alt E, Welte G, et al. Adipose tissue derived stem cells differentiate into carcinoma-associated fibroblast-like cells under the influence of tumor derived factors. *Cell Oncol (Dordr)* 2011;34:55-67.
133. Dirat B, Bochet L, Dabek M, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res* 2011;71:2455-65.

134. Peroni D, Scambi I, Pasini A, et al. Stem molecular signature of adipose-derived stromal cells. *Exp Cell Res* 2008;314:603-15.
135. Pachon-Pena G, Yu G, Tucker A, et al. Stromal stem cells from adipose tissue and bone marrow of age-matched female donors display distinct immunophenotypic profiles. *J Cell Physiol* 2011;226:843-51.
136. Tarnok A, Ulrich H, Bocsi J. Phenotypes of stem cells from diverse origin. *Cytometry A* 2010;77:6-10.
137. Suga H, Matsumoto D, Eto H, et al. Functional implications of CD34 expression in human adipose-derived stem/progenitor cells. *Stem Cells Dev* 2009;18:1201-10.
138. Maumus M, Sengenès C, Decaunes P, et al. Evidence of in situ proliferation of adult adipose tissue-derived progenitor cells: influence of fat mass microenvironment and growth. *J Clin Endocrinol Metab* 2008;93:4098-106.
139. Muehlberg FL, Song YH, Krohn A, et al. Tissue-resident stem cells promote breast cancer growth and metastasis. *Carcinogenesis* 2009;30:589-97.
140. Zhang Y, Daquinag A, Traktuev DO, et al. White adipose tissue cells are recruited by experimental tumors and promote cancer progression in mouse models. *Cancer Res* 2009;69:5259-66.
141. Prantl L, Muehlberg F, Navone NM, et al. Adipose tissue-derived stem cells promote prostate tumor growth. *Prostate* 2010;70:1709-15.
142. Walter M, Liang S, Ghosh S, Hornsby PJ, Li R. Interleukin 6 secreted from adipose stromal cells promotes migration and invasion of breast cancer cells. *Oncogene* 2009;28:2745-55.
143. Zimmerlin L, Donnenberg AD, Rubin JP, Basse P, Landreneau RJ, Donnenberg VS. Regenerative therapy and cancer: in vitro and in vivo studies of the interaction between adipose-derived stem cells and breast cancer cells from clinical isolates. *Tissue Eng Part A* 2011;17:93-106.
144. Donnenberg VS, Zimmerlin L, Rubin JP, Donnenberg AD. Regenerative therapy after cancer: what are the risks? *Tissue Eng Part B Rev* 2010;16:567-75.
145. Kristiansen OP, Nolsoe RL, Larsen L, et al. Association of a functional 17beta-estradiol sensitive IL6-174G/C promoter polymorphism with early-onset type 1 diabetes in females. *Hum Mol Genet* 2003;12:1101-10.
146. Hwang IR, Hsu PI, Peterson LE, et al. Interleukin-6 genetic polymorphisms are not related to *Helicobacter pylori*-associated gastroduodenal diseases. *Helicobacter* 2003;8:142-8.
147. Xita N, Georgiou I, Chatzikyriakidou A, et al. Effect of adiponectin gene polymorphisms on circulating adiponectin and insulin resistance indexes in women with polycystic ovary syndrome. *Clin Chem* 2005;51:416-23.
148. Escobar-Morreale HF, Calvo RM, Villuendas G, Sancho J, San Millan JL. Association of polymorphisms in the interleukin 6 receptor complex with obesity and hyperandrogenism. *Obes Res* 2003;11:987-96.
149. Snoussi K, Strosberg AD, Bouaouina N, Ben Ahmed S, Helal AN, Chouchane L. Leptin and leptin receptor polymorphisms are associated with increased risk and poor prognosis of breast carcinoma. *BMC Cancer* 2006;6:38.
150. Skoog T, van't Hooft FM, Kallin B, et al. A common functional polymorphism (C-->A substitution at position -863) in the promoter region of the tumour necrosis factor-alpha (TNF-alpha) gene associated with reduced circulating levels of TNF-alpha. *Hum Mol Genet* 1999;8:1443-9.
151. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988;44:837-45.
152. Pencina MJ, D'Agostino RB, Sr., D'Agostino RB, Jr., Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Stat Med* 2008;27:157-72; discussion 207-12.

153. Sundstrom J, Byberg L, Gedeberg R, Michaelsson K, Berglund L. Useful tests of usefulness of new risk factors: Tools for assessing reclassification and discrimination. *Scand J Public Health* 2011;39:439-41.
154. Cavadas V, Osorio L, Sabell F, Teves F, Branco F, Silva-Ramos M. Prostate cancer prevention trial and European randomized study of screening for prostate cancer risk calculators: a performance comparison in a contemporary screened cohort. *Eur Urol* 2010;58:551-8.
155. Mottet N, Bellmunt J, Bolla M, et al. EAU guidelines on prostate cancer. Part II: Treatment of advanced, relapsing, and castration-resistant prostate cancer. *Eur Urol* 2011;59:572-83.
156. Scher HI, Halabi S, Tannock I, et al. Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. *J Clin Oncol* 2008;26:1148-59.
157. Thalmann S, Juge-Aubry CE, Meier CA. Explant cultures of white adipose tissue. *Methods Mol Biol* 2008;456:195-9.
158. Bicho MC, Pereira da Silva A, Matos A, Silva RM, Bicho MD. Sex steroid hormones influence the risk for cervical cancer: modulation by haptoglobin genetic polymorphism. *Cancer Genet Cytogenet* 2009;191:85-9.
159. Song GJ, Lewis V. Mitochondrial DNA integrity and copy number in sperm from infertile men. *Fertil Steril* 2008;90:2238-44.
160. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003;31:e15.
161. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004;3:Article3.
162. Reiner-Benaim A, Yekutieli D, Letwin NE, et al. Associating quantitative behavioral traits with gene expression in the brain: searching for diamonds in the hay. *Bioinformatics* 2007;23:2239-46.
163. Neville MJ, Collins JM, Gloyn AL, McCarthy MI, Karpe F. Comprehensive human adipose tissue mRNA and microRNA endogenous control selection for quantitative real-time-PCR normalization. *Obesity (Silver Spring)* 2011;19:888-92.
164. Catalan V, Gomez-Ambrosi J, Rotellar F, et al. Validation of endogenous control genes in human adipose tissue: relevance to obesity and obesity-associated type 2 diabetes mellitus. *Horm Metab Res* 2007;39:495-500.
165. Albertsen PC, Hanley JA, Fine J. 20-year outcomes following conservative management of clinically localized prostate cancer. *JAMA* 2005;293:2095-101.
166. Harnden P, Shelley MD, Coles B, Staffurth J, Mason MD. Should the Gleason grading system for prostate cancer be modified to account for high-grade tertiary components? A systematic review and meta-analysis. *Lancet Oncol* 2007;8:411-9.
167. Okorodudu DO, Jumeau MF, Montori VM, et al. Diagnostic performance of body mass index to identify obesity as defined by body adiposity: a systematic review and meta-analysis. *Int J Obes (Lond)* 2010;34:791-9.
168. Ribeiro R, Lopes C, Medeiros R. The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives. *Prostate Cancer Prostatic Dis* 2006;9:19-24.
169. Ribeiro R, Monteiro C, Cunha V, et al. Human periprostatic adipose tissue promotes prostate cancer aggressiveness in vitro. *J Exp Clin Cancer Res* 2012;31:32.
170. Ribeiro RJ, Monteiro CP, Cunha VF, et al. Tumor Cell-educated Periprostatic Adipose Tissue Acquires an Aggressive Cancer-promoting Secretory Profile. *Cell Physiol Biochem* 2012;29:233-40.
171. Kratzik C, Womastek I, Bieglmayer C, et al. Lower serum total testosterone is associated with lymph node metastases in a radical prostatectomy cohort study. *Anticancer Res* 2011;31:3615-8.
172. Massengill JC, Sun L, Moul JW, et al. Pretreatment total testosterone level predicts pathological stage in patients with localized prostate cancer treated with radical prostatectomy. *J Urol* 2003;169:1670-5.

173. Severi G, Morris HA, MacInnis RJ, et al. Circulating steroid hormones and the risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2006;15:86-91.
174. Schatzl G, Madersbacher S, Thurnidl T, et al. High-grade prostate cancer is associated with low serum testosterone levels. *Prostate* 2001;47:52-8.
175. Garcia-Cruz E, Piqueras M, Huguet J, et al. Hypertension, dyslipidemia and overweight are related to lower testosterone levels in a cohort of men undergoing prostate biopsy. *Int J Impot Res* 2012.
176. Frederiksen L, Hojlund K, Hougaard DM, et al. Testosterone therapy decreases subcutaneous fat and adiponectin in aging men. *Eur J Endocrinol* 2012;166:469-76.
177. Doak CM, Wijnhoven TM, Schokker DF, Visscher TL, Seidell JC. Age standardization in mapping adult overweight and obesity trends in the WHO European Region. *Obes Rev* 2011.
178. do Carmo I, Dos Santos O, Camolas J, et al. Overweight and obesity in Portugal: national prevalence in 2003-2005. *Obes Rev* 2008;9:11-9.
179. Hussain M, Tangen CM, Higano C, et al. Absolute prostate-specific antigen value after androgen deprivation is a strong independent predictor of survival in new metastatic prostate cancer: data from Southwest Oncology Group Trial 9346 (INT-0162). *J Clin Oncol* 2006;24:3984-90.
180. Devlin HL, Mudryj M. Progression of prostate cancer: multiple pathways to androgen independence. *Cancer Lett* 2009;274:177-86.
181. Schroder FH. Progress in understanding androgen-independent prostate cancer (AIPC): a review of potential endocrine-mediated mechanisms. *Eur Urol* 2008;53:1129-37.
182. Saylor PJ, Kozak KR, Smith MR, et al. Changes in biomarkers of inflammation and angiogenesis during androgen deprivation therapy for prostate cancer. *Oncologist* 2012;17:212-9.
183. Smith MR, Lee H, Fallon MA, Nathan DM. Adipocytokines, obesity, and insulin resistance during combined androgen blockade for prostate cancer. *Urology* 2008;71:318-22.
184. Chen J. Multiple signal pathways in obesity-associated cancer. *Obes Rev* 2011;12:1063-70.
185. Chung TD, Yu JJ, Spiotto MT, Bartkowski M, Simons JW. Characterization of the role of IL-6 in the progression of prostate cancer. *Prostate* 1999;38:199-207.
186. Iwamura M, Sluss PM, Casamento JB, Cockett AT. Insulin-like growth factor I: action and receptor characterization in human prostate cancer cell lines. *Prostate* 1993;22:243-52.
187. Kaklamani V, Yi N, Zhang K, et al. Polymorphisms of ADIPOQ and ADIPOR1 and prostate cancer risk. *Metabolism* 2011;60:1234-43.
188. Dhillon PK, Penney KL, Schumacher F, et al. Common polymorphisms in the adiponectin and its receptor genes, adiponectin levels and the risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2011;20:2618-27.
189. Kesarwani P, Mandhani A, Mittal RD. Polymorphisms in tumor necrosis factor-A gene and prostate cancer risk in North Indian cohort. *J Urol* 2009;182:2938-43.
190. Danforth KN, Rodriguez C, Hayes RB, et al. TNF polymorphisms and prostate cancer risk. *Prostate* 2008;68:400-7.
191. Muller-Hermelink N, Braumuller H, Pichler B, et al. TNFR1 signaling and IFN-gamma signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis. *Cancer Cell* 2008;13:507-18.
192. Calzascia T, Pellegrini M, Hall H, et al. TNF-alpha is critical for antitumor but not antiviral T cell immunity in mice. *J Clin Invest* 2007;117:3833-45.
193. Maggio M, Basaria S, Ceda GP, et al. The relationship between testosterone and molecular markers of inflammation in older men. *J Endocrinol Invest* 2005;28:116-9.
194. Gallagher DJ, Vijai J, Cronin AM, et al. Susceptibility loci associated with prostate cancer progression and mortality. *Clin Cancer Res* 2010;16:2819-32.
195. Penney KL, Pyne S, Schumacher FR, et al. Genome-wide association study of prostate cancer mortality. *Cancer Epidemiol Biomarkers Prev* 2010;19:2869-76.
196. Wiklund FE, Adami HO, Zheng SL, et al. Established prostate cancer susceptibility variants are not associated with disease outcome. *Cancer Epidemiol Biomarkers Prev* 2009;18:1659-62.

197. Lin DW, FitzGerald LM, Fu R, et al. Genetic variants in the LEPR, CRY1, RNASEL, IL4, and ARVCF genes are prognostic markers of prostate cancer-specific mortality. *Cancer Epidemiol Biomarkers Prev* 2011;20:1928-36.
198. Mullberg J, Oberthur W, Lottspeich F, et al. The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. *J Immunol* 1994;152:4958-68.
199. Galicia JC, Tai H, Komatsu Y, Shimada Y, Akazawa K, Yoshie H. Polymorphisms in the IL-6 receptor (IL-6R) gene: strong evidence that serum levels of soluble IL-6R are genetically influenced. *Genes Immun* 2004;5:513-6.
200. Quinton ND, Lee AJ, Ross RJ, Eastell R, Blakemore AI. A single nucleotide polymorphism (SNP) in the leptin receptor is associated with BMI, fat mass and leptin levels in postmenopausal Caucasian women. *Hum Genet* 2001;108:233-6.
201. Sun Q, Cornelis MC, Kraft P, et al. Genome-wide association study identifies polymorphisms in LEPR as determinants of plasma soluble leptin receptor levels. *Hum Mol Genet* 2010;19:1846-55.
202. da Silva BA, Bjorbaek C, Uotani S, Flier JS. Functional properties of leptin receptor isoforms containing the gln-->pro extracellular domain mutation of the fatty rat. *Endocrinology* 1998;139:3681-90.
203. Skogsberg J, Kannisto K, Cassel TN, Hamsten A, Eriksson P, Ehrenborg E. Evidence that peroxisome proliferator-activated receptor delta influences cholesterol metabolism in men. *Arterioscler Thromb Vasc Biol* 2003;23:637-43.
204. Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 2004;145:2273-82.
205. Boquest AC, Shahdadfar A, Brinckmann JE, Collas P. Isolation of stromal stem cells from human adipose tissue. *Methods Mol Biol* 2006;325:35-46.
206. Toda S, Uchihashi K, Aoki S, et al. Adipose tissue-organotypic culture system as a promising model for studying adipose tissue biology and regeneration. *Organogenesis* 2009;5:50-6.
207. Khandekar MJ, Cohen P, Spiegelman BM. Molecular mechanisms of cancer development in obesity. *Nat Rev Cancer* 2011;11:886-95.
208. O'Hara A, Lim FL, Mazzatti DJ, Trayhurn P. Microarray analysis identifies matrix metalloproteinases (MMPs) as key genes whose expression is up-regulated in human adipocytes by macrophage-conditioned medium. *Pflugers Arch* 2009;458:1103-14.
209. Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* 2005;184:285-93.
210. Tokuda Y, Satoh Y, Fujiyama C, Toda S, Sugihara H, Masaki Z. Prostate cancer cell growth is modulated by adipocyte-cancer cell interaction. *BJU Int* 2003;91:716-20.
211. Hoda MR, Popken G. Mitogenic and anti-apoptotic actions of adipocyte-derived hormone leptin in prostate cancer cells. *BJU Int* 2008;102:383-8.
212. Mori S, Murakami-Mori K, Bonavida B. Interleukin-6 induces G1 arrest through induction of p27(Kip1), a cyclin-dependent kinase inhibitor, and neuron-like morphology in LNCaP prostate tumor cells. *Biochem Biophys Res Commun* 1999;257:609-14.
213. Mizokami A, Gotoh A, Yamada H, Keller ET, Matsumoto T. Tumor necrosis factor-alpha represses androgen sensitivity in the LNCaP prostate cancer cell line. *J Urol* 2000;164:800-5.
214. Chopra DP, Menard RE, Januszewski J, Mattingly RR. TNF-alpha-mediated apoptosis in normal human prostate epithelial cells and tumor cell lines. *Cancer Lett* 2004;203:145-54.
215. Mistry T, Digby JE, Chen J, Desai KM, Randeva HS. The regulation of adiponectin receptors in human prostate cancer cell lines. *Biochem Biophys Res Commun* 2006;348:832-8.
216. Zeyda M, Farmer D, Todoric J, et al. Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *Int J Obes (Lond)* 2007;31:1420-8.

217. Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhoof A, Brichard SM. Adipokines oversecreted by omental adipose tissue in human obesity. *Am J Physiol Endocrinol Metab* 2007;293:E656-65.
218. Kharait S, Dhir R, Lauffenburger D, Wells A. Protein kinase Cdelta signaling downstream of the EGF receptor mediates migration and invasiveness of prostate cancer cells. *Biochem Biophys Res Commun* 2006;343:848-56.
219. Wells A, Gupta K, Chang P, Swindle S, Glading A, Shiraha H. Epidermal growth factor receptor-mediated motility in fibroblasts. *Microsc Res Tech* 1998;43:395-411.
220. Desai B, Ma T, Chellaiah MA. Invadopodia and matrix degradation, a new property of prostate cancer cells during migration and invasion. *J Biol Chem* 2008;283:13856-66.
221. Subramaniam V, Vincent IR, Jothy S. Upregulation and dephosphorylation of cofilin: modulation by CD44 variant isoform in human colon cancer cells. *Exp Mol Pathol* 2005;79:187-93.
222. Tang CH, Lu ME. Adiponectin increases motility of human prostate cancer cells via adiponR, p38, AMPK, and NF-kappaB pathways. *Prostate* 2009;69:1781-9.
223. Im E, Motiejunaite R, Aranda J, et al. Phospholipase Cgamma activation drives increased production of autotaxin in endothelial cells and lysophosphatidic acid-dependent regression. *Mol Cell Biol* 2010;30:2401-10.
224. Takahashi M, Ota S, Hata Y, et al. Constitutive expression of hepatocyte growth factor may maintain the sheet construction of gastric epithelial cells through facilitating actin-myosin contractile system. *Biochem Biophys Res Commun* 1996;219:40-6.
225. Kinoshita M, Shimokado K. Autocrine FGF-2 is responsible for the cell density-dependent susceptibility to apoptosis of HUVEC : A role of a calpain inhibitor-sensitive mechanism. *Arterioscler Thromb Vasc Biol* 1999;19:2323-9.
226. Hoang MV, Nagy JA, Fox JE, Senger DR. Moderation of calpain activity promotes neovascular integration and lumen formation during VEGF-induced pathological angiogenesis. *PLoS One* 2010;5:e13612.
227. Taraboletti G, Roberts DD, Liotta LA. Thrombospondin-induced tumor cell migration: haptotaxis and chemotaxis are mediated by different molecular domains. *J Cell Biol* 1987;105:2409-15.
228. Wang JM, Taraboletti G, Matsushima K, Van Damme J, Mantovani A. Induction of haptotactic migration of melanoma cells by neutrophil activating protein/interleukin-8. *Biochem Biophys Res Commun* 1990;169:165-70.
229. Ferry G, Tellier E, Try A, et al. Autotaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation. Up-regulated expression with adipocyte differentiation and obesity. *J Biol Chem* 2003;278:18162-9.
230. Azare J, Doane A, Leslie K, et al. Stat3 mediates expression of autotaxin in breast cancer. *PLoS One* 2011;6:e27851.
231. Sung SY, Chung LW. Prostate tumor-stroma interaction: molecular mechanisms and opportunities for therapeutic targeting. *Differentiation* 2002;70:506-21.
232. George DJ, Halabi S, Shepard TF, et al. The prognostic significance of plasma interleukin-6 levels in patients with metastatic hormone-refractory prostate cancer: results from cancer and leukemia group B 9480. *Clin Cancer Res* 2005;11:1815-20.
233. Michalaki V, Syrigos K, Charles P, Waxman J. Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer. *Br J Cancer* 2004;90:2312-6.
234. Culig Z. New insights into the role of interleukin-6 in human prostate cancer. *J Urol* 2009;182:1255-6.
235. Chun JY, Nadiminty N, Dutt S, et al. Interleukin-6 regulates androgen synthesis in prostate cancer cells. *Clin Cancer Res* 2009;15:4815-22.
236. Weber GF, Lett GS, Haubein NC. Osteopontin is a marker for cancer aggressiveness and patient survival. *Br J Cancer* 2010;103:861-9.

237. Thalmann GN, Sikes RA, Devoll RE, et al. Osteopontin: possible role in prostate cancer progression. *Clin Cancer Res* 1999;5:2271-7.
238. Balkwill F. TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev* 2006;25:409-16.
239. Yan B, Wang H, Rabbani ZN, et al. Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. *Cancer Res* 2006;66:11565-70.
240. Bates RC, Mercurio AM. Tumor necrosis factor-alpha stimulates the epithelial-to-mesenchymal transition of human colonic organoids. *Mol Biol Cell* 2003;14:1790-800.
241. Yamauchi T, Kamon J, Waki H, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 2001;7:941-6.
242. Li H, Stampfer MJ, Mucci L, et al. A 25-year prospective study of plasma adiponectin and leptin concentrations and prostate cancer risk and survival. *Clin Chem* 2010;56:34-43.
243. Opdenakker G, Masure S, Grillet B, Van Damme J. Cytokine-mediated regulation of human leukocyte gelatinases and role in arthritis. *Lymphokine Cytokine Res* 1991;10:317-24.
244. Catalan V, Gomez-Ambrosi J, Rodriguez A, et al. Increased adipose tissue expression of lipocalin-2 in obesity is related to inflammation and matrix metalloproteinase-2 and metalloproteinase-9 activities in humans. *J Mol Med (Berl)* 2009;87:803-13.
245. Villarroya J, Giral M, Villarroya F. Mitochondrial DNA: an up-and-coming actor in white adipose tissue pathophysiology. *Obesity (Silver Spring)* 2009;17:1814-20.
246. Robin ED, Wong R. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J Cell Physiol* 1988;136:507-13.
247. Kaaman M, Sparks LM, van Harmelen V, et al. Strong association between mitochondrial DNA copy number and lipogenesis in human white adipose tissue. *Diabetologia* 2007;50:2526-33.
248. Yamauchi T, Kamon J, Waki H, et al. The mechanisms by which both heterozygous peroxisome proliferator-activated receptor gamma (PPARgamma) deficiency and PPARgamma agonist improve insulin resistance. *J Biol Chem* 2001;276:41245-54.
249. Wilson-Fritch L, Burkart A, Bell G, et al. Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* 2003;23:1085-94.
250. del Pozo CH, Calvo RM, Vesperinas-Garcia G, et al. Expression profile in omental and subcutaneous adipose tissue from lean and obese subjects. Repression of lipolytic and lipogenic genes. *Obes Surg* 2011;21:633-43.
251. Nieman KM, Kenny HA, Penicka CV, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med* 2011;17:1498-503.
252. Discacciati A, Orsini N, Wolk A. Body mass index and incidence of localized and advanced prostate cancer--a dose-response meta-analysis of prospective studies. *Ann Oncol* 2012.
253. Hoon Kim J, Lee SY, Myung SC, Kim YS, Kim TH, Kim MK. Clinical significance of the leptin and leptin receptor expressions in prostate tissues. *Asian J Androl* 2008;10:923-8.
254. Wurmbach JH, Hammerer P, Sevinc S, Huland H, Ergun S. The expression of angiopoietins and their receptor Tie-2 in human prostate carcinoma. *Anticancer Res* 2000;20:5217-20.
255. Satoh N, Yamada Y, Kinugasa Y, Takakura N. Angiopoietin-1 alters tumor growth by stabilizing blood vessels or by promoting angiogenesis. *Cancer Sci* 2008;99:2373-9.
256. Yang RB, Ng CK, Wasserman SM, et al. Identification of a novel family of cell-surface proteins expressed in human vascular endothelium. *J Biol Chem* 2002;277:46364-73.
257. Qiu W, David D, Zhou B, et al. Down-regulation of growth arrest DNA damage-inducible gene 45beta expression is associated with human hepatocellular carcinoma. *Am J Pathol* 2003;162:1961-74.
258. Hong YH, Nishimura Y, Hishikawa D, et al. Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. *Endocrinology* 2005;146:5092-9.
259. O'Carroll SJ, Mitchell MD, Faenza I, Cocco L, Gilmour RS. Nuclear PLCbeta1 is required for 3T3-L1 adipocyte differentiation and regulates expression of the cyclin D3-cdk4 complex. *Cell Signal* 2009;21:926-35.

260. Sun T, Fu M, Bookout AL, Kliewer SA, Mangelsdorf DJ. MicroRNA let-7 regulates 3T3-L1 adipogenesis. *Mol Endocrinol* 2009;23:925-31.
261. Bhindi B, Trottier G, Elharram M, et al. Measurement of peri-prostatic fat thickness using transrectal ultrasonography (TRUS): a new risk factor for prostate cancer. *BJU Int* 2012.
262. Buhe V, Loisel S, Pers JO, Le Ster K, Berthou C, Youinou P. Updating the physiology, exploration and disease relevance of complement factor H. *Int J Immunopathol Pharmacol* 2010;23:397-404.
263. Wilczek E, Rzepko R, Nowis D, et al. The possible role of factor H in colon cancer resistance to complement attack. *Int J Cancer* 2008;122:2030-7.
264. Rodeheffer MS, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells in vivo. *Cell* 2008;135:240-9.
265. Kidd S, Spaeth E, Watson K, et al. Origins of the tumor microenvironment: quantitative assessment of adipose-derived and bone marrow-derived stroma. *PLoS One* 2012;7:e30563.
266. Klopp AH, Zhang Y, Solley T, et al. Omental adipose tissue-derived stromal cells promote vascularization and growth of endometrial tumors. *Clin Cancer Res* 2012;18:771-82.
267. Joe AW, Yi L, Even Y, Vogl AW, Rossi FM. Depot-specific differences in adipogenic progenitor abundance and proliferative response to high-fat diet. *Stem Cells* 2009;27:2563-70.
268. Koul HK, Kumar B, Koul S, et al. The role of inflammation and infection in prostate cancer: Importance in prevention, diagnosis and treatment. *Drugs Today (Barc)* 2010;46:929-43.
269. Lee KL, Peehl DM. Molecular and cellular pathogenesis of benign prostatic hyperplasia. *J Urol* 2004;172:1784-91.
270. Cho JW, Kang MC, Lee KS. TGF-beta1-treated ADSCs-CM promotes expression of type I collagen and MMP-1, migration of human skin fibroblasts, and wound healing in vitro and in vivo. *Int J Mol Med* 2010;26:901-6.
271. Lin G, Yang R, Banie L, et al. Effects of transplantation of adipose tissue-derived stem cells on prostate tumor. *Prostate* 2010.
272. Waugh DJ, Wilson C, Seaton A, Maxwell PJ. Multi-faceted roles for CXC-chemokines in prostate cancer progression. *Front Biosci* 2008;13:4595-604.
273. Begley L, Monteleon C, Shah RB, Macdonald JW, Macoska JA. CXCL12 overexpression and secretion by aging fibroblasts enhance human prostate epithelial proliferation in vitro. *Aging Cell* 2005;4:291-8.
274. Bellows CF, Zhang Y, Chen J, Frazier ML, Kolonin MG. Circulation of Progenitor Cells in Obese and Lean Colorectal Cancer Patients. *Cancer Epidemiol Biomarkers Prev* 2011.
275. Ma J, Li H, Giovannucci E, et al. Prediagnostic body-mass index, plasma C-peptide concentration, and prostate cancer-specific mortality in men with prostate cancer: a long-term survival analysis. *Lancet Oncol* 2008;9:1039-47.
276. Mauer MM, Harris RB, Bartness TJ. The regulation of total body fat: lessons learned from lipectomy studies. *Neurosci Biobehav Rev* 2001;25:15-28.

8. APPENDIXES

PAPER I

REVIEW

The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives

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Obesity-associated prostate cancer (PCa) remains controversial, although most studies rely on body mass index evaluation, which is an indirect measure of fatness. Studies using body fat measurement and disease stratification according to PCa stage found stronger associations between obesity and PCa. Leptin is a pleiotrophic hormone mainly synthesized by adipocytes that acts in peripheral organs such as the prostate. This article reviews obesity-associated leptin's pathophysiological role in PCa progression. PCa development results from some known risk factors. Currently, there is enough evidence suggesting that leptin is an additional factor involved in advanced PCa occurrence, and obesity association with high-grade disease. Life-long exposure to genetic and/or environmental susceptibility factors that predispose to obesity and higher leptin levels may increase the risk for advanced PCa.

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Keywords: advanced disease; leptin; pathophysiology; prostate cancer; obesity

Introduction

The prevalence of obesity in developed countries markedly increased over the last decades to an extent considered epidemic.¹ The obesity-associated increased risk for cancer is well documented in many model systems,² including those for prostate cancer (PCa). The rising prevalence of overweight and obesity in developed countries probably will be in the near future responsible by a significant proportion of cancers. Furthermore, the diagnosis is difficult because lower levels of prostate-specific antigen (PSA) in obese and overweight men could mask consequential PCa.³

There has been an increasing interest on the study of cellular and molecular mechanism of cancer by energy restriction models.⁴ Berrigan *et al.*⁵ and Mai *et al.*⁶ observed that the relationship between energetic balance and cancer development could be explained to a great extent by caloric restriction, as mediated through leptin.

Leptin is a hormone with a central role in obesity and is secreted principally by adipocytes, which contributes to the concept of adipocyte and adipose tissue as an endocrine cell and an endocrine organ, respectively.

Recent reviews state that leptin is involved in increased susceptibility for cancer, particularly of the breast and prostate.^{7–10}

In this article we review the leptin pathway from the obesity state to PCa susceptibility and progression to advanced disease.

Obesity and PCa

The OECD Health Data 2004¹¹ shows that in Europe (19 countries with repeated measures of BMI since 1980 until 2003), the obesity prevalence is increasing. Among the USA population, there were about 20% of obese individuals.¹² Asian countries, historically nonobese populations, also had pronounced increase in obesity prevalence during the last years.¹³ Concomitantly, over the last years PCa incidence has also increased. In the United States, PCa incidence annual percent change increased 2.7 in the time period of 1973–1988, 16.2 in 1988–1992, and –11.7 in 1992–1995.¹⁴ It seems that PCa incidence follows obesity incidence (Figure 1), except for the years 1992–1995 when after a peak of PCa incidence attributable to implementation of PSA screening, there was an expected decrease to normal detection rate.

There are inconclusive results concerning PCa association with dietary fat and calories intake¹⁷ and with obesity and overweight.¹⁸ Nevertheless, there is considerable amount of evidence that favors the association of obesity to higher-grade or -stage prostatic tumors.^{19–23}

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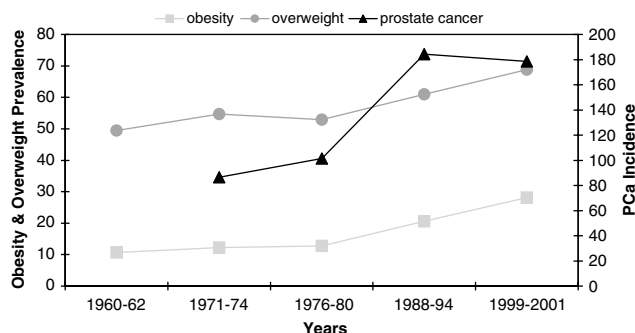


Figure 1 Similar trends in obesity and prostate cancer in the last 40 years in the USA; the increase in obesity and overweight prevalence is accompanied by incidence increase in PCa, in several time periods since 1962 through 2001, in the USA population. Data on overweight and obesity were obtained from CDC,¹⁵ and data about PCa incidence from Stanford *et al.*¹⁴ and Ries *et al.*¹⁶

The aforementioned inconsistencies among studies may be due to the methods used for assessing obesity. There are contradictory results from several studies using body mass index (BMI) to evaluate adiposity.²⁴⁻²⁹ However, studies using abdominal adiposity measurement, either by waist-to-hip ratio³⁰ or CT scan,³¹ found an increased risk for PCa.

An explanation for such conflicting results might be the failure to distinguish peripheral from central fat. Epidemiological reports of obesity have been based upon anthropometrical measures such as BMI, which are, particularly in men bearing greater muscle mass, inadequate estimates of adiposity. Among the elderly, the age group most affected by PCa, BMI could be a less valid indicator of adiposity, because they tend to have a shift of fat from peripheral to central sites with a concomitant waist-to-hip ratio at the same level of BMI.³²

Actually, there is agreement to expand the adipocyte role beyond fat storage, because of its crucial regulatory actions to whole body physiology. Nevertheless, the over storage of fat may contribute to the pathogeny of obesity-related diseases.³³ In fact, adipocytes secrete several cytokines known to contribute to an obesity-associated inflammatory state.^{34,35} This chronic inflammatory state may predispose to cancer etiopathogenesis, through adipokines roles that help to explain the molecular basis of obesity-associated PCa.

Several proteins secreted by adipocytes (leptin, TNF α , IL-6, TGF β , IGF-I) are important mediators in PCa development and/or progression.³⁶⁻⁴⁰ Here we describe the leptin pathway.

Pathophysiology of obesity: leptin's role

Research on obesity pathophysiology has been recently intensified, largely because of the discovery of leptin gene.⁴¹ Leptin is a 16-kDa protein, whose gene was previously cloned and sequenced.⁴¹ Its main roles are regulation of energy balance and mediation of the adaptation to fasting. Plasma leptin levels correlate with fat stores⁴² and the volume and number of adipocytes.^{43,44} Although leptin decreases body weight and fat content, hiperleptinemia found in obese subjects probably reflects leptin resistance⁴⁵ and fat depots.

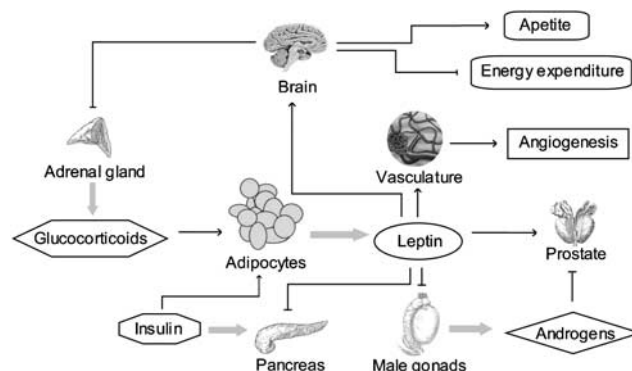


Figure 2 Schematic representations of leptin main peripheral and central actions; leptin has direct and indirect peripheral actions in the prostate, by interaction with the prostatic LEPR and at capillary endothelium inducing angiogenesis. Leptin levels are maintained by interaction with other physiological mediators like glucocorticoids, androgens and insulin. Leptin's central role is to regulate key molecules that control energy expenditure and appetite. Lines with point arrow – positive effect; lines without point arrows – negative effect; gray arrows – physiological effect.

Serum leptin levels seem to be dependent on body fat distribution. Some reports suggest it is better correlated with subcutaneous rather than visceral fat.^{46,47} However, a study conducted in monozygous twins discordant for obesity, demonstrated an association of the visceral fat area with leptin levels in men.⁴⁸ Furthermore, Tong *et al.*⁴⁹ found that circulating leptin level predicted visceral fat accumulation at 5 and 10 years follow-up. Cumulatively, omental adipose tissue expresses more leptin with aging,⁵⁰ while PCa risk increases with age. These results suggest that age and type of obesity can influence leptin concentration in serum.

Environmental factors such as diet are additional key regulators of leptin secretion by adipocytes. A westernized saturated fat-rich diet accounts for significant increases in plasma leptin.^{51,52}

The leptin gene (*LEP*) has several polymorphisms (SNPs) at the 5' flanking region. These polymorphisms might be responsible for functional abnormalities in leptin, and one (–2548 G/A) was shown to be associated with an overexpression of leptin mRNA⁵³ and higher serum levels.^{53,54}

Positional cloning of the leptin receptors (LEPR) confirmed their location in the hypothalamus and choroids plexus, areas known to regulate feeding behavior,⁵⁵ as well as in several peripheral tissues.⁵⁶ The presence of LEPR in prostatic tissue and prostate tumors has already been demonstrated,^{57,58} suggesting a plausible biological role for leptin in this gland (Figure 2). In fact, leptin seems to have a stimulatory role in prostate growth during puberty.⁵⁹

Whereas genetic characteristics predispose to lifelong exposure to higher leptin production in carriers of the overexpressing functional variant of the *LEP* gene (–2548 G/A), studies on adiposity usually reflect a one-moment evaluation, when the cumulative effect of fat content (all-life exposure) may be relevant in cancer development.

Early since fetal life, we are exposed to hormones and environmental factors that will influence health and obesity throughout life.⁶⁰ Leptin levels in cord blood derived from placenta and fetal tissues⁶¹ correlate with

body mass and fat mass of the neonate⁶² and with fetal fat depots.⁶³ Furthermore, autopsy studies found that microscopic foci of PCa and high-grade prostatic intraepithelial neoplasia occur earlier than clinical findings⁶⁴ and many could never been discovered in a screening program.^{64,65} We hypothesize that all-life exposure to obesity-associated genetic and environmental factors is relevant for PCa development. The shift towards clinical manifestations may depend upon the cumulative effect of time exposed to obesity, saturated fat intake and genetic susceptibility.

Leptin and PCa

Measurement of serum leptin in case-control studies with PCa patients have been used to demonstrate leptin involvement in PCa. The risk for PCa in individuals with higher levels of leptin has not been demonstrated.^{66,67} However, it was found that higher leptin levels were linked to tumor progression and advanced disease.^{38,68} We conclude from these investigations that leptin may be predominantly relevant in PCa progression. Studies on this issue should evaluate the grade and stage of PCa patients.

In vitro studies on PCa cell lines showed that leptin promotes proliferation of androgen-independent PCa^{69,70} and induces cell migration and the expression of growth factors in PCa cells.⁷¹ These results suggest that studies undertaken to ascertain leptin and/or obesity association to PCa should differentiate patients according to androgen resistance.

Molecular genetics studies on *LEP* and *LEPR* polymorphisms showed higher risk (OR = 4.67) for advanced disease in a functional variant in *LEP* gene.⁷² In the *LEPR* variant there was lack of association to PCa.⁷³ The frequencies of genotype distribution found for the *LEP* variant in normal controls from several countries, evidence a positive correlation of the risk genotype with PCa incidence in those populations, in the context of a gene/environmental model.⁷²

Leptin interaction with several hormones and/or growth factors that are involved in PCa is an additional pathway showing leptin indirect action in prostatic cells.

Tumor and host produced proinflammatory cytokines (IL-6, IL-1, TNF α) upregulate the *OB* gene in adipose tissue,⁷⁴ while leptin induces IL-6 and IGF-I production. This self-perpetuating loop allows tumor growth, involving leptin and other adipocytic molecules, contributing for tumor progression to advanced disease (Figure 3).

These lines of evidence from epidemiological, cell lines and genetics suggest that leptin has an important role in the development of advanced PCa.

There is evidence supporting this additional mechanism of PCa development, which relies on *LEP* gene interaction with life events, namely body fatness and saturated fat intake. This gene-environment relation is further supported by epidemiological data on geographic PCa incidence, dietary habits and genetic background. We recently identified the *LEP* -2548 AA as a risk genotype for PCa; however, the frequency of this homozygous in normal controls varies among populations (Table 1). Apparently, there is incongruence according to the risk genotype frequency in Japan

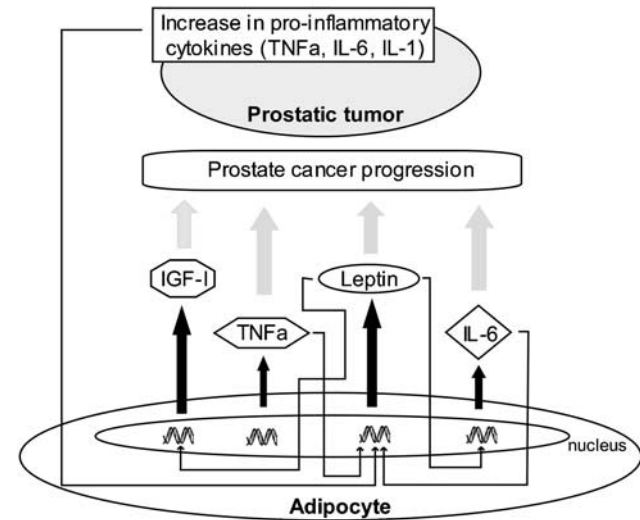


Figure 3 Role of adipocyte mediating tumor burden through self-regulatory loop and costimulatory action of adipokines; the tumor itself can perpetuate the growing stimulus through a stimulatory loop of tumor-derived cytokines on leptin synthesis. Leptin and several adipocytokines induce tumor growth, and exert some degree of control one over the others, mainly through stimulation of their synthesis. TNF α , tumor necrosis factor alpha; IL-6, interleucine 6; IL-1, interleucine 1; IGF-I, insulin growth factor I. Lines with point arrow – positive effect in gene expression; dark arrows – physiological effect that is regulated by gene expression; gray arrows – action of cytokines in prostatic tumor.

Table 1 Agreement between available genotypic frequencies for *LEP* risk homozygous AA and 2000 and 2002 PCa incidence, in several countries

	Genotype <i>LEP</i> –2548 AA (%) ^a	PCa incidence ASR (W), 2000 ^b	PCa incidence ASR (W), 2002 ^b
Japan	60.8	11.1	12.6
Portugal	10.2	36.7	46.8
Germany	15.8	53.6	60.5
France	20.1	56.5	59.3
USA	21.0	104.3	124.8

^aFrequencies of AA homozygous for *LEP* –2548 G/A gene polymorphism in healthy individuals from case-control studies. Data from Ribeiro *et al.*⁷² and Skibola *et al.*⁷⁵

^bData from GLOBOCAN 2000⁷⁶ and 2002,⁷⁷ respectively. ASR (W), Age standardized rate (World).

(60.8%) and the incidence of PCa in this country (11.1 ASR_W),⁷⁷ one of the lowest in the world. However, an earlier study by Wittemore *et al.*⁷⁸ showed that Japanese-Americans had even greater risk for PCa than African-American or Caucasian-American populations and that it was associated with saturated fat intake. Accordingly, Shibata *et al.*⁷⁹ observed higher incidence of PCa in Japanese-American compared to native Japanese. The association of genetic predisposition with lifestyle reflect the interaction of genes with environmental issues in PCa development.

Current hormonal therapy for PCa includes the use of antiandrogens, LHRH agonists and antagonists or combined androgen blockade.⁸⁰ The purpose of these pharmacological approaches is to lower androgen serum levels, which will induce a hormonal deregulation leading to leptin overexpression.⁹ The unbalanced serum

increase in leptin and decrease in androgens may facilitate androgen-independent cell growth, while downregulating androgen-dependent cells. The association of factors contributing to higher levels of leptin, for example, obesity, dietetic habits, leptin resistance, genetic variant in *LEP*, will increase further leptin production by adipocytes and gives ground for progression to androgen-independent PCa.

Thus, hormonal therapeutic intervention in advanced PCa or androgen-independent PCa should take into account individual genetic, hormonal and anthropometrical profile. Blocking peripheral leptin action, at either LEPR or intracellular pathways, without interfering with its physiological functions, will be a serious challenge to pharmacological sciences.

Conclusions

PCa is a multifactorial disease with some well-established mechanisms. Leptin's involvement in PCa development is an interesting emerging field of investigation.

Leptin is a key mediator of obesity and is involved in PCa progression. Its molecular, endocrinologic and genetic features may concur, directly or indirectly, to PCa development.

Results from epidemiological studies may be biased by measurement error, by not considering all-life exposure to obesity or by not adjusting for true body fat content. Most studies evaluating obesity in PCa patients use indirect methods to measure fat mass content or particularly visceral adiposity. These studies may contribute to conflicting results. Another source of confounding may arise from the lack of stratification of patients by PCa grade or androgen-sensitivity. Stratification could help find groups of patients with an obesity-associated risk.

Further studies, using disease stratification and adequate methods to evaluate fat content, are required to clarify obesity role in PCa carcinogenesis and/or progression. Longitudinal studies evaluating all-life exposure to obesity/diet and leptin's genetic/metabolic roles will help to elucidate the effect of such conditioning factors in PCa.

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References

- 1 James PT. Obesity: the worldwide epidemic. *Clin Dermatol* 2004; **22**: 276–280.
- 2 Bray GA. The underlying basis for obesity: relationship to cancer. *J Nutr* 2002; **132**: 3451S–3455S.

- 3 Baillargeon J, Pollock BH, Kristal AR, Bradshaw P, Hernandez J, Basler J *et al*. The association of body mass index and prostate-specific antigen in a population-based study. *Cancer* 2005; **103**: 1092–1095.
- 4 Zhu Z, Jiang W, Thompson HJ. An experimental paradigm for studying the cellular and molecular mechanisms of cancer inhibition by energy restriction. *Mol Carcinogenesis* 2002; **35**: 51–56.
- 5 Berrigan D, Perkins SN, Haines DC, Hursting SD. Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice. *Carcinogenesis* 2002; **23**: 817–822.
- 6 Mai V, Colbert LH, Berrigan D, Perkins SN, Pfeiffer R, Lavigne JA *et al*. Calorie restriction and diet composition modulate spontaneous intestinal tumorigenesis in Apc (min) mice through different mechanisms. *Cancer Res* 2003; **63**: 1752–1755.
- 7 Rose DP, Gilhooly EM, Nixon DW. Adverse effects of obesity on breast cancer prognosis, and the biological actions of leptin (review). *Int J Oncol* 2002; **21**: 1285–1292.
- 8 Markowska A, Malendowicz K, Drews K. The role of leptin in breast cancer. *Eur J Gynaecol Oncol* 2004; **25**: 192–194.
- 9 Ribeiro R, Lopes C, Medeiros R. Leptin and prostate: implications for cancer prevention – overview of genetics and molecular interactions. *Eur J Cancer Prev* 2004; **13**: 359–368.
- 10 Somasundar P, McFadden DW, Hileman SM, Vona-Davis L. Leptin is a growth factor in cancer. *J Surg Res* 2004; **116**: 337–349.
- 11 OECD Health Data 2004: A comparative analysis of 30 countries. Organisation for economic co-operation and development. 2004; 2004 edn. (<http://www.oecd.org/dataoecd/13/26/31963708.xls>).
- 12 Kuczmarski RJ, Flegal KM, Campbell SM, Johnson CL. Increasing prevalence of overweight among US adults. The National Health and Nutrition Examination Surveys, 1960–1991. *JAMA* 1994; **272**: 205–211.
- 13 Popkin BM. The nutrition transition in low-income countries: an emerging crisis. *Nutr Rev* 1994; **52**: 285–298.
- 14 Stanford JL, Stephenson RA, Coyle LM, Cerhan J, Correa R, Eley JW *et al*. *Prostate Cancer Trends 1973–1995*, SEER Program, National Cancer Institute. NIH Pub. No. 99-4543. Bethesda, MD, 1999.
- 15 National Center for Health Statistics. Health, United States, 2004. *With Chart Book on Trends in the Health of Americans*. Hyattsville, MD, 2004.
- 16 Ries LAG, Eisner MP, Kosary CL, Hankey BF, Miller BA, Clegg L, Mariotto A, Feuer EJ, Edwards BK (eds). *SEER Cancer Statistics Review, 1975–2000*. National Cancer Institute: Bethesda (MD), 2003.
- 17 Bosland MC, Oakley-Girvan I, Whittemore AS. Dietary fat, calories, and prostate cancer risk. *J Natl Cancer Inst* 1999; **91**: 489–491.
- 18 Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* 2004; **4**: 579–591.
- 19 Furuya Y, Akimoto S, Akakura K, Ito H. Smoking and obesity in relation to the etiology and disease progression of prostate cancer in Japan. *Int J Urol* 1998; **5**: 134–137.
- 20 Mydlo JH, Tieng NL, Volpe MA, Chaiken R, Kral JG. A pilot study analyzing PSA, serum testosterone, lipid profile, body mass index and race in a small sample of patients with and without carcinoma of the prostate. *Prostate Cancer Prostatic Dis* 2001; **4**: 101–105.
- 21 Amling CL, Riffenburgh RH, Sun L, Moul JW, Lance RS, Kusuda L *et al*. Pathologic variables and recurrence rates as related to obesity and race in men with prostate cancer undergoing radical prostatectomy. *J Clin Oncol* 2004; **22**: 439–445.
- 22 Freedland SJ, Aronson WJ, Kane CJ, Presti Jr JC, Amling CL, Elashoff D *et al*. Impact of obesity on biochemical control after radical prostatectomy for clinically localized prostate cancer: a report by the Shared Equal Access Regional Cancer Hospital Database Study Group. *J Clin Oncol* 2004; **22**: 446–453.
- 23 Kane CJ, Bassett WW, Sadetsky N, Silva S, Wallace K, Pasta DJ *et al*. Obesity and prostate cancer clinical risk factors at presentation: data from CaPSURE. *J Urol* 2005; **173**: 732–736.

- 24 Andersson SO, Wolk A, Bergstrom R, Adami HO, Engholm G, Englund A *et al.* Body size and prostate cancer: a 20-year follow-up study among 135006 Swedish construction workers. *J Natl Cancer Inst* 1997; **89**: 385–389.
- 25 Nilsen TI, Vatten LJ. Anthropometry and prostate cancer risk: a prospective study of 22,248 Norwegian men. *Cancer Causes Control* 1999; **10**: 269–275.
- 26 Rodriguez C, Patel AV, Calle EE, Jacobs EJ, Chao A, Thun MJ. Body mass index, height, and prostate cancer mortality in two large cohorts of adult men in the United States. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 345–353.
- 27 Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625–1638.
- 28 Giovannucci E, Rimm EB, Liu Y, Leitzmann M, Wu K, Stampfer MJ *et al.* Body mass index and risk of prostate cancer in U.S. health professionals. *J Natl Cancer Inst* 2003; **95**: 1240–1244.
- 29 Jonsson F, Wolk A, Pedersen NL, Lichtenstein P, Terry P, Ahlborn A *et al.* Obesity and hormone-dependent tumors: cohort and co-twin control studies based on the Swedish Twin Registry. *Int J Cancer* 2003; **106**: 594–599.
- 30 Hsing AW, Deng J, Sesterhenn IA, Mostofi FK, Stanczyk FZ, Benichou J *et al.* Body size and prostate cancer: a population-based case-control study in China. *Cancer Epidemiol Biomarkers Prev* 2000; **9**: 1335–1341.
- 31 von Hafe P, Pina F, Perez A, Tavares M, Barros H. Visceral fat accumulation as a risk factor for prostate cancer. *Obes Res* 2004; **12**: 1930–1935.
- 32 Borkan GA, Hulth DE, Gerzof SG, Robbins AH, Silbert CK. Age changes in body composition revealed by computed tomography. *J Gerontol* 1983; **38**: 673–677.
- 33 Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, Burrell MA. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 2001; **280**: E827–E847.
- 34 Browning LM. n-3 polyunsaturated fatty acids, inflammation and obesity-related disease. *Proc Nutr Soc* 2003; **62**: 447–453.
- 35 Ramos EJ, Xu Y, Romanova I, Middleton F, Chen C, Quinn R *et al.* Is obesity an inflammatory disease? *Surgery* 2003; **134**: 329–335.
- 36 Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P *et al.* Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* 1998; **279**: 563–566.
- 37 Giri D, Ozen M, Ittmann M. Interleukin-6 is an autocrine growth factor in human prostate cancer. *Am J Pathol* 2001; **59**: 2159–2165.
- 38 Saglam K, Aydur E, Yilmaz M, Goktas S. Leptin influences cellular differentiation and progression in prostate cancer. *J Urol* 2003; **169**: 1308–1311.
- 39 Michalaki V, Syrigos K, Charles P, Waxman J. Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer. *Br J Cancer* 2004; **90**: 2312–2316.
- 40 Danielpour D. Functions and regulation of transforming growth factor-beta (TGF-beta) in the prostate. *Eur J Cancer* 2005; **41**: 846–857.
- 41 Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425–432.
- 42 Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y *et al.* Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995; **1**: 1155–1161.
- 43 Hamilton BS, Paglia D, Aym K, Deitel M. Increased obese mRNA expression in omental fat cells from massively obese humans. *Nat Med* 1995; **1**: 953–956.
- 44 Lonnqvist F, Arner P, Nordfors L, Schalling M. Overexpression of the obese (ob) gene in adipose tissue of human obese subjects. *Nat Med* 1995; **1**: 950–953.
- 45 Ahima RS, Flier JS. Leptin. *Annu Rev Physiol* 2000; **62**: 413–437.
- 46 Lefebvre AM, Laville M, Vega N, Riou JP, van Gaal L, Auwerx J *et al.* Depot-specific differences in adipose tissue gene expression in lean and obese subjects. *Diabetes* 1998; **47**: 98–103.
- 47 Cnop M, Landchild MJ, Vidal J, Havel PJ, Knowles NG, Carr DR *et al.* The concurrent accumulation of intra-abdominal and subcutaneous fat explains the association between insulin resistance and plasma leptin concentrations. *Diabetes* 2002; **51**: 1005–1015.
- 48 Ronnema T, Karonen SL, Rissanen A, Koskenvuo M, Koivisto VA. Relation between plasma leptin levels and measures of body fat in identical twins discordant for obesity. *Ann Intern Med* 1997; **126**: 26–31.
- 49 Tong J, Fujimoto WY, Kahn SE, Weigle DS, McNeely MJ, Leonetti DL *et al.* Insulin, C-peptide, and leptin concentrations predict increased visceral adiposity at 5- and 10-year follow-ups in nondiabetic Japanese americans. *Diabetes* 2005; **54**: 985–990.
- 50 Hube F, Lietz U, Igel M, Jensen PB, Tornqvist H, Joost HG *et al.* Difference in leptin mRNA levels between omental and subcutaneous abdominal adipose tissue from obese humans. *Horm Metab Res* 1996; **28**: 690–693.
- 51 Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med* 1995; **1**: 1311–1314.
- 52 Chu NF, Stampfer MJ, Spiegelman D, Rifai N, Hotamisligil GS, Rimm EB. Dietary and lifestyle factors in relation to plasma leptin concentrations among normal weight and overweight men. *Int J Obes Relat Metab Disord* 2001; **25**: 106–114.
- 53 Hoffstedt J, Eriksson P, Mottagui-Tabar S, Arner P. A polymorphism in the leptin promoter region (–2548 G/A) influences gene expression and adipose tissue secretion of leptin. *Horm Metab Res* 2002; **34**: 355–359.
- 54 Mammès O, Betoulle D, Aubert R, Herbeth B, Siest G, Fumeron F. Association of the G-2548A polymorphism in the 5-prime region of the LEP gene with overweight. *Ann Hum Genet* 2000; **64**: 391–394.
- 55 Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000; **404**: 661–671.
- 56 Muoio DM, Lynis Dohm G. Peripheral metabolic actions of leptin. *Best Pract Res Clin Endocrinol Metab* 2002; **16**: 653–666.
- 57 Stattin P, Soderberg S, Hallmans G, Bylund A, Kaaks R, Stenman UH *et al.* Leptin is associated with increased prostate cancer risk: a nested case-referent study. *J Clin Endocrinol Metab* 2001; **86**: 1341–1345.
- 58 Vona-Davis L, Vedula G, McFadden DW, VanGilder R, Somasundar SMP, Hileman SM. Identification of leptin receptor isoforms in human prostate cancer cells. *J Surg Res* 2003; **114**: 302.
- 59 Nazian SJ, Cameron DF. Temporal relation between leptin and various indices of sexual maturation in the male rat. *J Androl* 1999; **20**: 487–491.
- 60 Oken E, Gillman MW. Fetal origins of obesity. *Obes Res* 2003; **11**: 496–506.
- 61 Schubring C, Kiess W, Englaro P, Rascher W, Dotsch J, Hanitsch S *et al.* Levels of leptin in maternal serum, amniotic fluid, and arterial and venous cord blood: relation to neonatal and placental weight. *J Clin Endocrinol Metab* 1997; **82**: 1480–1483.
- 62 Mantzoros CS, Varvarigou A, Kaklamani VG, Beratis NG, Flier JS. Effect of birth weight and maternal smoking on cord blood leptin concentrations of full-term and preterm newborns. *J Clin Endocrinol Metab* 1997; **82**: 2856–2861.
- 63 McMillen IC, Muhlhausler BS, Duffield JA, Yuen BS. Prenatal programming of postnatal obesity: fetal nutrition and the regulation of leptin synthesis and secretion before birth. *Proc Nutr Soc* 2004; **63**: 405–412.
- 64 Sanchez-Chapado M, Olmedilla G, Cabeza M, Donat E, Ruiz A. Prevalence of prostate cancer and prostatic intraepithelial neoplasia in Caucasian Mediterranean males: an autopsy study. *Prostate* 2003; **54**: 238–247.

- 65 Takahashi S, Shirai T, Hasegawa R, Imaida K, Ito N. Latent prostatic carcinomas found at autopsy in men over 90 years old. *Jpn J Clin Oncol* 1992; **22**: 117–121.
- 66 Hsing AW, Chua Jr S, Gao YT, Gentzsch E, Chang L, Deng J *et al*. Prostate cancer risk and serum levels of insulin and leptin: a population-based study. *J Natl Cancer Inst* 2001; **93**: 783–789.
- 67 Stattin P, Kaaks R, Johansson R, Gislefoss R, Soderberg S, Alfthan H *et al*. Plasma leptin is not associated with prostate cancer risk. *Cancer Epidemiol Biomarker Prev* 2003; **12**: 474–475.
- 68 Chang S, Hursting SD, Contois JH, Strom SS, Yamamura Y, Babaian RJ *et al*. Leptin and prostate cancer. *Prostate* 2001; **46**: 62–67.
- 69 Onuma M, Bub JD, Rummel TD, Iwamoto Y. Prostate cancer cell-adipocyte interaction: leptin mediates androgen-independent prostate cancer cell proliferation through c-Jun NH2-terminal kinase. *J Biol Chem* 2003; **278**: 42660–42667.
- 70 Somasundar P, Frankenberry KA, Skinner H, Vedula G, McFadden DW, Riggs D *et al*. Prostate cancer cell proliferation is influenced by leptin. *J Surg Res* 2004; **118**: 71–82.
- 71 Frankenberry KA, Somasundar P, McFadden DW, Vona-Davis LC. Leptin induces cell migration and the expression of growth factors in human prostate cancer cells. *Am J Surg* 2004; **188**: 560–565.
- 72 Ribeiro R, Vasconcelos A, Costa S, Pinto D, Morais A, Oliveira J *et al*. Overexpressing leptin genetic polymorphism (–2548 G/A) is associated with susceptibility to prostate cancer and risk of advanced disease. *Prostate* 2004; **59**: 268–274.
- 73 Kote-Jarai Z, Singh R, Durocher F, Easton D, Edwards SM, Ardern-Jones A *et al*. Association between leptin receptor gene polymorphisms and early-onset prostate cancer. *BJU Int* 2003; **92**: 109–112.
- 74 Grunfeld C, Zhao C, Fuller J, Pollack A, Moser A, Friedman J *et al*. Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters: a role for leptin in the anorexia of infection. *J Clin Invest* 1997; **97**: 2152–2157.
- 75 Skibola CF, Holly EA, Forrest MS, Hubbard A, Bracci PM, Skibola DR *et al*. Body mass index, leptin and leptin receptor polymorphisms, and non-hodgkin lymphoma. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 779–786.
- 76 Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2000: cancer incidence, mortality and prevalence worldwide, version 1.0. IARC CancerBase No. 4. IARC Press: Lyon 2001.
- 77 Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2002: Cancer incidence, mortality and prevalence worldwide, version 2.0. IARC CancerBase No. 5. IARC Press: Lyon 2004.
- 78 Whittemore AS, Kolonel LN, Wu AH, John EM, Gallagher RP, Howe GR *et al*. Prostate cancer in relation to diet, physical activity, and body size in Blacks, Whites, and Asians in the United States and Canada. *J Natl Cancer Inst* 1995; **87**: 652–661.
- 79 Shibata A, Whittemore AS, Imai K, Kolonel LN, Wu AH, John EM *et al*. Serum levels of prostate-specific antigen among Japanese-American and native Japanese men. *J Natl Cancer Inst* 1997; **89**: 1716–1720.
- 80 Hellerstedt BA, Pienta KJ. The current state of hormonal therapy for prostate cancer. *CA Cancer J Clin* 2002; **52**: 154–179.

PAPER II

Cancro da próstata

Porquê a preocupação com o estilo de vida e obesidade dos homens idosos?

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A obesidade afecta actualmente uma percentagem significativa de indivíduos, principalmente nos países desenvolvidos. Nestes países, o aumento da prevalência de obesidade é acompanhado por um aumento significativo de casos de cancro da próstata (CaP).

A evidência científica acumulada relativamente às associações entre a obesidade, estilo de vida e o risco de desenvolver CaP de pior prognóstico sugere que estão identificados alguns factores de risco susceptíveis de serem modificados, cuja alteração será recomendável aconselhar, de modo a diminuir a incidência e mortalidade do CaP.

À medida que o número de homens obesos e paralelamente com CaP aumenta devemos procurar intervenções no estilo de vida, para além de médicas, que possam ser eficientes na prevenção da instalação, recorrência ou progressão desta doença, e simultaneamente promover um bom estado de saúde geral.

A obesidade é um sério desafio que se coloca às sociedades ocidentais, sobretudo devido ao aumento dramático da sua prevalência nos últimos anos. Esta “epidemia” da obesidade reflecte a progressiva diminuição secular da actividade física, associada a alterações alimentares substanciais que se repercutem num consumo passivo de excesso de energia.¹

A obesidade está associada ao desenvolvimento de várias doenças crónicas como a doença arterial coronária, hipertensão e diabetes.^{1,2} Contudo, um estudo de coorte recente,³ que acompanhou 900 000 adultos durante 16 anos, demonstrou que um índice de massa corporal (IMC) mais elevado estava associado a risco de morte por cancro, incluindo o cancro da próstata nos

homens. De facto, alguns trabalhos de revisão mais recentes têm salientado a relação entre obesidade e cancro da próstata,^{4,5} estando o risco aumentado no idoso devido ao aumento da massa adiposa, diminuição da actividade física e alterações hormonais associados frequentemente ao envelhecimento.

A abordagem médica do cancro da próstata tem vindo a basear-se no rastreio através do doseamento sérico do antígeno específico da próstata (PSA) e nos tratamentos cirúrgico, hormonal, radio e quimioterápico.⁶ Os clínicos são reconhecidamente especialistas nas modalidades terapêuticas convencionais, embora aparentemente se mantenham afastados do aconselhamento sobre estilo de vida, actividade física, obesidade e dieta, com o objectivo de melhorar a prevenção e o prognóstico do cancro da próstata e de forma mais geral de várias outras doenças. De facto, apesar de a maioria dos estudos se centrar na repercussão do risco atribuível a factores de estilo de vida na incidência para cancro, existem hoje evidências que sugerem que estes factores também podem influenciar significativamente o prognóstico do cancro da próstata.⁷

Tomando estes dados em consideração, o caminho a percorrer parece mais claro e premente, na po-

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pulação idosa que possui maior risco de ser diagnosticado com neoplasia. Em vez de nos centrarmos exclusivamente nas queixas específicas anatomicamente localizadas, talvez devêssemos assumir uma perspectiva mais global e sermos os proponentes da saúde masculina. Como alternativa a prescrever apenas farmacoterapia para a patologia prostática ou perseguir inflexivelmente a causa de elevação ligeira dos valores de PSA, dever-se-ia considerar compreender, no todo, o estilo de vida e saúde do doente, pois podem interferir de modo significativo no percurso natural do cancro da próstata.

Obesidade e cancro da próstata

A obesidade é actualmente considerada uma "epidemia", principalmente nos países desenvolvidos, onde se verificam taxas de prevalência crescentes desde há alguns anos.⁸ Os resultados do relatório de saúde da OECD de 2004 (19 países com medidas repetidas de índice de massa corporal desde 1980) mostraram que a prevalência de obesidade na Europa está a aumentar.⁹ Em Portugal, um estudo recente conduzido em 6411 adultos de ambos os sexos demonstrou uma elevada prevalência de excesso de peso (38.6%) e de obesidade (13.8%) avaliados através de IMC.¹⁰ Comparando estes resultados com uma avaliação efectuada em 1995-1998, verificou-se que existe um aumento da prevalência de excesso de peso e obesidade nos últimos 10 anos no nosso país.¹⁰ Cumulativamente, Padez¹¹ demonstrou um aumento da prevalência de excesso de peso e obesidade em jovens recrutas militares portugueses do sexo masculino (n=850 081) entre 1986 (10.5% e 0.9%) e 2000 (21.3% e 4.2%, respectivamente). Até nos países Asiáticos, tradicionalmente populações não obesas, se tem verificado um au-

mento significativo na prevalência de obesidade.¹² Contudo, embora se saiba que a perda de peso efectiva e durável depende de alterações permanentes na qualidade da alimentação, ingestão energética e actividade física, os desafios do tratamento médico e da profilaxia social não têm sido cumpridos.¹

O aumento de prevalência da obesidade parece correlacionar-se nos últimos anos com o aumento da incidência de CaP. De facto, observou-se nos últimos anos um aumento da incidência e da mortalidade por CaP em vários países ocidentais,^{13,14} incluindo Portugal.¹⁵ Mesmo países com baixa incidência de cancro da próstata, como o Japão e China, têm um aumento de incidência desta neoplasia nos últimos anos, que parece acompanhar a adopção de estilos de vida ocidentais associados a altas prevalências de obesidade.¹⁶

Vários estudos epidemiológicos suportam a associação entre obesidade e susceptibilidade para CaP¹⁷⁻²³ e evidenciam risco aumentado para tumores prostáticos de grau ou estadios avançados, progressão da doença e mortalidade.²⁴⁻³⁰ Contudo, existem ainda resultados conflituosos,³¹ o que poderá dever-se à metodologia usada para avaliar a obesidade. Na maioria dos grandes estudos epidemiológicos com base populacional a obesidade é avaliada através de métodos indirectos de medição do conteúdo de gordura como o IMC, que não permite obter, particularmente nos homens que possuem maior massa muscular, uma correcta estimativa da adiposidade.

É interessante verificarmos que nos idosos, o grupo etário mais afectado por CaP, o IMC pode ser um indicador ainda menos válido, pois ocorre uma mudança dos depósitos de gordura, da periferia para locais

centrais, nomeadamente visceral. Em concordância, dois estudos caso-controlo mostraram que o risco é ainda mais acentuado quando se mede com fita métrica o perímetro abdominal (OR=2.71, IC95%=1.7-4.4)²⁰ ou a distribuição adiposa com tomografia axial computadorizada (OR=4.60, IC95%=2.6-8.2),²³ que são medidas mais específicas de adiposidade visceral (Quadro 1). Outro aspecto deficitário que condiciona a interpretação dos resultados é a avaliação do peso e altura através de questionário em alguns estudos coorte, pois foi previamente demonstrado que existe uma fraca correlação entre os valores de peso e altura auto-relatados e medidos objectivamente.³²

Além do papel da obesidade na susceptibilidade para CaP e risco de doença avançada, após diagnóstico e tratamento, o prognóstico parece igualmente ser influenciado pela obesidade. De facto, vários estudos demonstram que os homens obesos possuem maior risco de progressão bioquímica após prostatectomia radical ou após radioterapia externa.^{26,27,33-36}

Tem havido um interesse crescente no estudo de mecanismos moleculares e celulares do cancro através de modelos de restrição energética.³⁷ Berrigan et al.³⁸ e Mai et al.³⁹ observaram que a relação entre o equilíbrio energético e o desenvolvimento de cancro podia ser explicado pela restrição calórica, através da mediação de adipocitocinas. Vários estudos recentes evidenciam que algumas hormonas derivadas do tecido adiposo podem influenciar de modo significativo o crescimento e proliferação de tumores malignos. As adipocitocinas são moléculas produzidas principalmente, exclusivamente ou parcialmente no tecido adiposo, sabendo-se que algumas

Quadro 1 Magnitude do risco de CaP em indivíduos obesos de acordo com o método de avaliação da obesidade

Referência	País	Tipo Estudo	N	OR (IC95%)	Método
Moller et al. ¹⁷	Dinamarca	Coorte	43 965	1.30 (1.1-1.6) * ^d	IMC ^b
Andersson et al. ¹⁸	Suécia	Coorte	135 006	1.40 (1.1-1.8) **	IMC ^b
Hsing et al. ²⁰	China	Caso-controlo	238 / 471	2.71 (1.7-4.4) * ^d	Perímetro abd ^a
Rodriguez et al. ²¹	EUA	Coorte	816 268	1.27 (1.0-1.6) **	IMC ^b
Irani et al. ²²	França	Caso-controlo	194 / 194	2.47 (1.4-4.3) * ^d	IMC ^a
Von Hafe et al. ²³	Portugal	Caso-controlo	63 / 63	4.60 (2.6-8.2) * ^d	TAC
Gong et al. ³⁰	EUA	Coorte	10 258	1.78 (1.1-2.9) * ^d	IMC ^b

OR – Odds ratio; IC95% – Intervalo de confiança de 95 %; IMC – Índice de massa corporal (peso/altura-2); TAC – Tomografia axial computadorizada.

^aAvaliação do peso e altura ou perímetro abdominal por medição directa; ^bAvaliação do peso e altura efectuada através de questionário; ^cRisco para doença avançada; ^dRisco para doença localizada; *Risco para ser diagnosticado com CaP; ** Risco para morte por CaP.

possuem um papel relevante na regulação da angiogénese e do crescimento tumoral.^{40,41}

Actualmente, o adipócito já não é considerado um componente passivo do metabolismo humano. Tem havido um reconhecimento crescente de que o tecido adiposo funciona como um órgão endócrino e parácrino, que exerce efeitos biológicos diversos através da produção de factores de crescimento, polipéptidos e citocinas.⁴² Estas moléculas biologicamente activas secretadas pelos adipócitos e células estromais de suporte, foram denominadas adipocitocinas.⁴³ De facto, a importância conceptual da interacção entre células cancerígenas e células do estroma envolvente tem vindo a ser crescentemente aceite.⁴⁴ Estas interacções serão particularmente proeminentes em ambientes ricos em adipócitos.⁴⁵

De entre as cerca de 20 produzidas no tecido adiposo, a leptina, adiponectina, interleucina 6 (IL-6), factor de necrose tumoral alfa (TNFα), factor de crescimento vascular endotelial (VEGF) e inibidor do activador de plasminogénio 1 (PAI-1), têm sido progressivamente reconhecidos pelos seus múltiplos efeitos no comportamento biológico

das células tumorais.^{41,46,47} Existe actualmente evidência do seu envolvimento na angiogénese, um processo crítico para o desenvolvimento dos tumores sólidos,⁴⁸ na proliferação celular, metastização e alteração dos níveis de hormonas sexuais.⁴¹

Por outro lado, tem sido descrita a existência de um estado inflamatório associado à obesidade,⁴⁹ e a inflamação crónica é um factor comum e importante na patogénese das neoplasias.⁵⁰ Parece estar bem estabelecida a ideia de que as respostas inflamatórias persistentes observadas em neoplasias, ou envolvendo-as, regulam muitos aspectos do desenvolvimento dos tumores, nomeadamente a remodelação da matriz extra-celular, angiogénese ou potencial maligno, devido ao envolvimento de mediadores implicados na homeostasia dos tecidos.⁵¹ As adipocitocinas poderão estar envolvidas neste mecanismo, pois possuem funções imunomoduladoras e de regulação da inflamação.^{52,53}

Neste artigo, apresentamos argumentos que envolvem a obesidade, particularmente o tecido adiposo, no risco para CaP, sugerindo que os clínicos perante pacientes idosos com queixas urológicas comuns ou em consultas de rotina, considerem

medir o peso, estatura, perímetro abdominal ou solicitar avaliações mais específicas de adiposidade, através de DEXA, TAC abdominal ou bioimpedância eléctrica. A obesidade, requer avaliação clínica cuidada para identificar factores subjacentes e para permitir um tratamento coerente.

Estilo de vida e cancro da próstata

Estudos epidemiológicos sugerem que factores ambientais podem mediar a transformação de CaP latente em tumores clinicamente aparentes e que a dieta parece influenciar esta progressão. Apesar dos factores confundidores presentes em estudos clínicos que avaliam o efeito da alimentação no risco para cancro, os dados mantêm-se consistentes quanto ao facto de que vários nutrientes podem prevenir o desenvolvimento e progressão do CaP.^{54,55}

Alimentos ricos em licopeno, tal como o tomate e seus derivados, têm vindo a ser discutidos como potenciais efectores na prevenção e tratamento no CaP. Alguns estudos demonstraram que o consumo diário de uma porção de tomate ou seus derivados confere protecção para carcinogénese, devido à sua intervenção no mecanismo oncogénico

mediado por espécies reactivas de oxigénio (ROS).⁵⁶ Esta evidência é suportada por estudos coorte que demonstraram que o consumo frequente de derivados de tomate tem um efeito protector risco para CaP e por estudos caso-controlo que confirmaram a associação inversa entre licopeno e CaP e a existência de uma relação dose-resposta significativa.⁵⁷⁻⁵⁹ Cumulativamente, numa coorte de 1202 homens diagnosticados inicialmente com CaP localizado e tratamento idêntico, Chan et al.⁶⁰ analisaram a dieta pós-diagnóstico e concluíram que o consumo de derivados de tomate e peixe conferiam protecção contra a progressão da doença. Resultados de estudos no modelo animal e em humanos sugerem que a ingestão de gorduras representa um factor de risco adicional para CaP, através do seu papel na carcinogénese e progressão tumoral.⁶¹⁻⁶³

Perante o corpo de evidência, é importante informar/aconselhar sobre a dieta adequada e como aumentar o gasto de energia através do exercício físico. Os clínicos sentir-se-ão particularmente encorajados a tomar esta posição, perante as recentes descobertas de Giovannucci et al.,⁶⁴ que verificaram uma redução significativa (~70%) do risco para desenvolver cancro da próstata avançado e para morrer da doença em homens com mais de 3 h de exercício vigoroso por semana.

Vários estudos coorte conduzidos em vários países mostraram uma associação entre exercício e diminuição do risco de CaP.^{19, 65-68} Cumulativamente, vários estudos caso-controlo evidenciaram uma associação entre risco diminuído de CaP e níveis mais elevados de actividade física.⁶⁹⁻⁷¹ A média de redução do risco é de magnitude 10-30%. Em conjunto, estas evidências sugerem

uma provável ligação entre mais exercício físico e diminuição do risco de CaP. A capacidade do exercício modular níveis hormonais, prevenir a obesidade, melhorar a função imunitária e permitir lidar melhor com o stress oxidativo, foram postulados como mecanismos que suportam o efeito protector do exercício.⁷² O exercício pode também beneficiar homens com CaP, durante o período de tratamento.⁷³

O calcitriol (1,25-dihidroxitamina D3) que é a forma activa da vitamina D, promove a inibição do crescimento e a diferenciação de células tumorais prostáticas.^{75,77} Segundo Moreno et al.⁷⁶ a acção do calcitriol parece diminuir a expressão genética de ciclooxigenase-2 (COX-2) e dos receptores de prostaglandinas (PGs) EP2 e FP, protegendo as células do estímulo proliferativo das PGs. A acção do calcitriol nas células é mediada pelo receptor nuclear de vitamina D (VDR), cujo gene possui variantes que alteram a função da proteína com repercussões no risco para CaP.⁷⁷⁻⁷⁹

Actualmente, parece bem estabelecido que existe uma correlação positiva entre a exposição solar e os níveis séricos de vitamina D e entre a idade e a concentração de vitamina D em circulação. Alguns autores verificaram que era frequente encontrar uma redução dos níveis séricos de 1,25-dihidroxitamina D3 nos idosos,⁸⁰⁻⁸² aumentando a sua predisposição para o desenvolvimento e progressão de CaP.

A influência da exposição solar no prognóstico do CaP, é suportada por vários estudos ecológicos, caso-controlo e coorte, que analisaram a associação entre a exposição ao sol (avaliada por gradiente norte-sul da radiação solar UV, diferenças climáticas geográficas e exposição solar ocupacional) e mortalidade por

CaP.⁸³⁻⁸⁷ Apesar de a radiação solar em contacto com a pele poder ser uma importante fonte de vitamina D, a ingestão frequente de alimentos ricos em vitamina D ou a ingestão de suplementos de calcitriol podem representar um aporte vitamínico significativo.⁸⁸

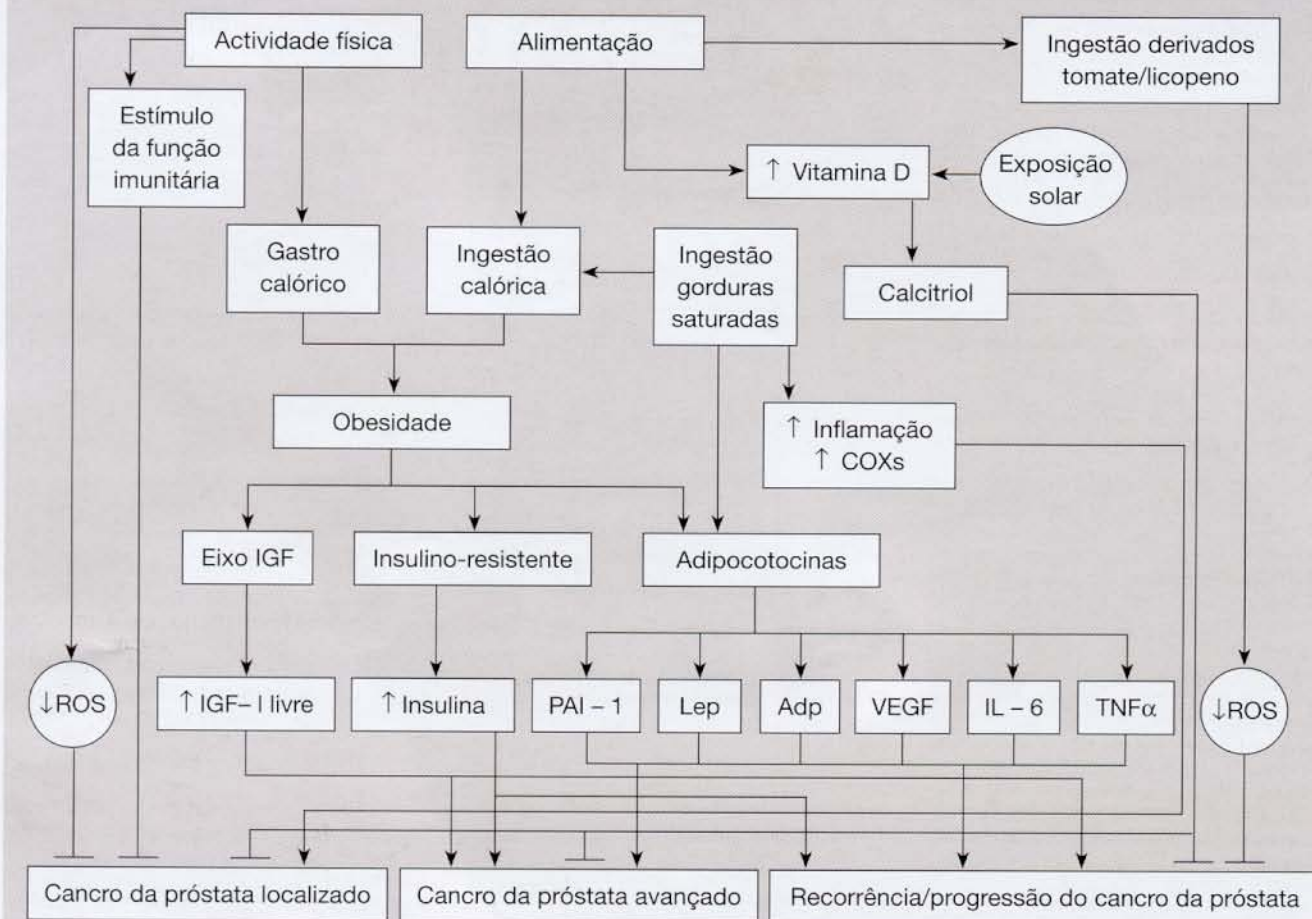
As evidências pré-clínicas apresentadas motivaram a realização de estudos clínicos com o objectivo de avaliar o potencial terapêutico do calcitriol no cancro. Estudos em que é administrado calcitriol semanalmente em doentes com CaP, parecem apresentar a melhor relação de eficácia vs. toxicidade.⁸⁹ Beer & Myrthue⁹⁰ sugerem que a combinação de calcitriol com quimioterápicos, p.e. docetaxel, poderá contribuir para um tratamento mais eficaz do CaP.

Conclusão

O aumento de risco para cancro associado à obesidade está bem documentado em vários modelos oncológicos incluindo o cancro da próstata (CaP). O aumento do número de indivíduos obesos ou com excesso de peso poderá ser responsável, no futuro próximo, por uma proporção significativa de casos de cancro. Adicionalmente, o diagnóstico é difícil devido à existência de valores mais baixos de antígeno específico da próstata (PSA) em circulação no sangue de homens obesos ou com excesso de peso, podendo mascarar a existência de neoplasia (Baillargeon et al., 2005).

Considerando o papel da alimentação no risco para CaP e para a progressão da doença, é razoável recomendar à população masculina o consumo de produtos com tomate (cerca de 5 porções por semana) e diminuir a ingestão de gordura, como parte de hábitos alimentares saudáveis que podem reduzir o

Figura 1 Mecanismos patofisiológicos e de protecção do cancro da próstata associados à obesidade, actividade física, alimentação e exposição solar



risco de ocorrência destes eventos e eventualmente melhorar o prognóstico em doentes diagnosticados com CaP.

De acordo com algumas incertezas relativamente à fiabilidade do PSA como marcador de cancro da próstata e ansiedades devido ao risco de sobre-diagnóstico do cancro da próstata assim como a tendência demográfica no sentido do aumento da população obesa, estamos perante argumentos para que todos os prestadores de cuidados médicos comecem a ter uma visão mais ampla sobre a abordagem do CaP, onde o aconselhamento sobre uma dieta adequada, obesidade e

aumento do exercício físico deve constar da consulta de cuidados primários e da consulta de seguimento pós-diagnóstico de CaP.

A acumulação de evidências sobre os efeitos benéficos da luz solar em vários tipos de cancro com elevada taxa de mortalidade, como o CaP, torna necessário reconsiderar as recomendações médicas de exposição solar, baseadas actualmente apenas no risco acrescido para cancro da pele.

Para a definição de estratégias de prevenção e optimização do tratamento do cancro da próstata, a atitude clínica e terapêutica deverá considerar não só o tratamento cen-

trado no tumor mas também outras variáveis individuais que influenciam a sua história natural (Figura 1). Num mundo em rápida mudança, esta abordagem poderá proporcionar aos médicos e em especial aos urologistas intervenções menos dependentes das intervenções cirúrgica ou rádio-terapêutica, concedendo aos doentes uma via baseada-na-evidência para atingir melhorias na longevidade e qualidade de vida. ■

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Referências Bibliográficas

- Haslam DW, James WP. Obesity. *Lancet* 2005;366:1197-209.
- Kopelman PG. Obesity as a medical problem. *Nature* 2000;404:635-43.
- Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003;348:1625-38.
- Freedland SJ, Aronson WJ. Examining the relationship between obesity and prostate cancer. *Rev Urol* 2004;6:73-81.
- Ribeiro R, Lopes C, Medeiros R. The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives. *Prostate Cancer Prostatic Dis* 2006;9:19-24.
- Pomerantz M, Kantoff P. Advances in the Treatment of Prostate Cancer. *Annu Rev Med* 2006 Sep 20; [Epub ahead of print]
- Bassett WW, Cooperberg MR, Sadetsky N, Silva S, DuChane J, Pasta DJ, Chan JM, Anast JW, Carroll PR, Kane CJ. Impact of obesity on prostate cancer recurrence after radical prostatectomy: data from CaPSURE. *Urology* 2005;66:1060-5.
- Dames PT. Obesity: the worldwide epidemic. *Clin Dermatol* 2004;22:276-80.
- OECD Health Data 2004: A comparative analysis of 30 countries. Organization for Economic Co-operation and Development, 2004; 2004 edn. (<http://www.oecd.org/dataoecd/13/26/31963708.xls>)
- do Carmo I, dos Santos O, Camolas J, Vieira J, Carreira M, Medina L, Reis L, Galvão-Teles A. Prevalence of obesity in Portugal. *Obes Rev* 2006;7(3):233-7.
- Padez C. Trends in overweight and obesity in Portuguese conscripts from 1986 to 2000 in relation to place of residence and educational level. *Public Health* 2006;120(10):946-52.
- Popkin BM. The nutrition transition in low-income countries: an emerging crisis. *Nutr Rev* 1994; 52: 285-98.
- Stanford JL, Stephenson RA, Coyle LM, Cerhan J, Correa R, Eley JW, Gilliland F, Hankey B, Kolonel LN, Kosary C, Ross R, Severson R, West D. Prostate Cancer Trends 1973-1995, SEER Program, National Cancer Institute. NIH Pub. No. 99-4543. Bethesda, MD, 1999.
- Hsing AW, Chokkalingam AP. Prostate cancer epidemiology. *Front Biosci* 2006;11:1388-413.
- Pinheiro PS, Tyczynski JE, Bray F, Amado J, Matos E, Parkin DM. Cancer incidence and mortality in Portugal. *Eur J Cancer* 2003;39:2507-20.
- Sim HG, Cheng CWS. Changing demography of prostate cancer in Asia. *Eur J Cancer* 2005;41:834-845.
- Moller H, Møllemaard A, Lindvik K, Olsen JH. Obesity and cancer risk: a Danish record-linkage study. *Eur J Cancer* 1994;30A:344-50.
- Andersson SO, Wolk A, Bergstrom R, Adami HO, Engholm G, Englund A, Nyren O. Body size and prostate cancer: a 20-year follow-up study among 135006 Swedish construction workers. *J Natl Cancer Inst* 1997;89:385-9.
- Cerhan JR, Torner JC, Lynch CF, Rubenstein LM, Lemke JH, Cohen MB, Lubartoff DM, Wallace RB. Association of smoking, body mass, and physical activity with risk of prostate cancer in the Iowa 65+ Rural Health Study (United States). *Cancer Causes Control* 1997; 8:229-38.
- Hsing AW, Deng J, Sesterhenn IA, Mostofi FK, Stanczyk FZ, Benichou J, Xie T, Gao YT. Body size and prostate cancer: a population-based case-control study in China. *Cancer Epidemiol Biomarkers Prev* 2000;9:1335-41.
- Rodriguez C, Patel AV, Calle EE, Jacobs EJ, Chao A, Thun MJ. Body mass index, height, and prostate cancer mortality in two large cohorts of adult men in the United States. *Cancer Epidemiol Biomarkers Prev* 2001;10:345-53.
- Irani J, Lefebvre O, Murat F, Dahmani L, Dore B. Obesity in relation to prostate cancer risk: comparison with a population having benign prostatic hyperplasia. *BJU Int* 2003; 91: 482-4.
- von Hafe P, Pina F, Perez A, Tavares M, Barros H. Visceral fat accumulation as a risk factor for prostate cancer. *Obes Res* 2004; 12: 1930-5.
- Furuya A, Kimoto S, Akakura K, Ito H. Smoking and obesity in relation to the etiology and disease progression of prostate cancer in Japan. *Int J Urol* 1998; 5: 134-7.
- Mydlo JH, Tieng NL, Volpe MA, Chaiken R, Kral JG. A pilot study analyzing PSA, serum testosterone, lipid profile, body mass index and race in a small sample of patients with and without carcinoma of the prostate. *Prostate Cancer Prostatic Dis* 2001; 4: 101-105.
- Ameling CL, Riffenburgh RH, Sun L, Moul JW, Lance RS, Kusuda L, Sexton WJ, Soderdahl DW, Donahue TF, Foley JP, Chung AK, McLeod DG. Pathologic variables and recurrence rates as related to obesity and race in men with prostate cancer undergoing radical prostatectomy. *J Clin Oncol* 2004; 22: 439-45.
- Freedland SJ, Aronson WJ, Kane CJ, Presti JC Jr, Ameling CL, Elashoff D, Terris MK. Impact of obesity on biochemical control after radical prostatectomy for clinically localized prostate cancer: a report by the Shared Equal Access Regional Cancer Hospital database study group. *J Clin Oncol* 2004; 22: 446-53.
- Kane CJ, Bassett WW, Sadetsky N, Silva S, Wallace K, Pasta DJ, Cooperberg MR, Chan JM, Carroll PR. Obesity and prostate cancer clinical risk factors at presentation: data from CaPSURE. *J Urol* 2005; 173:732-6.
- Ma J, Li H, Mucci LA, Gaziano JM, Stampfer MJ. Prediagnostic body mass index (BMI) and prostate cancer (CaP) mortality: a 21-year survival analysis in the Physicians Health Study. Multidisciplinary Prostate Cancer Symposium, 17-19 February 2005; Orlando, Florida.
- Gong Z, Neuhaus ML, Goodman PJ, Albanes D, Chi C, Hsing AW, Lippman SM, Platz EA, Pollak MN, Thompson IM, Kristal AR. Obesity, diabetes, and risk of prostate cancer: results from the prostate cancer prevention trial. *Cancer Epidemiol Biomarkers Prev* 2006; 15: 1977-83.
- Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* 2004;4:579-91.
- Clemente C, Moreira P, Oliveira B, Vaz de Almeida MD. Índice de massa corporal - sensibilidade e especificidade. *Acta Med Port* 2004;17:353-8.
- Bassett WW, Cooperberg MR, Sadetsky N, Silva S, DuChane J, Pasta DJ, Chan JM, Anast JW, Carroll PR, Kane CJ. Impact of obesity on prostate cancer recurrence after radical prostatectomy: data from CaPSURE. *Urology* 2005;66:1060-5.
- Freedland SJ, Isaacs WB, Mangold LA, Yiu SK, Grubb KA, Partin AW, Epstein JI, Walsh PC, Platz EA. Stronger association between obesity and biochemical progression after radical prostatectomy among men treated in the last 10 years. *Clin Cancer Res* 2005; 11:2883-8.
- Freedland SJ, Grubb KA, Yiu SK, Humphreys EB, Nielsen ME, Mangold LA, Isaacs WB, Partin AW. Obesity and risk of biochemical progression following radical prostatectomy at a tertiary care referral center. *J Urol* 2005;174:919-22.
- Strom SS, Kamat AM, Gruschus SK, Gu Y, Wen S, Cheung MR, Pisters LL, Lee AK, Rosser CJ, Kuban DA. Influence of obesity on biochemical and clinical failure after external-beam radiotherapy for localized prostate cancer. *Cancer* 2006;107:631-9.
- Zhu Z, Jiang W, Thompson HJ. An experimental paradigm for studying the cellular and molecular mechanisms of cancer inhibition by energy restriction. *Mol Carcinog* 2002;35:51-6.
- Berrigan D, Perkins SN, Haines DC, Hursting SD. Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice. *Carcinogenesis* 2002;23:817-22.
- Mai V, Colbert LH, Berrigan D, Perkins SN, Pfeiffer R, Lavigne JA, Lanza E, Haines DC, Schatzkin A, Hursting SD. Calorie restriction and diet composition modulate spontaneous intestinal tumorigenesis in Apc(Min) mice through different mechanisms. *Cancer Res* 2003;63:1752-5.
- Ribeiro R, Lopes C, Medeiros R. Leptin and prostate: implications for cancer prevention-overview of genetics and molecular interactions. *Eur J Cancer Prev* 2004;13:359-68.
- Housa D, Housova J, Vernerova Z, Haluzik M. Adipocytokines and cancer. *Physiol Res* 2006;55(3): 233-44.
- Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, Burrell MA. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 2001;280:E827-E847.
- Matsuzawa Y, Funahashi T, Nakamura T. Molecular mechanism of metabolic syndrome X: contribution of adipocytokines adipocyte-derived bioactive substances. *Ann N Y Acad Sci* 1999; 892: 146-54.
- Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp Cell Res* 2001;264: 169-184.
- Rajala MW, Scherer PE. Minireview: The adipocyte - at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* 2003;144: 3765-73.
- Ribeiro R, Vasconcelos A, Costa S, Pinto D, Morais A, Oliveira J, Lobo F, Lopes C, Medeiros R. Overexpressing leptin genetic polymorphism (-2548 G/A) is associated with susceptibility to prostate cancer and risk of advanced disease. *Prostate* 2004; 59:268-74.
- Rose DP, Komninou D, Stephenson GD. Obesity, adipocytokines, and insulin resistance in breast cancer. *Obes Rev* 2004;5:153-65.
- Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995;1: 1155-61.
- Ramos EJ, Xu Y, Romanova I, Middleton F, Chen C, Quinn R, Inui A, Das U, Meguid MM. Is obesity an inflammatory disease? *Surgery* 2003;134:329-35.
- Schottenfeld D, Beebe-Dimmer J. Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. *CA Cancer J Clin* 2006; 56: 69-83.
- van Kempen LC, de Visser KE, Coussens LM. Inflammation, proteases and cancer. *Eur J Cancer* 2006;42:728-34.
- Juge-Aubry CE, Henrichot E, Meier CA. Adipose tissue: a regulator of inflammation. *Best Pract Res Clin Endocrinol Metab* 2005;19:547-66.
- Ribeiro R, Araújo A, Lopes C, Medeiros R. Immunoinflammatory mechanisms in lung cancer - is leptin a mediator? *J Thorac Oncol* (in press).
- Cohen LA. Nutrition and prostate cancer: a review. *Ann N Y Acad Sci* 2002;963:148-55.
- Sonn GA, Aronson W, Litwin MS. Impact of diet on prostate cancer: a review. *Prostate Cancer Prostatic*

- Dis 2005;8:304-10.
56. Ellinger S, Ellinger J, Stehle P. Tomatoes, tomato products and lycopene in the prevention and treatment of prostate cancer: do we have the evidence from intervention studies? *Curr Opin Clin Nutr Metab Care* 2006;9:722-7.
57. Lu QY, Hung JC, Heber D, Go VL, Reuter VE, Cordon-Cardo C, Scher HI, Marshall R, Zhang ZF. Inverse associations between plasma lycopene and other carotenoids and prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2001;10:749-56.
58. Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. A prospective study of tomato products, lycopene, and prostate cancer risk. *J Natl Cancer Inst* 2002;94:391-8.
59. Jian L, Du CJ, Lee AH, Binns CW. Do dietary lycopene and other carotenoids protect against prostate cancer? *Int J Cancer* 2005;113:1010-4.
60. Chan JM, Holick CN, Leitzmann MF, Rimm EB, Willett WC, Stampfer MJ, Giovannucci EL. Diet after diagnosis and the risk of prostate cancer progression, recurrence, and death (United States). *Cancer Causes Control* 2006;17:199-208.
61. Wang Y, Corr JG, Thaler HT, Tao Y, Fair WR, Heston WD. Decreased growth of established human prostate LNCaP tumors in nude mice fed a low-fat diet. *J Natl Cancer Inst* 1995;87:1456-62.
62. Andersson SO, Wolk A, Bergstrom R, Giovannucci E, Lindgren C, Baron J. Energy, nutrient intake and prostate cancer risk: a population-based case-control study in Sweden. *Int J Cancer* 1996;68:716-22.
63. Meyer F, Bairati I, Shadmani R, Fradet Y, Moore L. Dietary fat and prostate cancer survival. *Cancer Causes Control* 1999;10:245-51.
64. Giovannucci EL, Liu Y, Leitzmann MF, Stampfer MJ, Willett WC. A prospective study of physical activity and incident and fatal prostate cancer. *Arch Intern Med* 2005;165:1005-10.
65. Oliveria SA, Kohl HW 3rd, Trichopoulos D, Blair SN. The association between cardiorespiratory fitness and prostate cancer. *Med Sci Sports Exerc* 1996;28:97-104.
66. Clarke G, Whittemore AS. Prostate cancer risk in relation to anthropometry and physical activity: the National Health and Nutrition Examination Survey I Epidemiological Follow-Up Study. *Cancer Epidemiol Biomarkers Prev* 2000;9:875-81.
67. Patel AV, Rodriguez C, Jacobs EJ, Solomon L, Thun MJ, Calle EE. Recreational physical activity and risk of prostate cancer in a large cohort of U.S. men. *Cancer Epidemiol Biomarkers Prev* 2005;14:275-9.
68. Nilsen TI, Romundstad PR, Vatten LJ. Recreational physical activity and risk of prostate cancer: A prospective population-based study in Norway (the HUNT study). *Int J Cancer* 2006;119:2943-7.
69. Bairati I, Larouche R, Meyer F, Moore L, Fradet Y. Lifetime occupational physical activity and incidental prostate cancer (Canada). *Cancer Causes Control* 2000;11:759-64.
70. Pierotti B, Altieri A, Talamini R, Montella M, Tavani A, Negri E, Franceschi S, La Vecchia C. Lifetime physical activity and prostate cancer risk. *Int J Cancer* 2005;114:639-42.
71. Jian L, Shen ZJ, Lee AH, Binns CW. Moderate physical activity and prostate cancer risk: a case-control study in China. *Eur J Epidemiol* 2005;20:155-60.
72. Torti DC, Matheson GO. Exercise and prostate cancer. *Sports Med* 2004;34:363-9.
73. Windsor PM, Nicol KF, Potter J. A randomized, controlled trial of aerobic exercise for treatment-related fatigue in men receiving radical external beam radiotherapy for localized prostate carcinoma. *Cancer* 2004;101:550-7.
74. Tokar EJ, Webber MM. Cholecalciferol (vitamin D3) inhibits growth and invasion by up-regulating nuclear receptors and 25-hydroxylase (CYP27A1) in human prostate cancer cells. *Clin Exp Metastasis* 2005;22:275-84.
75. Wang YR, Wigington DP, Strugnell SA, Knutson JC. Growth inhibition of cancer cells by an active metabolite of a novel vitamin D prodrug. *Anticancer Res* 2005;25:4333-9.
76. Moreno J, Krishnan AV, Peehl DM, Feldman D. Mechanisms of vitamin D-mediated growth inhibition in prostate cancer cells: inhibition of the prostaglandin pathway. *Anticancer Res* 2006;26:2525-30.
77. Chokkalingam AP, McGlynn KA, Gao YT, Pollak M, Deng J, Sesterhenn IA, Mostofi FK, Fraumeni JF Jr, Hsing AW. Vitamin D receptor gene polymorphisms, insulin-like growth factors, and prostate cancer risk: a population-based case-control study in China. *Cancer Res* 2001;61:4333-6.
78. Medeiros R, Morais A, Vasconcelos A, Costa S, Pinto D, Oliveira J, Lopes . The role of vitamin D receptor gene polymorphisms in the susceptibility to prostate cancer of a southern European population. *J Hum Genet* 2002;47:413-8.
79. Huang SP, Huang CY, Wu WJ, Pu YS, Chen J, Chen YY, Yu CC, Wu TT, Wang JS, Lee YH, Huang JK, Huang CH, Wu MT. Association of vitamin D receptor FokI polymorphism with prostate cancer risk, clinicopathological features and recurrence of prostate specific antigen after radical prostatectomy. *Int J Cancer* 2006;119:1902-7.
80. Perry HM 3rd, Horowitz M, Morley JE, Patrick P, Vellas B, Baumgartner R, Garry PJ. Longitudinal changes in serum 25-hydroxyvitamin D in older people. *Metabolism* 1999;48:1028-32.
81. Dixon T, Mitchell P, Beringer T, Gallacher S, Moniz C, Patel S, Pearson G, Ryan P. An overview of the prevalence of 25-hydroxy-vitamin D inadequacy amongst elderly patients with or without fragility fracture in the United Kingdom. *Curr Med Res Opin* 2006;22:405-15.
82. Visser M, Deeg DJ, Puts MT, Seidell JC, Lips P. Low serum concentrations of 25-hydroxyvitamin D in older persons and the risk of nursing home admission. *Am J Clin Nutr* 2006;84:616-22.
83. Hanchette CL, Schwartz GG. Geographic patterns of prostate cancer mortality. Evidence for a protective effect of ultraviolet radiation. *Cancer* 1992;70:2861-9.
84. Grant WB. A multicountry ecologic study of risk and risk reduction factors for prostate cancer mortality. *Eur Urol* 2004;45:271-9.
85. Rødsahl TE, Tretli S, Dahlback A, Moan J. Vitamin D3 from sunlight may improve the prognosis of breast, colon- and prostate cancer (Norway). *Cancer Causes Control* 2004;15:149-58.
86. Krickler A, Armstrong B. Does sunlight have a beneficial influence on certain cancers? *Prog Biophys Mol Biol* 2006;92:132-9.
87. Schwartz GG, Hanchette CL. UV, latitude, and spatial trends in prostate cancer mortality: all sunlight is not the same (United States). *Cancer Causes Control* 2006;17:1091-101.
88. Peehl DM. Vitamin D and prostate cancer risk. *Eur Urol* 1999;35:392-4.
89. Beer TM, Myrthue A. Calcitriol in cancer treatment: from the lab to the clinic. *Mol Cancer Ther* 2004;3:373-81.
90. Beer TM, Myrthue A. Calcitriol in the treatment of prostate cancer. *Anticancer Res* 2006;26:2647-51.

PAPER III

Immunoinflammatory Mechanisms in Lung Cancer Development: Is Leptin a Mediator?

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and Rui Medeiros, PhD*†

Abstract: This is a short review focusing on leptin immunoinflammatory mechanisms that ultimately may contribute to lung cancer development. We explored the complex and intricate interaction of leptin with immune cells to propose a pathway of inflammation-associated lung cancer development.

Key Words: Immunity, Leptin, Lung cancer, Inflammation.

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IMMUNOINFLAMMATORY RESPONSE AND LUNG CANCER

Immune cells constitute a critical component of host response to cancer,¹ although their role in cancer pathogenesis remains incompletely established. Epidemiological data indicate that chronic inflammation increases the risk of malignant transformation² and, therefore, that unresolved host immune reactivity may promote tumor development.³ In fact, persistent or recurrent immunoinflammatory up-regulation may induce or influence susceptibility to carcinogenesis by some known mechanisms (Fig. 1).⁴

Most lung cancer patients are smokers,⁵ and tobacco consumption is a well-established risk factor.⁶ A chronic inflammatory state is known to correlate with the decline in lung function among smokers^{7,8} and with lung cancer etiology.⁹ Chronic cigarette smoking retards mucociliary clearance of foreign particulates and secretions that favor a persistent inflammation, whereas the inhaled particles evoke vigorous lung and airway inflammatory responses.^{10–12} Initiation of the immunoinflammatory lung response is induced by exposure to inhaled antigenic particles and is followed by an expression pattern of chemokines and cytokines that may be influenced by the individual genetic background.^{13,14} This mechanism may predispose a patient to an amplified and longer immune response. The role of chemokines in lung cancer biology has been highlighted in a previous review.¹⁵

LEPTIN, INFLAMMATION, AND LUNG CANCER

Investigators have found that a functional polymorphism in the leptin gene (*LEP*-2548 G/A) was associated with increased risk for developing non-small cell lung cancer (odds ratio, 1.97; 95% confidence interval, 1.13–3.43) and to earlier onset of disease, and that carriers of the risk genotype simultaneously smokers had even higher risk for developing cancer of the lung (odds ratio, 4.82; 95% confidence interval, 1.05–22.17).¹⁶ These results support leptin's role in exacerbating existing cigarette smoke-induced lung inflammation that may lead to increased amplitude and duration of reaction, which has a major role in lung cancer pathogenesis.

Leptin is an adipocytokine that has been consistently implicated in lung physiology and pathophysiology. It is involved in fetal lung development and in adult normal lung cells' physiology or malignant proliferation.^{17–19} Besides a possible direct effect of leptin in normal and tumor lung cells, most importantly, there is strong evidence of leptin's up-regulatory role in the immunoinflammatory system,²⁰ supporting its role as a prominent interplay between inflammation and lung cancer (Figure 1). It is now well established that fat depots' function goes beyond structural, metabolic, and heat-insulating properties because of cytokines, growth factors, and hormone production that may prolong the pro-inflammatory microenvironment.

LEPTIN, INNATE IMMUNITY, AND CANCER

Although the immune surveillance hypothesis has received some experimental support, the net effect of inflammation and/or innate immune system activation is stimulation of tumor growth in most cases.²¹ Leptin affects both innate and adaptive immunity. In innate immunity, leptin directly and indirectly modulates the activity and function of neutrophils by increasing chemotaxis and production of oxygen radicals,²² increases phagocytosis by monocytes/macrophages, and activates and promotes macrophage cell chemotaxis.²³ Cumulatively, it was shown that recombinant human leptin administration increased both C-reactive protein and P-selectin,²⁴ whereas an association was observed between the concentration of VCAM-1 and ICAM-1 and circulating leptin levels.^{25,26} These evidences support an indirect role for leptin in chemotaxis and, consequently, in attraction of inflammatory cells through an action in adhesion molecules, further increasing the magnitude of inflammation.

The inflammatory process initiated by infection, cellular damage, tumor cells, reactive oxygen species production,

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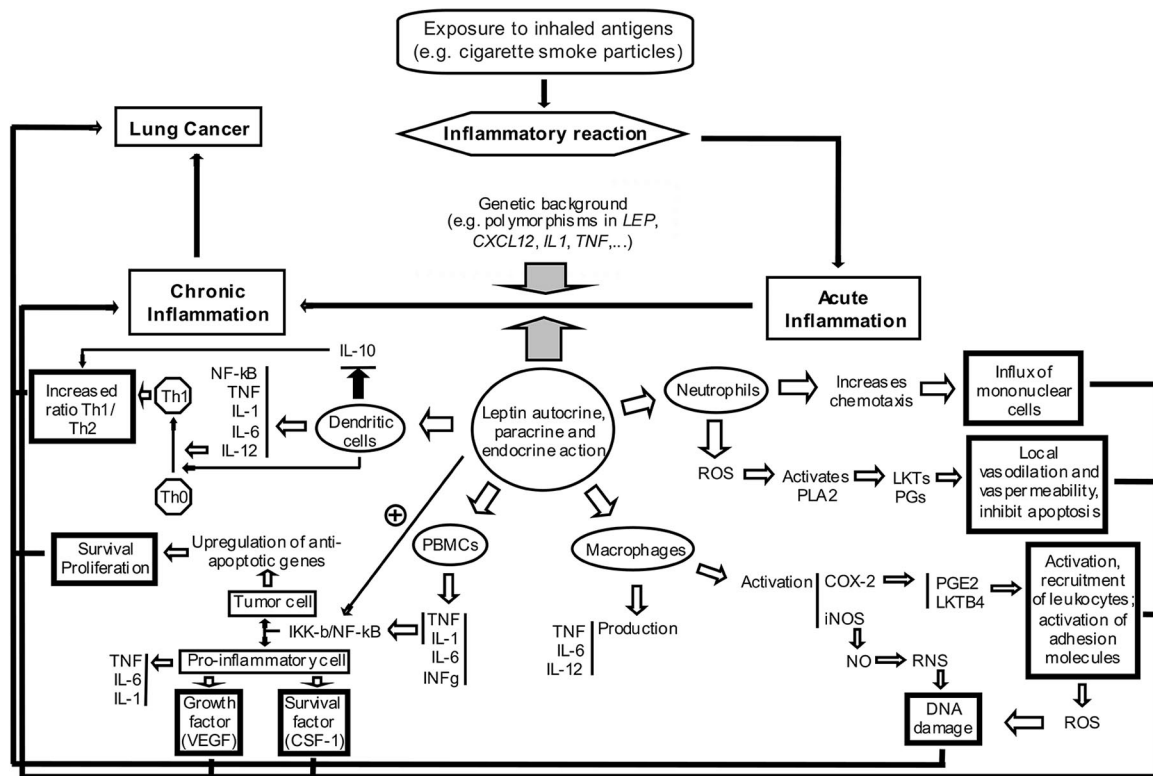


FIGURE 1. Leptin's role in immunoinflammatory-mediated lung cancer development. After exposure to inhaled antigens (e.g., smoke particles) follows acute inflammation that under normal circumstances is self-limited. However, depending on the magnitude and duration of exposure to pro-inflammatory inhaled antigens and on the individual's genetic background (polymorphisms in genes that express pro-inflammatory molecules), the inflammatory reaction may last longer and/or may be increased. Leptin may have a central role in chronic inflammation development and maintenance of chronic inflammation, through autocrine, paracrine, and endocrine mechanisms. There is a broad range of action for leptin in immune cells. Leptin stimulates and activates neutrophils, macrophages, peripheral blood mononuclear cells, dendritic cells, and T cells, whose products, ultimately, may induce chronic inflammation and lung cancer carcinogenesis. Th0 = naive T cell, Th1 = T helper 1, Th2 = T helper 2, NF- κ B = nuclear factor κ B, TNF = tumor necrosis factor- α , VEGF = vascular endothelial growth factor, PLA2 = phospholipase A2, LKTs = leukotrienes, PGs = prostaglandins, IFN γ = interferon- γ , COX-2 = cyclooxygenase 2, iNOS = inducible nitric oxide synthase, NO = nitric oxide, CSF = colony stimulating factor, ROS = reactive oxygen species, RNS = reactive nitrogen species, PBMCs = peripheral blood mononuclear cells.

inhaled particles from tobacco, or environment pollutants stimulates the synthesis of interleukin (IL)-1, IL-6, leptin, and tumor necrosis factor (TNF) by macrophages and stromal and endothelial cells.^{27,28} These molecules are partially responsible for the expression of acute-phase inflammatory response elements, such as cyclooxygenase-2, nuclear factor κ B, and C-reactive protein,²⁹ and are associated with the induction of leukocyte recruitment and activating-adhesion molecules, P-selectin, E-selectin, VCAM and ICAM.^{28,30} Activated leukocytes produce large quantities of reactive oxygen species that will cause oxidative damage to surrounding cells and enhance risk for inflammation-mediated cytotoxicity and DNA damage in normal cells. The role of leptin in up-regulating reactive oxygen species production through an effect in monocytes or indirectly in endothelial cells by an increase in monocyte-chemoattractant protein 1 is well known.^{31,32} Reactive oxygen species derived from inflammation is an important endogenous carcinogenic factor, which is increased by long-term chronic inflammation.

Santos-Alvarez et al.³³ observed a stimulatory effect of leptin on peripheral blood mononuclear cell production of TNF and IL-6. Furthermore, leptin's role in peripheral blood mononuclear cell proliferation and activation is mediated by activation of the leptin receptor in these cells,³⁴ inducing a pattern of cytokine release compatible with the induction of a T-helper 1 immune response,³⁵ which is associated with a negative prognostic factor in patients with non-small cell lung cancer. Leptin mediates the up-regulation of dendritic cells' function and survival and decreases production of IL-10, which further contributes further to T-helper 1 immune phenotype.³⁶ Simultaneously, leptin up-regulates the secretion of IL-1, IL-6, IL-12, TNF, and MIP-1 α by dendritic cells.³⁶

Activation of the inhibitor of nuclear factor- κ B kinase β -dependent nuclear factor- κ B molecular pathway is a molecular mechanism that connects inflammation and cancer. Its activation increases tumor growth and progression in different cell types through activation of gene targets of pro-inflammatory cytokines and chemokines, such as TNF, IL-1,

and IL-6.³⁷ The up-regulation of immunomodulators TNF, IL-1, IL-6, and prostaglandin E₂ through leptin via the nuclear factor- κ B pathway³⁸ further strengthens the up-regulated pro-inflammatory profile in the tumor microenvironment. Furthermore, TNF and IL-1 stimulate leptin production by adipocytes,^{39,40} further contributing to prolonged leptin-induced inflammation.

After leptin's stimulatory effect, macrophages also increase the production of the pro-inflammatory enzyme cyclooxygenase 2 and their products leukotriene (LKT) B₄ and prostaglandin (PG) E₂, and augments inducible nitric oxide synthase (iNOS) activity.^{41,42} Production of reactive nitrogen species (RNS) in response to inflammation-induced iNOS overexpression might induce generation and accumulation of additional mutations that drive tumor progression.⁴³ Several studies support a stimulatory action of leptin in endothelial NOS (eNOS) and inducible NOS (iNOS) activities, which results in increased NO production by adipocytic and endothelial cells.^{44,45} Raso et al⁴² showed that leptin is a potent synergistic factor that cooperates with IFN- γ to increase the expression of iNOS and cyclooxygenase 2. The cyclooxygenase-2 enzyme up-regulates the production of the vasoactive prostaglandins (PGs) and leukotrienes (LKTs) responsible for increasing the amount of local vasodilation and vasopermeability, and cumulatively, for enhanced inflammation through leukocytes accumulation. The PGs also inhibit apoptosis and stimulate angiogenesis and invasiveness.⁴⁶ As a result of chronic inflammation, constant exposure to cyclooxygenase 2-derived prostaglandins may also enhance carcinogenic risk by reducing apoptosis and increasing the likelihood of mutant cell survival and cancer development.

LEPTIN, ADAPTIVE IMMUNITY, AND CANCER

Apparently, T cells may also contribute to tumor growth because, in the progression phase, they are the main source of IL-6,⁴⁷ and their overall contribution might depend on the balance between tumor-promoting cytokines, such as IL-6, and tumor-suppressor cytokines, such as IL-10 and TGF- β .³⁷ Leptin, which increases IL-6 production and decreases IL-10 secretion, provides an interesting clue to the understanding of leptin's association with inflammation and cancer, although much research in inflammatory cancer models is required to clarify this issue.

In adaptive immunity, leptin affects the generation, maturation, and survival of thymic T cells by reducing their rate of apoptosis.⁴⁸ Leptin orients naive T-cell proliferation and differentiation to TH1 phenotype³⁶ and promotes the switch toward TH1 immune responses on memory T cells by increasing IFN γ and TNF secretion and stimulating the production of IgG₂ α by B cells.⁴⁹ The TH1/TH2 cytokine balance is preserved in normal individuals, but during inflammation, the imbalance in the TH1/TH2 cytokine response overcomes. In patients with non-small cell lung cancer, a high TH1/TH2 ratio in peripheral blood is a negative prognostic factor.⁵⁰

SUMMARY

Recent developments in leptin immunological pathways suggest a previously unappreciated complexity of cancer cell-

normal cell-immunoinflammatory cell cross-talk. This interaction found in the inflammatory medium may deregulate and increase the magnitude and duration of inflammation, promoting tumor development. Leptin, a pleiotrophic hormone synthesized mainly by adipocytes, may be an important interplay between immunoinflammatory up-regulation and lung cancer development, warranting further studies in this field.

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REFERENCES

1. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. *Nat Rev Cancer* 2004;4:11–22.
2. Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. *Proc Natl Acad Sci USA* 1995;92:5258–5265.
3. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860–867.
4. Schottenfeld D, Beebe-Dimmer J. Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. *CA Cancer J Clin* 2006;56:69–83.
5. Boyle P, Autier P, Bartelink H, et al. European Code Against Cancer and scientific justification: third version (2003). *Ann Oncol* 2003;14:973–1005.
6. Crispo A, Brennan P, Jockel KH, et al. The cumulative risk of lung cancer among current, ex- and never-smokers in European men. *Br J Cancer* 2004;91:1280–1286.
7. Burchfiel CM, Marcus EB, Curb JD, et al. Effects of smoking and smoking cessation on longitudinal decline in pulmonary function. *Am J Respir Crit Care Med* 1995;151:1778–1785.
8. Chinn S, Jarvis D, Melotti R, et al. Smoking cessation, lung function, and weight gain: a follow-up study. *Lancet* 2005;365:1629–1635.
9. Ballaz S, Mulshine JL. The potential contributions of chronic inflammation to lung carcinogenesis. *Clin Lung Cancer* 2003;5:46–62.
10. Wanner A, Salathe M, O'Riordan TG. Mucociliary clearance in the airways. *Am J Respir Crit Care Med* 1996;154:1868–1902.
11. Matsumoto K, Aizawa H, Inoue H, et al. Eosinophilic airway inflammation induced by repeated exposure to cigarette smoke. *Eur Respir J* 1998;12:387–394.
12. Castro P, Legora-Machado A, Cardilo-Reis L, et al. Inhibition of interleukin-1 β reduces mouse lung inflammation induced by exposure to cigarette smoke. *Eur J Pharmacol* 2004;498:279–286.
13. Coelho A, Calçada C, Catarino R, Pinto D, Fonseca G, Medeiros R. CXCL12-3' A polymorphism and lung cancer metastases protection: new perspectives in immunotherapy? *Cancer Immunol Immunother* 2006;55:639–643.
14. Lind H, Zienoldiny S, Ryberg D, Skaug V, Phillips DH, Haugen A. Interleukin 1 receptor antagonist gene polymorphism and risk of lung cancer: a possible interaction with polymorphisms in the interleukin 1 β gene. *Lung Cancer* 2005;50:285–290.
15. Arenberg D. Chemokines in the biology of lung cancer. *J Thorac Oncol* 2006;1:287–288.
16. Ribeiro R, Araujo AP, Coelho A, et al. A functional polymorphism in the promoter region of leptin gene increases susceptibility for non-small cell lung cancer. *Eur J Cancer* 2006;42:1188–1193.
17. Hoggard N, Hunter L, Duncan JS, Williams LM, Trayhurn P, Mercer JG. Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta. *Proc Natl Acad Sci USA* 1997;94:11073–11078.
18. Tsuchiya T, Shimizu H, Horie T, Mori M. Expression of leptin receptor in lung: leptin as a growth factor. *Eur J Pharmacol* 1999;365:273–279.

19. Meissner U, Hanisch C, Ostreicher I, et al. Differential regulation of leptin synthesis in rats during short-term hypoxia and short-term carbon monoxide inhalation. *Endocrinology* 2005;146:215–220.
20. La Cava A, Matarese G. The weight of leptin in immunity. *Nat Rev Immunol* 2004;4:371–379.
21. Philip M, Rowley DA, Schreiber H. Inflammation as a tumor promoter in cancer induction. *Semin Cancer Biol* 2004;14:433–439.
22. Caldefie-Chezet F, Poulin A, Vasson MP. Leptin regulates functional capacities of polymorphonuclear neutrophils. *Free Radic Res* 2003;37:809–814.
23. Fenton JI, Hursting SD, Perkins SN, Hord NG. Leptin induces an Apc genotype-associated colon epithelial cell chemokine production pattern associated with macrophage chemotaxis and activation. *Carcinogenesis* 2006 Aug 4 [Epub ahead of print].
24. Canavan B, Salem RO, Schurgin S, et al. Effects of physiological leptin administration on markers of inflammation, platelet activation, and platelet aggregation during caloric deprivation. *J Clin Endocrinol Metab* 2005;90:5779–5785.
25. Kent JW, Comuzzie AG, Mahaney MC, et al. Intercellular adhesion molecule-1 concentration is genetically correlated with insulin resistance, obesity, and HDL concentration in Mexican Americans. *Diabetes* 2004;53:2691–2695.
26. Porreca E, Di Febbo C, Fusco L, Moretta V, Di Nisio M, Cuccurullo F. Soluble thrombomodulin and vascular adhesion molecule-1 are associated to leptin plasma levels in obese women. *Atherosclerosis* 2004;172:175–180.
27. Mastronardi CA, Yu WH, Rettori V, McCann S. Lipopolysaccharide-induced leptin release is not mediated by nitric oxide, but is blocked by dexamethasone. *Neuroimmunomodulation* 2000;8:91–97.
28. Rahman A, Anwar KN, Malik AB. Protein kinase C-zeta mediates TNF-alpha-induced ICAM-1 gene transcription in endothelial cells. *Am J Physiol Cell Physiol* 2000;279:C906–C914.
29. Feng L, Xia Y, Garcia GE, Hwang D, Wilson CB. Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysaccharide. *J Clin Invest* 1995;95:1669–1675.
30. Manning AM, Bell FP, Rosenbloom CL, et al. NF-kappa B is activated during acute inflammation in vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. *J Inflamm* 1995;45:283–296.
31. Bouloumie A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. *FASEB J* 1999;13:1231–1238.
32. Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem* 2001;276:25096–25100.
33. Santos-Alvarez J, Goberna R, Sanchez-Margalet V. Human leptin stimulates proliferation and activation of human circulating monocytes. *Cell Immunol* 1999;194:6–11.
34. Sanchez-Margalet V, Martin-Romero C, Santos-Alvarez J, Goberna R, Najib S, Gonzalez-Yanes C. Role of leptin as an immunomodulator of blood mononuclear cells: mechanisms of action. *Clin Exp Immunol* 2003;133:11–19.
35. Zarkesh-Esfahani H, Pockley G, Metcalfe RA, et al. High-dose leptin activates human leukocytes via receptor expression on monocytes. *J Immunol* 2001;167:4593–4599.
36. Mattioli B, Straface E, Quaranta MG, Giordani L, Viora M. Leptin promotes differentiation and survival of human dendritic cells and licenses them for Th1 priming. *J Immunol* 2005;174:6820–6828.
37. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005;5:749–759.
38. Lappas M, Permezel M, Rice GE. Leptin and adiponectin stimulate the release of proinflammatory cytokines and prostaglandins from human placenta and maternal adipose tissue via nuclear factor-kappaB, peroxisomal proliferator-activated receptor-gamma and extracellularly regulated kinase 1/2. *Endocrinology* 2005;146:3334–3342.
39. Janik JE, Curti BD, Considine RV, et al. Interleukin 1 alpha increases serum leptin concentrations in humans. *J Clin Endocrinol Metab* 1997;82:3084–3086.
40. Zhang HH, Kumar S, Barnett AH, Eggo MC. Tumour necrosis factor-alpha exerts dual effects on human adipose leptin synthesis and release. *Mol Cell Endocrinol* 2000;159:79–88.
41. Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. *J Leukoc Biol* 2000;68:437–446.
42. Raso GM, Pacilio M, Esposito E, Coppola A, Di Carlo R, Meli R. Leptin potentiates IFN-gamma-induced expression of nitric oxide synthase and cyclo-oxygenase-2 in murine macrophage J774A. 1. *Br J Pharmacol* 2002;137:799–804.
43. Goodman JE, Hofseth LJ, Hussain SP, Harris CC. Nitric oxide and p53 in cancer-prone chronic inflammation and oxyradical overload disease. *Environ Mol Mutagen* 2004;44:3–9.
44. Mastronardi CA, Yu WH, McCann SM. Resting and circadian release of nitric oxide is controlled by leptin in male rats. *Proc Natl Acad Sci USA* 2002;99:5721–5726.
45. Vecchione C, Maffei A, Colella S, et al. Leptin effect on endothelial nitric oxide is mediated through Akt-endothelial nitric oxide synthase phosphorylation pathway. *Diabetes* 2002;51:168–173.
46. Fosslien E. Biochemistry of cyclooxygenase (COX)-2 inhibitors and molecular pathology of COX-2 in neoplasia. *Crit Rev Clin Lab Sci* 2000;37:431–502.
47. Becker C, Fantini MC, Schramm C, et al. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 2004;21:491–501.
48. Howard JK, Lord GM, Matarese G, et al. Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice. *J Clin Invest* 1999;104:1051–1059.
49. Martin-Romero C, Santos-Alvarez J, Goberna R, Sanchez-Margalet V. Human leptin enhances activation and proliferation of human circulating T lymphocytes. *Cell Immunol* 2000;199:15–24.
50. Ito N, Suzuki Y, Taniguchi Y, Ishiguro K, Nakamura H, Ohgi S. Prognostic significance of T helper 1 and 2 and T cytotoxic 1 and 2 cells in patients with non-small cell lung cancer. *Anticancer Res* 2005;25:2027–2031.

PAPER IV

Genetic Polymorphism in *EGF* Is Associated with Prostate Cancer Aggressiveness and Progression-Free Interval in Androgen Blockade – Treated Patients

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Abstract **Purpose:** Most prostate cancer patients develop resistance to androgen deprivation treatment, resulting in hormone resistance. Epidermal growth factor (EGF) activates several pro-oncogenic intracellular pathways inducing proliferation, differentiation, and tumorigenesis in epithelial cells. The EGF-EGF receptor pathway seems to be especially relevant in hormone-resistant prostate cancer stage. A single nucleotide polymorphism G>A in +61 locus of *EGF* gene has been described, in which A homozygous carriers express significantly less EGF protein compared with G allele carriers. Our purpose was to investigate the potential prognostic and predictive role of *EGF* functional genetic variant +61G>A in prostate cancer patients submitted to androgen blockade therapy (ABT). **Experimental Design:** We conducted a case-control study in prostate cancer patients treated with ABT ($n = 123$) and in healthy controls without evidence of cancer ($n = 152$). Cumulatively, a follow-up study (median follow-up, 37 months) was undertaken to evaluate response to ABT therapy in prostate cancer patients. *EGF* +61G>A genotypes were detected by PCR-RFLP. **Results:** We found increased risk in G carriers, after age-adjusted regression analysis, for being diagnosed with Gleason ≥ 7 and with metastatic disease compared with control group (CG; age-adjusted odds ratio, 3.37, $P = 0.004$ and age-adjusted odds ratio, 2.61, $P = 0.043$, respectively). Kaplan-Meier survival analysis and log-rank test showed an influence of *EGF* +61G>A polymorphism in time to relapse during ABT ($P = 0.018$). **Conclusions:** *EGF* functional polymorphism may contribute to earlier relapse in ABT patients, supporting the involvement of *EGF* as an alternative pathway in hormone-resistant prostatic tumors. Furthermore, our results lend support to EGF-EGF receptor pathway as an additional therapeutic target during hormonal treatment.

Prostate cancer is the most common nonskin cancer among men in most Western populations (1). Initial treatment in early stages of disease is usually prostatectomy or radiation, which removes or destroys tumoral cells that are confined within the

prostate capsule. Patients diagnosed in advanced stages are frequently submitted to endocrine manipulation with androgen blockade therapy (ABT; ref. 2), although most men will eventually fail this therapy and die from recurrent hormone-resistant prostate cancer (HRPC). HRPC is a common lethal form of prostate cancer that frequently metastasizes and eventually leads to death (3). Therefore, it is important to understand the mechanisms involved in androgen-independent progression.

The androgen pathway is commonly accepted to have a critical role in survival of prostatic cells. Nevertheless, progression into advanced prostate cancer and incurable forms has also been associated with the activation of other cascades mediated by growth factors responsible for the balance between cell growth rate and apoptosis (4). Several pathways have been proposed to be involved in HRPC development, and their understanding will pave the way to more effective therapies. In fact, prostate cell growth and differentiation in the absence of androgens may be due to alternative intracellular signaling pathways, such as the ErbB pathway, which implicates epidermal growth factor (EGF) as a probable ligand (5). Additionally, cross-talk between these signaling transduction pathways has been implicated in prostate cancer survival in an androgen-poor environment (6, 7).

Cumulatively, studies by Ye et al. (8) showed that there is a positive feedback stimulation loop between androgen-induced

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Table 1. Allelic and genotype frequencies and OR analysis in prostate cancer patients versus controls according to *EGF* +61 G>A polymorphism

	Prostate cancer, n (%)	Controls, n (%)	aOR (95% CI)	P
Genotypes				
AA	33 (0.27)	57 (0.38)	1.94 (0.99-3.79)	0.053
AG/GG	90 (0.73)	95 (0.62)		
Alleles				
A	124 (0.50)	167 (0.55)	1.22 (0.84-1.70)	0.290
G	122 (0.50)	137 (0.45)		

gene transcription and EGF-stimulated signal transduction, as one could stimulate the synthesis of the receptors for the other.

The EGF receptor (EGFR) is proposed to participate in the pathogenesis or maintenance of several human cancers of epithelial origin. In prostate cancer cells, EGFR ligands are frequently elevated and EGFR itself is commonly overexpressed (9). Furthermore, EGFR expression increases during progression to a hormone-resistant stage (10).

EGF is encoded by *EGF* gene, located in chromosome 4q25-q27. Recently, Shahbazi et al. (11) identified a functional G>A single nucleotide polymorphism at position +61 in the 5' untranslated region of the *EGF* gene. *In vitro* studies showed that G carriers have an increased EGF production in cultured peripheral blood mononuclear cells, glioma cells, glioblastoma cells, and breast cancer cells (11–13).

Previous studies found controversial results about the association between *EGF* polymorphism and cancer development and prognosis (11–16). Nevertheless, the present study is the first to evaluate the relevance the *EGF* functional polymorphism in HRPC patients.

Materials and Methods

Study population. One hundred and twenty-three patients (ages 71.7 ± 7.4 y) submitted to ABT between 1995 and 2006 at Lisbon Medical Centre (Central Region) were included in the study. All cases were submitted to prostate biopsy and had histopathologically confirmed diagnosis; the median follow-up time was 37 mo (range, 2-137 mo). All cases were considered with complete data for statistical analysis, from which 35% of the cases had localized prostate disease (T₁-T_{2b}), 36% of the cases had locally advanced disease (T₃-T₄), and 29% of the cases had metastatic disease (N⁺ and/or M⁺). The types of hormonal treatment were as follows: antiandrogens plus luteinizing hormone-releasing hormone agonist combination therapy (84.9%), luteinizing hormone-releasing hormone agonist alone (9.2%), and antiandrogens alone (5.9%). Hormone resistance was evaluated through prostate-specific antigen recurrence, which was defined as

two consecutive increasing prostate-specific antigen values >1.0 ng·mL⁻¹ and differing by >0.2 ng·mL⁻¹.

Men ages >45 y, without known history of cancer, were recruited from the Portuguese Institute of Oncology-Porto Centre blood donor's bank (ages 55.6 ± 8.5 y) and included in the control group ($n = 152$). Study was conducted according to Helsinki Declaration principles. Antecubital peripheral venous blood sample was collected from each subject enrolled in the study followed by DNA extraction from WBC fraction according to salting out procedure (17).

EGF +61 G>A polymorphism genotyping. *EGF* +61 G>A variants were analyzed through PCR-RFLP method, as described in a previous report (16). Briefly, DNA was amplified in a 50-μL reaction mixture containing *EGF* +61 G>A primers (forward, 5'-TGTCCTAAAGGAAAGGAGGT-3'; reverse, 5'-TTCACAGAGTTTAAACAGCCC-3'), 1× PCR buffer, 1 unit Taq polymerase, 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleotide triphosphates, and 100 ng DNA.

PCR products (242 bp) were incubated overnight with *AluI* restriction endonuclease at 37°C. The polymorphism was defined by the presence (A) or absence (G) of an additional restriction site.

Quality control procedures implemented for the *EGF* genotype analyses included double sampling in ~10% of the samples to assess reliability and the use of negative controls to step-away false positives. Two authors obtained the results independently, and the ambiguous results were reanalyzed.

Statistical analysis. Genotype proportions among groups were compared with Pearson χ^2 test. Central tendency measures and comparisons between groups were evaluated through independent sample Mann-Whitney *U* test. Unconditional logistic regression analysis was used to compute odds ratio (OR) and 95% confidence interval (95% CI) estimating the association of genotypes to prostate cancer susceptibility, and to stratified Gleason score, metastatic disease, and hormone resistance development, while adjusting for age in all these variables [age-adjusted OR (aOR)]. The Kaplan-Meier method and log-rank test were used to compare genotype influence in the disease-free survival interval.

Results

The functional *EGF* +61 G>A polymorphism genotype distribution in patients and controls is described in Table 1. Using

Table 2. Risk for being diagnosed with higher Gleason grade (≥ 7) and with metastatic prostate cancer in *EGF* +61 G carriers

	Controls, n (%)	Metastatic prostate cancer, n (%)	Gleason ≥ 7 , n (%)
AA	57 (0.38)	8 (0.22)	13 (0.19)
AG/GG	95 (0.62)	28 (0.78)	54 (0.81)
aOR*		2.61	3.37
95% CI		1.03-6.60	1.47-7.73
P		0.043	0.004

*aOR in comparison with control group.

Table 3. Prostate cancer patient genotype frequencies according to tumor staging and prostate-specific antigen level

	Genotypes		P
	AA, n (%)	AG/GG, n (%)	
Staging			
Localized (T ₁ -T _{2b} stage)	11 (0.26)	32 (0.74)	
Locally advanced (T ₃ -T ₄ stage)	14 (0.32)	30 (0.68)	
Metastatic (N ⁺ and/or M ⁺ stage)	8 (0.22)	28 (0.78)	0.612*
Prostate-specific antigen level at diagnosis (ng·mL ⁻¹) [†]	33.2 ± 9.7	62.7 ± 16.1	0.901 [‡]

* χ^2 test.[†]Average ± SE.[‡]Mann-Whitney U test.

the recessive model, frequencies for homozygous AA and AG/GG genotypes were, respectively, 0.27 and 0.73 for prostate cancer patients and 0.38 and 0.62 in the control group. Although the association is not statistically significant, there is a trend to an overrepresentation of AG/GG genotype in prostate cancer group compared with normal controls (aOR, 1.94; 95% CI, 0.99-3.79; $P = 0.053$).

Table 2 presents EGF +61 G>A genotype distribution and age-adjusted risk for being diagnosed with an aggressive prostate cancer phenotype (e.g., presence of metastatic disease or Gleason grade >7). Carriers of the G allele have a significantly higher age-adjusted risk for being diagnosed with metastatic prostate cancer (aOR, 2.61; 95% CI, 1.03-6.60; $P = 0.043$) and with higher Gleason grade tumors (≥ 7 ; aOR, 3.37; 95% CI, 1.47-7.73; $P = 0.004$).

Within the group of patients, EGF +61 G>A functional polymorphism was not associated with disease stage and prostate-specific antigen serum level at diagnosis (Table 3). Table 4 shows the influence of EGF +61 G>A polymorphism in the response to ABT. Prostate cancer patients who developed hormone resistance had similar genotype frequencies to hormone respondents ($P = 0.468$).

Kaplan-Meier survival curves (Fig. 1) and log-rank test (Mantel-Cox) show that EGF +61 G>A polymorphism is associated with progression-free interval cumulative probability. Median estimated cumulative survival was significantly lower in AG/GG carriers compared with AA homozygous (15.2 and 87.4 months, respectively; $P = 0.018$), showing an earlier relapse in prostate cancer patients following hormone ablation therapy.

Table 4. Hormone resistance in prostate cancer patients submitted to ABT according to EGF +61 G>A polymorphism

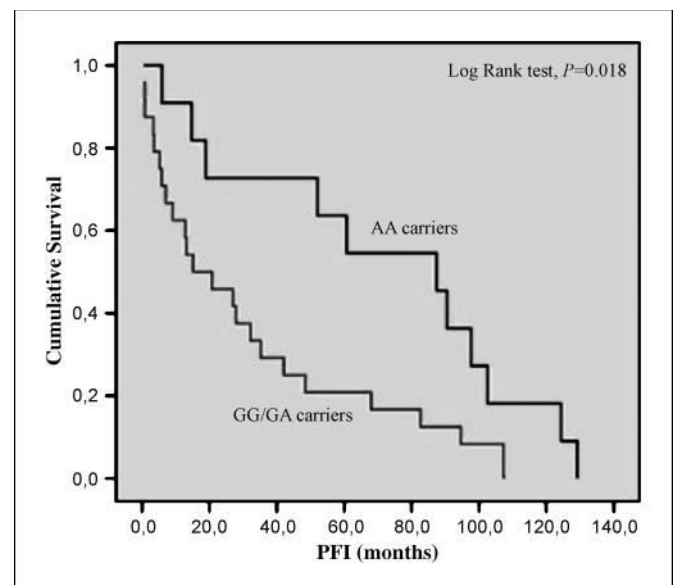
	Genotypes		P
	AA, n (%)	AG/GG, n (%)	
Hormone resistance			
No	22 (0.67)	66 (0.73)	
Yes	11 (0.33)	24 (0.27)	0.468*

* χ^2 test.

Discussion

Patients with local or distant metastatic prostate cancer are usually treated primarily through pharmacologic androgen suppression (2). This hormonal therapy is initially efficient, although the majority of patients will subsequently become unresponsive to androgen inhibition (18). HRPC is a complex and heterogeneous form of prostate cancer with a high capacity of progression and metastization (4).

EGF has been described as one of the most influent mitogenic factors in prostate function regulation (19). Furthermore, its receptor is overexpressed in prostate tumors, and the EGF-EGFR pathway is involved in hormone resistance development (9). In prostate cancers, EGFR is weakly expressed in the benign gland and in areas of low-grade prostatic intraepithelial neoplasia, whereas it is highly expressed in high-grade prostatic intraepithelial neoplasia and in neoplastic cells (20). Expression levels of EGF and EGFR in prostate cancer cells seem to be enhanced during disease progression to HRPC and metastatic prostate cancer (10).

**Fig. 1.** Kaplan-Meier analysis of progression-free interval (PFI) in prostate cancer androgen blockade – treated patients according to EGF +61 G>A polymorphism.

Through overproduction of EGFR ligands such as EGF, transforming growth factor- α , amphiregulin, heparin-binding EGF, and betacellulin, cancer cells can stimulate their own proliferation through an autocrine mechanism (21–23). *In vitro* studies show that some EGFR ligands, such as amphiregulin, are overexpressed in prostate cancer cells (PC3; ref. 24), emphasizing the relevance of further studies in other ligands. Cumulatively, *in vitro* studies revealed that EGFR signaling pathway inhibition reduces androgen-independent DU145 and PC3 prostate cancer cell growth through interference in G₁-S cell cycle progression phase (25).

Although the *EGF* +61 G>A polymorphism is localized in a noncoding region and the exact mechanism by which it affects EGF production is still undetermined, this locus seems to be involved in *EGF* regulation because it affects *EGF* expression (11, 13). It is hypothesized that this polymorphism might influence EGF through differential *EGF* mRNA processing and degradation or by providing a binding site for transcription factors.

Conversely, it is expected that G carriers may have a higher EGF availability in tumor environment. *EGF* +61 G>A polymorphism has been the subject of investigation in several case-control studies, involving other cancer types, with controversial results (11–15).

A recent investigation using prostatic tumor cell expression signatures showed an attenuated androgen signaling signature in high-grade and metastatic prostate cancer, eventually reflecting and providing support for the clinical association of grade with prognosis (26). In our study, risk for being diagnosed with a high Gleason grade is significantly increased in G carriers (aOR, 3.37; 95% CI, 1.47–7.73). Concomitantly, we observed a trend toward an increased risk to develop prostate cancer in *EGF* +61 G carriers (aOR, 1.94; 95% CI, 0.99–3.79), although this association was not statistically significant. Several studies have previously shown that growth factors promote tissue proliferation and malignant transformation (27). Accordingly, results from our study support that this genetic variant, which influences *EGF* expression and serum levels, may have an effect in prostate cancer development and in higher grade at diagnosis. In fact, some authors (28) refer that aberrant growth and differentiation are due to inappropriate cellular environment. Our results suggest that *EGF* +61 G>A polymorphism, which represents a functional modification in *EGF* expression since birth and throughout life, exposes AG/GG carriers to higher EGF concentrations, inducing modifications in prostate normal and tumoral cell microenvironment, leading to different patterns of cell differentiation, and contributing to a higher Gleason grade at diagnosis.

We found a significantly higher risk for metastasis in G carriers ($P = 0.043$). Growth factors, such as EGF, have an important role in migration and invasion of cancer cells. They can disrupt different pathways and contribute to metastatization. The EGFR can interact with the integrin $\alpha_6\beta_4$ and promote cell migration through activation of phosphatidylinositol 3-kinase and other downstream pathways (29–31). Cumulatively, previous studies

showed a significant association between EGFR activation and the acquisition of the invasive phenotype (32–34).

We hypothesize that in G carriers, which express significantly more *EGF*, the EGF-EGFR pathway may be up-regulated, inducing a metastatic tumor phenotype.

The HRPC is a multistep and multievent process with different molecular patterns throughout development (35), involving changes in growth factor signaling. Several reports support an interaction of EGFR and EGF-EGFR pathway with androgen receptor (AR) pathway in prostate cancer cells, although the role of AR in hormone resistance development is still controversial. Furthermore, AR is also overexpressed in hormone-resistant tumors (36). Recently, Bonaccorsi et al. (37) showed that expression of AR affects clathrin-mediated endocytosis pathway of EGFR, suggesting that AR-positive prostatic cells have a less aggressive phenotype due to interference in proinvasive EGFR signaling (37). Moreover, studies in prostate cancer cell cultures indicated that androgen deprivation could favor the progression to HRPC states by up-regulating the EGFR pathway (38).

Our results of earlier hormone resistance in hormone-treated prostate cancer patients agree with these previous findings because the lack of ligands to AR due to ABT will decrease AR signaling, inducing a more aggressive phenotype due to EGF-EGFR alternative signaling pathway. Alternatively, we hypothesize that because *EGF* AG/GG carriers have increased expression of *EGF* and because the AR pathway can be activated by other growth factors, including EGF, tumoral cells of G carriers may be exposed to higher EGF concentrations through both autocrine and paracrine mechanisms, inducing a faster progression to hormone resistance.

Taken together, these results suggest that simultaneous blockade of AR and EGFR pathways may be appropriate for prostate cancer treatment with effect in the time to HRPC development and in prognosis. Accordingly, *in vitro* and *in vivo* studies evidence growth suppression in HRPC cells, after tyrosine kinase inhibitor or anti-EGFR monoclonal antibody therapies combined with other chemotherapeutic drugs (39–42). Nevertheless, clinical trials of tyrosine kinase inhibitor in hormone-refractory metastatic prostate cancer patients did not effectively increase clinical response (43, 44). Further studies in larger populations and varying chemotherapeutic regimens to include tyrosine kinase inhibitor or EGFR monoclonal antibodies should be undertaken to ascertain the relevance of EGF-EGFR pathway in HRPC progression and the effectiveness of anti-EGFR treatments in these patients.

The present study suggests that the *EGF* functional polymorphism may contribute to the establishment of a genetic profile to ABT efficacy in prostate cancer patients before their involvement in hormonal treatment and supports the involvement of EGF as an alternative molecular pathway in hormone-resistant prostatic tumors. Further studies in larger samples are required to strengthen these results and guide future investigations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Hsing AW, Chokkalingam AP. Prostate cancer epidemiology. *Front Biosci* 2006;11:1388–413.
2. Pronzato P, Rondini M. Hormonotherapy of advanced prostate cancer. *Ann Oncol* 2005;4:iv80–4.
3. Calabro F, Sternberg CN. Current indications for chemotherapy in prostate cancer patients. *Eur Urol* 2007; 51:17–26.
4. Harper ME, Goddard L, Glynn-Jones E, et al. Multiple responses to EGF receptor activation and their abrogation by a specific EGF receptor tyrosine kinase inhibitor. *Prostate* 2002;52:59–68.
5. Feldman BJ, Feldman D. The development of

- androgen-independent prostate cancer. *Nat Rev Cancer* 2001;1:34–45.
6. El Sheikh SS, Domin J, Abel P, Stamp G, Lalani E-N. Androgen-independent prostate cancer: potential role of androgen and ErbB receptor signal transduction crosstalk. *Neoplasia* 2003;5:99–109.
 7. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat Med* 1999;5:280–5.
 8. Ye D, Mendelsohn J, Fan Z. Androgen and epidermal growth factor down-regulate cyclin-dependent kinase inhibitor p27Kip1 and costimulate proliferation of MDA PCa 2a and MDA PCa 2b prostate cancer cells. *Clin Cancer Res* 1999;5:2171–7.
 9. Vicentini C, Festuccia C, Gravina GL, Angellucci A, Marronaro A, Bologna M. Prostate cancer cell proliferation is strongly reduced by the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 *in vitro* on human cell lines and primary cultures. *J Cancer Res Clin Oncol* 2003;129:165–75.
 10. Di Lorenzo G, Tortora G, D'Armiento F, et al. Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clin Cancer Res* 2002;8:3438–44.
 11. Shahbazi M, Pravica V, Nasreen N, et al. Association between functional polymorphism in EGF gene and malignant melanoma. *Lancet* 2002;359:397–401.
 12. Bhowmick D, Zhuang Z, Wait SD, Weil R. A functional polymorphism in the EGF gene is found with increased frequency in glioblastoma multiforme patients and is associated with more aggressive disease. *Cancer Res* 2004;64:1220–3.
 13. Costa B, Ferreira P, Costa P, et al. Association between functional EGF+61 polymorphism and glioma risk. *Clin Cancer Res* 2007;13:2621–5.
 14. Hamai Y, Matsumura S, Matsusaki K, et al. A single nucleotide polymorphism 5' untranslated region of the EGF gene is associated with occurrence and malignant progression of gastric cancer. *Pathobiology* 2004;72:133–8.
 15. Okamoto I, Roka F, Kröger J, et al. The EGF A61G polymorphism is associated with disease-free period and survival in malignant melanoma. *J Invest Dermatol* 2006;126:2242–6.
 16. Ribeiro R, Soares A, Pinto D, Catarino R, Lopes C, Medeiros R. EGF genetic polymorphism is associated with clinical features but not malignant phenotype in neurofibromatosis type 1 patients. *J Neurooncol* 2007;81:225–9.
 17. Mullenbach R, Lagoda PJ, Welter C. An efficient salt-chloroform extraction of DNA from blood and tissues. *Trends Genet* 1989;5:391.
 18. Angelucci A, Schenone S, Gravina G, et al. Pyrazolo [3,4-*d*] pyrimidines c-Src inhibitors reduce epidermal growth factor-induced migration in prostate cancer cells. *Eur J Cancer* 2006;42:2838–45.
 19. El Sheikh SS, Domin J, Abel P, Stamp G, Lalani E-N. Phosphorylation of both EGFR and ErbB2 is a reliable predictor of prostate cancer cell proliferation in response to EGF. *Neoplasia* 2004;6:846–53.
 20. Harper ME, Glynn-Jones E, Goddard L, Mathews P, Nicholson RI. Expression of androgen receptor and growth factors in premalignant lesions of the prostate. *J Pathol* 1998;186:169–77.
 21. Sato B. Can an autocrine loop explain sex-hormone-dependent tumour growth? A brief overview. *Oncology* 1999;57:3–6.
 22. Normanno N, Kim N, Wen D, et al. Expression of messenger RNA for amphiregulin, heregulin, and cripto-1, three new members of the epidermal growth factor family, in human breast carcinomas. *Breast Cancer Res Treat* 1995;35:293–7.
 23. Miguel P, Royuela M, Bethencourt R, Ruiz A, Fraile B, Paniagua R. Immunohistochemical comparative analysis of transforming growth factor α , epidermal growth factor, and epidermal growth factor receptor in normal, hyperplastic and neoplastic human prostates. *Cytokine* 1999;11:722–7.
 24. Yotsumoto F, Yagi H, Suzuki SO, et al. Validation of HB-EGF and amphiregulin as target for human cancer therapy. *Biochem Biophys Res Commun* 2008;365:555–61.
 25. Karashima T, Sweeney P, Slaton J, et al. Inhibition of angiogenesis by the anti-epidermal growth factor receptor antibody Imclone c225 in androgen-independent prostate cancer growing orthotopically in nude mice. *Clin Cancer Res* 2002;8:1253–64.
 26. Tomlins SA, Mehra R, Rhodes DR, et al. Integrative molecular concept modelling of prostate cancer progression. *Nat Genet* 2007;39:41–51.
 27. Aaronson SA. Growth factors and cancer. *Science* 1991;254:1146–53.
 28. Long R, Morrissey C, Fitzpatrick J. Prostate epithelial cell differentiation and its relevance to the understanding of prostate cancer therapies. *Clin Sci* 2005;108:1–11.
 29. Mercurio AM, Rabinovitz I, Shaw LM. The $\alpha 6 \beta 4$ integrin and epithelial cell migration. *Curr Opin Cell Biol* 2001;13:541–5.
 30. Shaw LM, Rabinovitz I, Wang HH, Tokar A, Mercurio AM. Activation of phosphoinositide 3-OH kinase by the $\alpha 6 \beta 4$ integrin promotes carcinoma invasion. *Cell* 1997;91:949–60.
 31. Tampellini M, Longo M, Cappia S, et al. Co-expression of EGF receptor, TGF α and S6 kinase is significantly associated with colorectal carcinomas with distant metastases at diagnosis. *Virchows Arch* 2007;450:321–8.
 32. Zolfaghari A, Djakiew D. Inhibition of chemomigration of a human prostatic carcinoma cell (TSU-pr1) line by inhibition of epidermal growth factor receptor function. *Prostate* 1996;28:232–8.
 33. Kondapaka SB, Fridman R, Reddy KB. Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells. *Int J Cancer* 1997;70:722–6.
 34. Angelucci A, Gravina GL, Rucci N, et al. Suppression of EGF-R signaling reduces the incidence of prostate cancer metastasis in nude mice. *Endocr Relat Cancer* 2006;13:197–210.
 35. Taille A, Vacherot F, Salomon L, et al. Hormone-refractory prostate cancer: a multi-step and multi-event process. *Prostate Cancer Prostatic Dis* 2001;4:204–12.
 36. Visakorpi T, Hyytinen E, Koivisto P, et al. *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. *Nat Genet* 1995;9:401–6.
 37. Bonaccorsi L, Nosi D, Muratori M, Formigli L, Forti G, Baldi E. Altered endocytosis of epidermal growth factor receptor in androgen receptor positive prostate cancer cell lines. *J Mol Endocrinol* 2007;38:51–66.
 38. Festuccia G, Gravina GL, Angelucci A, et al. Additive antitumor effects of the epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (Iressa), and the nonsteroidal antiandrogen, bicalutamide (Casodex), in prostate cancer cells *in vitro*. *Int J Cancer* 2005;115:630–40.
 39. Belochitski O, Ariad S, Shany S, Fridman V, Gavrilov V. Efficient dual treatment of the hormone-refractory prostate cancer cell line DU145 with cetuximab and 1,25-dihydroxyvitamin D3. *In Vivo* 2007;21:371–6.
 40. Mimeault M, Johansson SL, Vankatraman G, et al. Combined targeting of epidermal growth factor receptor and hedgehog signaling by gefitinib and cyclopamine cooperatively improves the cytotoxic effects of docetaxel on metastatic prostate cancer cells. *Mol Cancer Ther* 2007;6:967–78.
 41. Mimeault M, Venkatraman G, Johansson SL, et al. Novel combination therapy against metastatic and androgen-independent prostate cancer by using gefitinib, tamoxifen and etoposide. *Int J Cancer* 2007;120:160–9.
 42. Busby JE, Kim SJ, Yazici S, et al. Therapy of multi-drug resistant human prostate tumors in the prostate of nude mice by simultaneous targeting of the epidermal growth factor receptor and vascular endothelial growth factor receptor on tumor-associated endothelial cells. *Prostate* 2006;66:1788–98.
 43. Salzberrg M, Rochlitz C, Morant R, et al. An open-label, noncomparative phase II trial to evaluate the efficacy and safety of docetaxel in combination with gefitinib in patients with hormone-refractory metastatic prostate cancer. *Onkologie* 2007;30:355–60.
 44. Curigliano G, Pelosi G, De Pas T, et al. Absence of epidermal growth factor receptor gene mutations in patients with hormone refractory prostate cancer not responding to gefitinib. *Prostate* 2007;67:603–4.

PAPER V

Combined analysis of *EGF* + 61G > A and *TGFB1* + 869T > C functional polymorphisms in the time to androgen independence and prostate cancer susceptibility

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Proliferative mechanisms involving the epidermal growth factor (EGF) and transforming growth factor beta (TGF- β_1) ligands are potential alternative pathways for prostate cancer (PC) progression to androgen independence (AI). Thus, the combined effect of *EGF* and *TGFB1* functional polymorphisms might modulate tumor microenvironment and consequently its development. We studied *EGF* + 61G > A and *TGFB1* + 869T > C functional polymorphisms in 234 patients with PC and 243 healthy individuals. Intermediate- and high-proliferation genetic profile carriers have increased risk for PC (odds ratio (OR) = 3.76, $P = 0.007$ and OR = 3.98, $P = 0.004$, respectively), when compared with low proliferation individuals. Multivariate analysis showed a significantly lower time to AI in the high proliferation group, compared with the low/intermediate proliferation genetic profile carriers (HR = 2.67, $P = 0.039$), after adjustment for age, metastasis and stage. Results suggest that combined analysis of target genetic polymorphisms may contribute to the definition of cancer susceptibility and pharmacogenomic profiles. Combined blockage of key molecules in proliferation signaling pathways could be one of the most promising strategies for androgen-independent prostate cancer.

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Keywords: *EGF*/*TGFB1* functional polymorphisms; prostate cancer; SNP variations; androgen independence

Introduction

Prostate cancer (PC) is one of the most common malignancies among men in the Western world and a major health problem in many industrialized countries.¹

Despite recent advances in the detection of early PC there is little effective therapy for patients with locally advanced and/or metastatic disease. Patients diagnosed in advanced stages are frequently submitted to hormonal treatment with androgen deprivation therapy (ADT),² although most men will eventually fail this therapy and die from recurrent androgen-independent prostate cancer (AIPC). AIPC is an invariably lethal condition associated with significant deterioration of the quality of life.^{3,4} Therefore, it is important to understand the mechanisms involved in AI progression.

It is known that the androgen pathway has a critical role in the survival of prostatic cells; however, progression into advanced PC and incurable forms has

⁷These authors contributed equally to this study.

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been associated with the activation of other cascades mediated by growth factors responsible for the balance between cell growth rate and apoptosis. Cell proliferation is normally regulated by the concerted action of both mitogenic growth signals and antiproliferative signals that converge on regulators of the cell cycle. In fact, the prostate is known to be dependent not exclusively on androgens but also on growth factors.⁵ Some authors suggest that aberrant growth and differentiation are because of inappropriate cellular environment.⁶

The epidermal growth factor (EGF) and the transforming growth factor-beta 1 (TGF-β₁) are the key players, with opposite roles, in cell proliferation. EGF activates several pro-oncogenic intracellular pathways leading to proliferation, differentiation and tumorigenesis of epithelial cells.^{7,8} Cumulatively, its receptor (EGFR (epidermal growth factor receptor)) is proposed to participate in the pathogenesis and growth of several epithelial human cancers. In PC cells, EGFR ligands are frequently elevated and EGFR itself is commonly overexpressed.⁹ Furthermore, EGFR expression increases during progression to AI.¹⁰

Epidermal growth factor is encoded by the *EGF* gene, located on chromosome 4q25-q27. Shabazi *et al.*¹¹ identified a functional G>A single nucleotide polymorphism at position +61 in the 5'-untranslated region of the *EGF* gene (rs4444903). *In vitro* studies showed that G-carriers have an increased EGF production in both normal and tumoral cells.^{11–13} This functional polymorphism has been associated with several malignancies,^{11–15} including an earlier report from our group in PC.¹⁶

Transforming growth factor-β₁ is a multifunctional regulatory polypeptide that controls many aspects of cellular function, such as cellular proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis, immune surveillance and survival.¹⁷ Nevertheless, TGF-β₁ has been suggested to play a dual role, acting as a suppressor in the early stages and as a tumor promoter in the later stages, by enhancing tumor cell motility and invasiveness.^{18,19} Recently, a functional polymorphism was described in *TGFβ1* gene (*TGFβ1* + 869T>C), responsible for a T-to-C substitution at nucleotide 29 of codon 10 (rs1982073). This variant is located in the hydrophobic core of the signal peptide, resulting in the replacement of a hydrophobic leucine with a small, neutral proline. This transition has been associated with higher circulating levels of TGF-β₁ (in homozygous C).^{20,21}

Genetic variants, which influence *EGF* and *TGFβ1* expressions and protein serum levels, may impact PC development and prognosis. Our purpose was to investigate the combination of *EGF* + 61G>A and *TGFβ1* + 869T>C functional polymorphisms in PC and AIPC in response to ADT.

Results

Using the recessive model, frequencies for homozygous AA and AG/GG genotypes of *EGF* + 61G>A polymorphism were, respectively, 0.32 and 0.68 for PC patients and 0.34 and 0.66 in the control group. The *TGFβ1* + 869T>C polymorphism frequencies for homozygous CC and CT/TT were 0.14 and 0.86 in PC group and 0.22 and 0.78 in the control group, respectively. Observed versus expected genotype frequencies were calculated, and no deviation from Hardy–Weinberg equilibrium was observed, except for the *TGFβ1* polymorphism in control group (*EGF* + 61G>A: PC group, *P* = 0.082, control group, *P* = 0.073; *TGFβ1* + 869T>C: PC group, *P* = 0.761, control group, *P* = 0.020).

High- and intermediate-proliferation genetic profiles' distributions were overrepresented in PC (0.56 and 0.42, respectively) and control (0.52 and 0.40, respectively) groups compared with the low-proliferation functional genetic profile (Table 1). The present results show a significantly higher risk for developing PC in the intermediate- and high-proliferation functional genetic profile carriers (odds ratio (OR) = 3.76, 95% confidence interval (CI) = 1.26–12.03 and OR = 3.98, 95% CI = 1.35–12.59, respectively). The population attributable risk (PAR) for intermediate and high proliferation groups was 30.8 and 42%, respectively.

The analysis of clinico-pathological characteristics according to the combined proliferation genetic profile showed no statistically significant associations of the combined polymorphisms with Gleason grade, distant metastasis and prostate specific antigen (PSA) at the time of diagnosis (*P* = 0.319, *P* = 0.572 and *P* = 0.254, respectively).

Concerning AI-free interval after the beginning of ADT, we found a significantly reduced time-to-AI in high-proliferation functional genetic profile carriers (93.99 (6.87) months in low/intermediate proliferation group and 76.51 (6.15) months in high proliferation group), using a multivariate Cox regression model with age (*P* = 0.299), tumor stage (*P* < 0.0001), surgery (*P* = 0.982) and hormonal

Table 1 Frequencies distribution and OR analysis in control and PC groups according to *EGF*+61G>A and *TGFβ1*+869T>C combined proliferation functional genetic profile

	Control group	PC group	OR	95% CI	P-value
<i>Combined genetic profile</i>					
Low proliferation	19 (0.08)	5 (0.02)	—		
Intermediate proliferation	98 (0.40)	97 (0.42)	3.76	1.26–12.03	0.007
High proliferation	126 (0.52)	132 (0.56)	3.98	1.35–12.59	0.004

Abbreviations: OR, odds ratio; PC, prostate cancer.

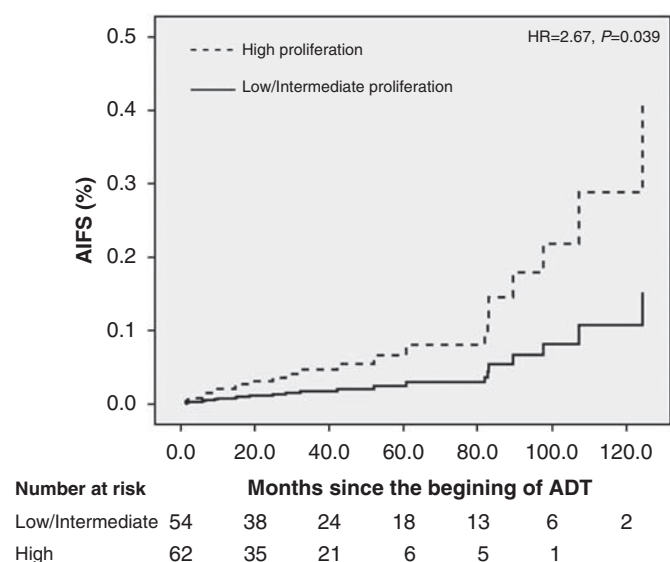


Figure 1 Androgen independence-free survival (AIFS) according to EGF-TGFB1 combined proliferation genetic profile in patients submitted to ADT.

treatment type ($P=0.487$) as covariates (hazard ratio (HR) = 2.67, 95% CI = 1.05–6.79, $P=0.039$) (Figure 1).

Discussion

The intricate balance between cell growth and proliferation factors versus apoptosis-inducing factors is mandatory for prostate growth regulation. Conversely, homeostatic disruptions in PC often trigger the loss of apoptosis and the overexpression of factors promoting cell survival and proliferation. A common deregulated mechanism in PC cells is distinguished by apoptotic evasion, uncontrolled proliferation and loss of differentiation.¹⁷

Growth factors play a significant role in the growth of normal, hyperplastic and malignant prostatic epithelium. There is a significant amount of evidence supporting that the EGF and the transforming growth factor-beta (TGF- β) families are among the most relevant mediators of proliferation in this type of cancer.^{22,23} In line with these findings, our results suggest that TGF- β 1 and EGF combined effect may impact significantly in the individual PC risk, as well as in ADT outcome. However, a limitation to the study on PC susceptibility association with combined EGF and TGFB1 variants, resides in the significantly different mean age between PC and control groups, and the lack of information and subsequent adjustment for potentially relevant environmental factors. Therefore, conclusions on this issue should be interpreted cautiously owing to limitations inherent to the design.

Other line of evidence unfocused in target genes showed that in genome-wide association studies,^{24–26} PC susceptibility loci do not reside within or near identifiable genes. It has been hypothesized that they exist in regulatory regions of DNA that control gene expression, or alternatively, in

regions of DNA that code for microRNAs or other regulatory transcripts, as recently conceptualized by Glinsky.^{27,28} Ultimately, our results and these new lines of research will encourage future studies to increase our understanding of the biological basis of PC, providing an opportunity to design new therapies.

Several lines of evidence support the involvement of EGF in PC development. The normal and tumoral prostatic epithelium produces large amounts of EGF,²⁹ its receptor was found to be overexpressed in prostatic tumors, and the EGF/EGFR pathway has been associated with AI development.^{9,30} Cumulatively, it was shown that EGF and EGFR expression levels in PC cells are enhanced during disease progression to AI and metastatic PC.¹⁰

Transforming growth factor- β 1 exerts a wide variety of biological actions, through both autocrine and paracrine mechanisms. It's role has been associated with advanced disease and metastasis, through the induction of extracellular proteolysis, angiogenesis and immune suppression.³¹ However, in the earlier stages of tumor development TGF- β 1 can act as an inhibitor of tumor progression.³² According to Tang *et al.*,³³ the suppression of autocrine TGF- β 1 actions leads to the activation of tumorigenic properties. In fact, it was observed a dual role for TGF- β 1 in PC cells, with both an inhibitory or a stimulatory growth effect.³⁴ These apparently paradoxical findings can be attributed to TGF- β 1 concentration, which leads to proliferation in low TGF- β 1 environments, and induces growth arrest in the presence of high concentrations of TGF- β 1.³⁵ This ligand that potentially inhibit epithelial, endothelial and hematopoietic cell proliferation, is able to prevent progression through the cell cycle by inducing expression of cyclin kinase inhibitors p15, p21 and p27.¹⁷ Furthermore, it regulates the expression of several key proteins in the control of cell-cycle progression from G1-to-S phase,¹⁷ including *c-myc*. It was shown that TGF- β 1 can rapidly inhibit the transcription of *c-myc* in epithelial cells.³⁶ TGF- β 1 is also produced in prostatic stromal cells, inducing apoptosis through a paracrine mechanism in prostate epithelial cell. In fact, it was already shown that the TGF- β signaling pathway may have prognostic significance in PC patients and that *in vitro* restoration of TGF- β 1 signaling pathway in PC cells inhibits proliferation.^{37,38}

Case-control molecular epidemiology studies from our group and others have shown promising results concerning the development of molecular markers for PC susceptibility and aggressiveness.^{39–42} Specifically, it was hypothesized that functional polymorphisms with impact in growth factor and cytokine expression and circulating levels may influence individual susceptibility to PC, the response to treatment and prognosis significance.

The EGF + 61G>A polymorphism encodes a significant functional difference in EGF expression.^{11–13} Conversely, it is expected that G-carriers will have a higher EGF availability in tumoral environment. EGF + 61G>A polymorphism has been the subject of investigation in case-control studies, involving other cancer types.^{11–16} Recently, we have shown that this functional polymorphism was associated with

increased risk for PC, being diagnosed with aggressive disease and worst response to ADT.¹⁶

The *TGF β 1* +869T>C functional polymorphism is responsible for significantly higher TGF- β ₁ circulating levels in C-carriers and *in vitro* transfection experiments showed that the signal peptide in C-carriers caused a 2.8-fold increase in the secretion of TGF- β ₁ compared with T-carriers.⁴³ The combined lower TGF- β ₁ production in the presence of T-allele²¹ and higher levels of EGF associated with the presence of G-allele, might contribute to a favorable long-term proliferative potential in prostate epithelial cells, which may increase PC risk.

Although interactions between cancer cells and the extracellular environment are important in processes such as invasion, angiogenesis and metastization, and TGF- β ₁ and EGF are known to play a role in these mechanisms,^{17,44} our results do not support this hypothesis. We suggest that, as we used a combined genetic profile of *EGF* and *TGF β 1* based on the proliferation phenotype, we were unable to find an association with aggressive PC.

Patients with local or distant metastatic PC are usually treated primarily through pharmacological androgen suppression.² This hormonal therapy is initially efficient, although the majority of patients will subsequently become unresponsive to androgen inhibition⁴⁵ and consequently the development of AIPC is a clinical problem of major concern.^{2,3} In fact, AIPC is a complex and heterogeneous form of PC with a high capacity of progression and metastization.⁴ Conversely, the comprehension of molecular pathways underlying this disease is imperative.

The AIPC is a multistep/multievent process with different molecular patterns throughout development, involving changes in signaling pathways of growth suppressing or promoting factors.⁴⁶ It was hypothesized that EGF/EGFR and TGF- β ₁/TGF- β ₁RII pathways are involved in the acquisition of AIPC phenotype, either through an independent alternative proliferative stimulus, or through the interference with androgen receptor (AR) axis.^{46,47}

The prostate is an androgen-dependent (AD) organ that undergoes involution after castration. Isaacs and Cooffey⁴⁸ suggested that the shift from AD-to-AIPC may be because of residual stem cells not responsive to androgens, which will emerge after ADT under the appropriate growth stimulus. It is well established that the microenvironment surrounding PC cells after ADT may play an important role in their behavior. Stem cells are usually quiescent and reside surrounded by a microenvironment that maintains the balance between quiescence and self-renewal stem cell population. TGF- β ₁ and EGF have been implicated as modulators of stem cell proliferation, thereby regulating their homeostasis.⁴⁹

In addition to the proposed mechanism for EGF and TGF- β ₁ in AI development, we suggest that by selecting AI cell clones, ADT creates an opportunity for these undifferentiated stem cells to grow according to the involving microenvironment. Accordingly, carriers of a high-proliferation constitutive genetic profile will likely be exposed to an increased proliferative stimulus, thus contributing to AI

disease. However, the small sample size in our study may limit the ability to discern meaningful differences. Further research is needed to evaluate the associations reported here in more details. In particular large, well-designed studies of ethnically diverse populations and functional studies on PC cells may help clarify which variants are truly causal for this disease.

Present results support that combined analysis of genetic polymorphisms might reinforce the clinical capacity to predict the response to treatment. Furthermore, these findings also support the need of other studies to ascertain the therapeutic value of targeted-combined therapies directed against both EGF/EGFR and TGF- β ₁/TGF- β ₁RII pathways.

In summary, we observed a statistically significant increased risk for developing PC in *EGF* and *TGF β 1* combined high- and intermediate-proliferation functional genetic profile carriers. Cumulatively, the high-proliferation functional genetic profile carriers were more prone to develop AI.

Materials and methods

Study population

This case-control study was undertaken in 234 patients, with a mean age of 69.1 (7.48), with histopathologically diagnosed PC. The median follow-up time was 32 months (range 2.5–137 months). Patients distribution according to the stage at the time of diagnosis was 43.4% presenting localized disease (T₁–T_{2b}), 37.9% with locally advanced disease (T₃–T₄) and 18.7% with metastatic disease (N⁺ and/or M⁺). The types of hormonal treatment were as follows: anti-androgens plus luteinizing hormone-releasing hormone agonists (aLHRH) combination therapy (81.7%); aLHRH alone (8.7%) and anti-androgens alone (9.6%). Hormone resistance was evaluated through PSA recurrence, which was defined as two consecutive increasing PSA values more than 1.0 ng ml⁻¹ and differing by more than 0.2 ng ml⁻¹.

Men older than 40 years of age, without known history of cancer were recruited from the Portuguese Institute of Oncology—Porto Centre Blood Donor's Bank and included in the control group (*n* = 243), with a mean age of 44.7 (11.55). Study was conducted according to the Helsinki Declaration principles. A venous blood sample (8 ml) was obtained from each subject by forearm venipuncture. White cell fraction was used to extract DNA according to salting-out procedure.⁵⁰

EGF + 61G>A and TGF β 1 + 869T>C genotyping

The *EGF* + 61G>A polymorphism was analyzed through polymerase chain reaction (PCR) followed by RFLP (restriction fragment length polymorphism), as described in earlier reports.^{15,16} Briefly, DNA was amplified in a 50- μ l reaction mixture containing *EGF* + 61G>A specific primers, PCR buffer 1 $\times$, Taq Polymerase 1 U, MgCl₂ 1.5 mM, dNTPs 0.2 mM, DNA 100 ng. PCR products (242 bp) were incubated overnight with *AluI* restriction endonuclease at 37°C. The restricted fragments were separated by electrophoresis on 3% agarose gels with ethidium bromide staining.

Table 2 Genotypes of the two polymorphisms combined into three functional categories according to functional proliferation outcome

Combination	EGF+61G>A	TGFB1+869T>C	Genetic proliferation profile
AA-CC	AA	CC	Low proliferation
AA-CT/TT	AA	CT/TT	Intermediate proliferation
AG/GG-CC	AG/GG	CC	Intermediate proliferation
AG/GG-CT/TT	AG/GG	CT/TT	High proliferation

The polymorphism was defined by presence (A) or absence (G) of an additional restriction site.

The *TGFB1* + 869T>C polymorphism was analyzed by allelic discrimination using 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR were carried out using a 6-μl reaction mixture, containing 1 × Master Mix (Applied Biosystems), with 1 × probes (TaqMan assay C_22272997_10__, Applied Biosystems) and 90 ng of the DNA sample.

Quality control procedures implemented for genotyping included double sampling in about 10% of the samples to assess reliability and the use of negative controls to step-away false-positives. In PCR-RFLP method, two authors obtained the results independently, and the ambiguous were reanalysed.

Statistical analysis

Genotypes of the two polymorphisms were combined into three categories according to the functional consequences in cell proliferation: low-, intermediate- and high-proliferation genetic profile (Table 2). The rationale for defining high-proliferation functional genetic profile was to associate the overexpressing G-allele from *EGF* + 61G>A polymorphism with the T-allele from *TGFB1* + 869T>C variant related to lower TGF-β₁ production. In the intermediate-functional genetic profile, we have combined *EGF* + 61G>A and *TGFB1* + 869T>C polymorphisms (AA plus CT/TT carriers, and AG/GG plus CC, respectively). The combination of *EGF* + 61G>A homozygous A with *TGFB1* + 869T>C homozygous C polymorphism corresponded to the low-proliferation genetic profile.

Genetic profiles proportions among groups were compared using the Pearson's χ^2 -test. OR and 95% confidence interval (CI) were calculated as a measure of association between *EGF*/*TGFB1* combined genetic profiles in cases and controls. A Cox proportional hazard model was used to analyze the time to AI (determined by the interval of time since the beginning of ADT until AI or the last clinical visit), considering as covariates, age at diagnosis (≥ 69 vs < 69 years old), tumor stage (localized vs locally advanced vs distant metastases), surgery (radical prostatectomy vs none) and hormonal treatment type (anti-androgens plus aLHRH combination therapy vs aLHRH alone vs anti-androgens alone). Hardy-Weinberg equilibrium was tested using Pearson's χ^2 -analysis to compare observed versus expected genotype frequencies.

We calculated the PAR, using the following formula: $PAR = PRF \times (1 - 1/OR)$. The PAR is the fraction of disease attributable to the risk factor, PRF is the percentage of the risk factor in case subjects, and OR is the odds ratio. All analyses were performed with SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA) considering a level of significance < 0.05 .

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Duality of interest

The authors disclose any commercial or other associations with acknowledged institutions that might pose a conflict of interest in connection with submitted material.

References

- Hsing AW, Chokkalingam AP. Prostate cancer epidemiology. *Front Biosci* 2006; **11**: 1388–1413.
- Pronzato P, Rondini M. Hormonotherapy of advanced prostate cancer. *Ann Oncol* 2005; **4**: iv80–iv84.
- Hellerstedt B, Pienta K. The current state of hormonal therapy for prostate cancer. *CA Cancer J Clin* 2002; **52**: 154–179.
- Catalona WJ. Management of cancer of the prostate. *N Engl J Med* 1994; **331**: 996–1003.
- Martín-Orozco R, Almaroz-Pro C, Rodríguez-Ubreva FJ, Cortés MA, Ropero S, Colomer R et al. EGF prevents the neuroendocrine differentiation of LNCaP Cells induced by serum deprivation: the modulator role of PI3K/Akt. *Neoplasia* 2007; **9**: 614–624.
- Long R, Morrissey C, Fitzpatrick J. Prostate epithelial cell differentiation and its relevance to the understanding of prostate cancer therapies. *Clin Sci* 2005; **108**: 1–11.
- Fisher DA, Lakshmanan J. Metabolism and effects of epidermal growth factor and related growth factors in mammals. *Endocr Rev* 1990; **11**: 418–442.
- Groenen LC, Nice EC, Burgess AW. Structure-function relationships for the EGF/TGF- family of mitogens. *Growth Factors* 1994; **11**: 235–257.

- 9 Vicentini C, Festuccia C, Gravina GL, Angelucci A, Marronaro A, Bologna M. Prostate cancer cell proliferation is strongly reduced by epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 *in vitro* on human cell lines primary cultures. *J Cancer Res Clin Oncol* 2003; **129**: 165–175.
- 10 Di Lorenzo G, Tortora G, D'Armiento FP, De Rosa G, Staibano S, Autorino R et al. Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clin Cancer Res* 2002; **8**: 3438–3444.
- 11 Shahbazi M, Pravica V, Nasreen N, Fakhoury H, Fryer AA, Strange RC et al. Association between functional polymorphism in EGF gene and malignant melanoma. *Lancet* 2002; **359**: 397–401.
- 12 Bhowmick D, Zhuang Z, Wait SD, Weil RJ. A functional polymorphism in the EGF gene is found with increased frequency in glioblastoma multiforme patients and is associated with more aggressive disease. *Cancer Res* 2004; **64**: 1220–1223.
- 13 Costa B, Ferreira P, Costa P, Canedo P, Oliveira P, Silva A et al. Association between functional EGF+61 polymorphism and glioma risk. *Clin Cancer Res* 2007; **13**: 2621–2625.
- 14 MacCarron S, Bateman A, Theaker J, Howell WM. EGF+61 gene polymorphism and susceptibility to and prognostic markers in cutaneous malignant melanoma. *Int J Cancer* 2003; **107**: 673–675.
- 15 Ribeiro R, Soares A, Pinto D, Catarino R, Lopes C, Medeiros R. EGF genetic polymorphism is associated with clinical features but not malignant phenotype in neurofibromatosis type 1 patients. *J Neurooncol* 2007; **81**: 225–229.
- 16 Teixeira AL, Ribeiro R, Cardoso D, Pinto D, Lobo F, Fraga A et al. Genetic Polymorphism in EGF is associated with prostate cancer aggressiveness and progression-free-interval in androgen blockade-treated patients. *Clin Cancer Res* 2008; **14**: 3367–3371.
- 17 Elliott R, Blobe G. Role of transforming growth factor beta in human cancer. *J Clin Oncol* 2005; **23**: 2078–2093.
- 18 Reiss M, Barcellos-Hoft MH. Transforming growth factor-b in breast cancer: a working hypothesis. *Breast Cancer Res Treat* 1997; **45**: 81–95.
- 19 Kretschmar M. Transforming growth factor-b and breast cancer. Transforming growth factor-b/SMAD signaling effects and cancer. *Breast Cancer Res* 2000; **2**: 107–115.
- 20 Grainger DJ, Heathcote K, Chiano M, Snieder H, Kemp PR, Metcalfe JC et al. Genetic control of the circulating concentration of transforming growth factor type beta1. *Hum Mol Genet* 1999; **8**: 93–97.
- 21 Yokota M, Ichihara S, Lin TL, Nakashima N, Yamada Y. Association of a T29C polymorphism of the transforming growth factor-beta1 gene with genetic susceptibility to myocardial infarction in Japanese. *Circulation* 2000; **101**: 2783–2787.
- 22 Soultiz N, Karyotis I, Delakas D, Spandidos DA. Expression analysis of peptide growth factors VEGF, FGF2, TGF β 1, EGF and IGF1 in prostate cancer and benign prostatic hyperplasia. *Int J Oncol* 2006; **29**: 305–314.
- 23 Jakowlew S. Transforming growth factor- β in cancer and metastasis. *Cancer Metastasis Rev* 2006; **25**: 435–457.
- 24 Gudmundsson J, Sulem P, Steinthorsdottir V, Bergthorsson JT, Thorleifsson G, Manolescu A et al. Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes. *Nat Genet* 2007; **39**: 977–983.
- 25 Zheng SL, Sun J, Wiklund F, Smith S, Stattin P, Li G et al. Cumulative association of five genetic variants with prostate cancer. *N Engl J Med* 2008; **358**: 910–919.
- 26 Haiman CA, Patterson N, Freedman ML, Myers SR, Pike MC, Waliszewska A et al. Multiple regions within 8q24 independently affect risk for prostate cancer. *Nat Genet* 2007; **39**: 638–644.
- 27 Glinsky GV. Phenotype-defining functions of multiple non-coding pathway. *Cell Cycle* 2008; **7**: 1630–1639.
- 28 Glinsky GV. An SNP-guided microRNA map of fifteen common human disorders identifies a consensus disease phenocode aiming at principal components of the nuclear import pathway. *Cell Cycle* 2008; **7**: 2570–2583.
- 29 Gregory H, Willshire IR, Kavanagh JP, Blacklock NJ, Chowdury S, Richards RC. Urogastrone-epidermal growth factor concentrations in prostatic fluid of normal individuals and patients with benign prostatic hypertrophy. *Clin Sci (Lond)* 1986; **70**: 359–363.
- 30 Harper ME, Glynne-Jones E, Goddard L, Mathews P, Nicholson RI. Expression of androgen receptor and growth factors in premalignant lesions of the prostate. *J Pathol* 1998; **186**: 169–177.
- 31 Stearns ME, Garcia FU, Fudge K, Rhim J, Wang M. Role of interleukin 10 and transforming growth factor β 1 in the angiogenesis and metastasis of human prostate primary tumor lines from orthotopic implants in severe combined immunodeficiency mice. *Clin Cancer Res* 1999; **5**: 711–720.
- 32 Kaklamani V, Pasche B. the role of TGF- β in cancer and the potential for therapy and prevention. *Expert Rev Anticancer Ther* 2004; **4**: 649–661.
- 33 Tang B, de Castro K, Barnes HE, Parks WT, Stewart L, Böttinger EP et al. Loss of responsiveness of transforming growth factor beta induces malignant transformation of nontumorigenic rat prostate epithelial cells. *Cancer Res* 1999; **59**: 4834–4842.
- 34 Brierie B, Moses H. TGF- β : the molecular jekyll and Hyde of cancer. *Nat Rev* 2006; **6**: 506–520.
- 35 Zhou W, Park I, Pins M, Kozlowski JM, Jovanovic B, Zhang J et al. Dual regulation of proliferation and growth arrest in prostatic stromal cells by transforming growth factor- β 1. *Endocrinology* 2003; **144**: 4280–4284.
- 36 Paterson IC, Davies M, Stone A, Huntley S, Smith E, Pring M et al. TGF- β 1 acts as a tumor suppressor of human malignant keratinocytes independently of Smad 4 expression and ligand-induced G1 arrest. *Oncogene* 2002; **21**: 1616–1624.
- 37 Brierie B, Moses HL. TGF-beta and cancer. *Cytokine Growth Factor Rev* 2006; **17**: 29–40.
- 38 Guo Y, Kyprianou N. Restoration of transforming growth factor beta signaling pathway in human prostate cancer cells suppresses tumorigenicity via induction of caspase-1-mediated apoptosis. *Cancer Res* 1999; **59**: 1366–1371.
- 39 Medeiros R, Vasconcelos A, Costa S, Pinto D, Ferreira P, Lobo F et al. Metabolic susceptibility genes and prostate cancer risk in a southern European population: the role of glutathione S-transferases GSTM1, GSTM3, And GSTT1 genetic polymorphisms. *Prostate* 2004; **58**: 414–420.
- 40 Medeiros R, Morais A, Vasconcelos A, Costa S, Pinto D, Oliveira J et al. The role of vitamin D receptor gene polymorphisms in the susceptibility to prostate cancer of a southern European population. *J Hum Genet* 2002; **47**: 413–418.
- 41 Ribeiro R, Vasconcelos A, Costa S, Pinto D, Morais A, Oliveira J et al. Overexpressing leptin genetic polymorphism (–2548G/A) is associated with susceptibility to prostate cancer and risk of advanced disease. *Prostate* 2004; **59**: 268–274.
- 42 Zheng SL, Sun J, Wiklund F, Smith S, Stattin P, Li G et al. Cumulative association of five genetic variants with prostate cancer. *N Engl J Med* 2008; **358**: 910–919.
- 43 Dunning AM, Ellis PD, McBride S, Kirschenlohr HL, Healey CS, Kemp PR et al. A transforming growth factor β 1 signal peptide variant increases secretion *in vitro* and is associated with increased incidence of invasive breast cancer. *Cancer Res* 2003; **63**: 2610–2615.
- 44 Bonaccorsi L, Carloni V, Muratori M, Formigli L, Zecchi S, Forti G et al. EGF receptor (EGFR) signalling promoting invasion is disrupted in androgen-sensitive prostate cancer cells by an interaction between EGFR and androgen receptor (AR). *Int J Cancer* 2004; **112**: 78–86.
- 45 Angelucci A, Schenone S, Gravina G, Muzi P, Festuccia C, Vicentini C et al. Pyrazolo [3,4-d] pyrimidines c-Src inhibitors reduce epidermal growth factor-induced migration in prostate cancer cells. *Eur J Cancer* 2006; **42**: 2838–2845.
- 46 Taille A, Vacherot F, Salomon L, Druel C, Gil Diez De Medina S, Abbou C et al. Hormone-refractory prostate cancer: a multi-step and multi-event process. *Prostate Cancer Prostatic Dis* 2001; **4**: 204–212.
- 47 Zhu M-L, Partin JV, Bruckheimer EM, Strup SE, Kyprianou N. TGF- β signaling and androgen receptor status determine apoptotic cross-talk in human prostate cancer cells. *Prostate* 2008; **68**: 287–295.
- 48 Isaacs JT, Coffey DS. Etiology and disease process of benign prostatic hyperplasia. *Prostate Suppl* 1989; **2**: 33–50.
- 49 Fortunel NO, Hatzfeld A, Hatzfeld JA. Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. *Blood* 2000; **96**: 2022–2036.
- 50 Mullenbach R, Lagoda PJ, Welter C. An efficient salt-chloroform extraction of DNA from blood and tissues. *Trends Genet* 1989; **5**: 391.

PAPER VI

PERFORMANCE OF AN ADIPOKINE PATHWAY-BASED MULTILOCUS GENETIC
RISK SCORE FOR PROSTATE CANCER RISK PREDICTION

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ABSTRACT

Few biomarkers are available to predict prostate cancer risk. Single nucleotide polymorphisms (SNPs) tend to have weak individual effects but, in combination, they have stronger predictive value. Adipokine pathways have been implicated in the pathogenesis.

We used a candidate pathway approach to investigate 29 functional SNPs in key genes from relevant adipokine pathways in a sample of 1006 men eligible for prostate biopsy. We used stepwise multivariate logistic regression and bootstrapping to develop a multilocus genetic risk score by weighting each risk SNP empirically based on its association with disease.

Seven common functional polymorphisms were associated with overall and high-grade prostate cancer ($\text{Gleason} \geq 7$), whereas three variants were associated with high metastatic-risk prostate cancer ($\text{PSA} \geq 20\text{ng/mL}$ and/or $\text{Gleason} \geq 8$). The addition of genetic variants to age and PSA improved the predictive accuracy for overall and high-grade prostate cancer, using either the area under the receiver-operating characteristics curves ($P < 0.02$), the net reclassification improvement ($P < 0.001$) and integrated discrimination improvement ($P < 0.001$) measures.

These results suggest that functional polymorphisms in adipokine pathways may act individually and cumulatively to affect risk and severity of prostate cancer, supporting the influence of adipokine pathways in the pathogenesis of prostate cancer. Use of such adipokine multilocus genetic risk score can enhance the predictive value of PSA and age in estimating absolute risk, which supports further evaluation of its clinical significance.

INTRODUCTION

Prostate cancer is a complex and unpredictable disease, with risk being affected by advancing age, ethnic background and family history. Although the causes of prostate cancer are not yet fully understood, genetic variation influences disease risk [1]. Prostate cancer is usually accompanied by a rise in the concentration of serum PSA, which has been used for decades as a sensitive but poorly specific biomarker, and a controversial predictor of prostate cancer mortality [2,3]. Many prostatic biopsies are unnecessary [4], which underscores the need for better prediction models with increased specificity to aid clinicians decide whether or not to recommend prostate biopsy. Furthermore, this is especially relevant in men with mildly elevated PSA values (3-10 ng/mL), but where the risk for being diagnosed with prostate cancer is only about 20-25% [5]. After diagnosis, some cancers are indolent and cause no clinical problems, whereas others progress and may be fatal [6]. Therefore, it is important to search for biomarkers of aggressive clinical outcome. Genetic markers provide good candidates for such a role.

Single-nucleotide polymorphisms (SNPs) identified as loci associated with prostate cancer in genome-wide association studies (GWAS) are common but confer only small increases in risk and the mechanisms underlying their association with prostate cancer risk remain unknown [7,8]. Recently, selected SNPs from GWAS were analyzed and converted into a genetic risk score, which was shown to reduce the number of biopsies although it did not discriminate aggressive cases [9].

The association between body mass and risk of prostate cancer is supported by meta-analyses that suggest increased risk of aggressive prostate cancer in the obese [10], and by studies using methods to estimate abdominal adiposity [11]. Recent work has focused on the role of adipokines and obesity-related molecules in the etiology of prostate cancer [12,13]. Variants in genes encoding components of these pathways have been evaluated for prostate

cancer risk and promising candidates have been identified [14,15,16,17]. These candidate genes code for molecules found to be over- or under-expressed in obesity [18,19,20] and are involved in several biological mechanisms that modulate tumor proliferation, apoptosis, angiogenesis, motility, migration, and immunity [12,21], i.e., traits that ultimately influence tumor behavior. Thus, common polymorphisms in adipokine pathways are plausible candidates that may help predict prostate cancer susceptibility. However, few studies have examined prostate cancer risk in the context of multi-loci SNPs in different adipokine pathways. In this report, we tested the hypothesis that SNPs in candidate genes involved in adipokine pathways may contribute to prostate cancer susceptibility and aggressiveness in a population of men referred for diagnostic surveillance. We also assessed the clinical utility of an adipokine genetic risk score to enhance the predictive value of age and PSA to predict high-risk individuals for screening and therapeutic management.

RESULTS

A total of 449 histologically confirmed prostate cancer and 557 non-prostate cancer patients were included in the analyses. Prostate cancer patients were older ($P<0.0001$) and presented with significantly higher circulating levels of PSA and a lower free/total PSA ratio ($P<0.0001$ and $P<0.0001$, respectively) (Table 1).

We evaluated the associations between each individual SNP on prostate cancer susceptibility (Table S2). In the dominant effect models (referent: wild-type homozygote) there were significant decreases in risk for *LEPR* Gln223Arg (aOR=0.6, 95%CI: 0.5-0.8, aOR=0.6, 95%CI: 0.5-0.8 and aOR=0.5, 95%CI: 0.4-0.8, for all, high-grade and high-risk prostate cancer for metastasis, respectively) and for *FGF2*+223 C>T (aOR=0.7, 95%CI: 0.5-1.0 in high-grade prostate cancer). An increase in risk of high-grade prostate cancer was found in carriers of the *IL6R* Asp358Ala variant (aOR=1.3, 95%CI: 1.0-1.7). In the recessive

effect models (referent: wild-type homozygotes and heterozygotes) a significantly increased risk was observed for *IGF1R*+3174 G>A (aOR=1.3, 95%CI: 1.0-1.9 for overall prostate cancer), *IGFBP3*-202 A>C (aOR=1.3, 95%CI: 1.0-1.8 and aOR=1.3, 95%CI: 1.0-1.8, for overall and high-grade prostate cancer, respectively) and with *OPN*-66 T>C (aOR=1.8, 95%CI: 1.1-3.0, aOR=1.9, 95%CI: 1.1-3.2 and aOR=2.4, 95%CI: 1.2-4.8, in overall, high-grade and high-risk prostate cancer for metastasis, respectively). Likewise, a significant protective effect for high-grade prostate cancer was observed for carriers of the *IL6*-597 G>A variant (aOR=0.7, 95%CI: 0.4-1.0). Age-stratification on the aforementioned seven SNPs indicated that effects were mostly restricted to subjects below the median age (of non-cancer group, Table S3).

Figure 1 shows that among prostate cancer cases there was a shorter waiting time-to-onset in *IL6R* Asp358Ala C-allele carriers (P=0.026) and in *IGF1R*+3174 AA homozygous (P=0.002). None of the other five SNPs influenced the time to onset of disease (data not shown).

To test our hypothesis that genetic variability in SNPs from adipokine pathways may contribute a combined effect for prostate cancer risk and/or aggressiveness, we estimated the overall mutually-adjusted effects by stepwise multivariate logistic regression. The SNPs in *LEPR* Gln223Arg, *OPN*-66 T>G, *IGF1R*+3174 G>A, *IGFBP3*-202 A>C, *FGF2*+223 C>T and *IL6*-597 G>A, plus age and PSA remained independently associated with risk for overall, and for high-grade prostate cancer (Table 2). In the prostate cancer group with high risk for metastasis, only the *LEPR* Gln223Arg, *OPN*-66 T>G and *FGF2*+223 C>T genetic variants, age and PSA persisted (Table 2). Within all groups, bootstrap analysis confirmed results (Table 2).

Age-adjusted logistic regression showed increased risk for prostate cancer (aOR=2.5, 95%CI: 2.0-3.2) and high grade prostate cancer (aOR=2.8, 95%CI: 2.2-3.5) using the

inclusive risk score as continuous variable (Table 3). The logistic regression models (inclusive versus PSA and age) were significantly different, for all prostate cancers ($P=0.0002$) and high-grade prostate cancers ($P=0.0001$), after likelihood ratio test.

The inclusive (age and PSA added to the multi-locus genetic set) linear risk score computed on the basis of the above logistic regression model was validated as overall risk predictor by MonteCarlo simulation in the original ($n=1006$) and subsample ($n=300$) datasets. As shown in Table 3, after 10000 permutations there was good reliability in odds ratios and confidence intervals for prostate cancer and high-grade prostate cancer.

Figure 2 shows the ROC curves for the all-inclusive genetic risk score and for the age and PSA-based risk score. The AUC estimates for both outcomes (all prostate cancers and high-grade prostate cancers) were significantly higher for the all-inclusive score than with the age plus PSA predictor, $P=0.0099$ and $P=0.0196$, respectively (Figure 2). The statistically superior predictive value of the all-inclusive score was confirmed via the NRI (all prostate cancers: 9.5%, $P<0.0001$, high-grade prostate cancer: 13.3%, $P<0.0001$) and IDI (all prostate cancers: 0.021, $P<0.0001$, high-grade prostate cancer: 0.024, $P<0.0001$) comparisons.

Genotype distributions in four SNPs deviated from Hardy-Weinberg equilibrium (Table S1). In sensitivity analysis of three relevant SNPs, equilibrium was achieved after restricting the control group to constrained conditions, whereas the trend towards increased risk remained stable, regardless of control group used (restricted or unrestricted) (Table S4). Three of these four deviated SNPs ended up in the all-inclusive risk score. Therefore, as an additional step to clarify the relative importance of these SNPs we tested a four SNPs risk score (excluding the 3 SNPs that were not in equilibrium). The risk for prostate cancer and high-grade prostate cancer remained significantly associated with the four SNPs risk score ($OR=2.6$, 95%CI: 2.0-3.4 and $OR=2.4$, 95%CI: 1.5-4.0, respectively), still after bootstrap validation using 10000 permutations ($OR=2.6$, 95%CI: 2.0-3.4 and $OR=3.1$, 95%CI: 2.3-4.1,

respectively). Furthermore, the logistic regression models (all-inclusive and PSA plus age) also persisted significantly different with the 4 SNPs risk score, for all prostate cancers (P=0.0107) and high-grade prostate cancers (P=0.0057), after likelihood ratio test. Despite AUC estimates for the all-inclusive score compared to the age plus PSA didn't reach statistical significance (all prostate cancer, P=0.0683 and high-grade prostate cancer, P=0.0723), the predictive value of the all-inclusive score remained higher in the NRI (all prostate cancers: 7.3%, P=0.0002, high-grade prostate cancer: 6.7%, P=0.0004) and IDI (all prostate cancers: 0.011, P=0.0012, high-grade prostate cancer: 0.013, P=0.0008) comparisons. These findings show that the predictive and discriminative ability of the genetic score based on 4 SNPs decreased but remained significant. Therefore, we used the all inclusive score.

DISCUSSION

Adipose tissue deregulation has been proposed as a relevant mechanism underlying obesity-related cancer, due to inappropriate release of biologically active adipokines. Thus, functional SNPs in genes coding for molecules involved in adipokine pathways may modulate the expression, transport, or signaling of adipokines, thereby influencing prostate cancer risk and biology. Our findings show that SNPs in genes from adipokine pathways (leptin, interleukin-6, fibroblast growth factor 2, osteopontin, and insulin growth factor) may influence the development of prostate cancer and aggressive disease. Interestingly, we found that both the *LEPR* Gln223Arg homozygous A and *OPN-66* homozygous G were significantly associated with all outcomes (risks of overall, high-grade, and high metastatic-risk prostate cancers).

The pleiotrophic effects of leptin, namely in tumor development and progression are mediated by its receptor [12,13]. Studies of SNPs affecting this pathway provided inconsistent results in prostate cancer. The leptin SNP at position -2548 was proposed as a susceptibility

locus for prostate cancer [14,15], albeit our data do not support this contention. Conversely, we found an increased risk in *LEPR* Gln223Arg homozygous A for prostate cancer, whereas others observed no such association [14,16]. *LEPR* Gln223Arg AA carriers have lower leptin binding affinity to soluble leptin receptor and have increased circulating free leptin and soluble leptin receptor levels [22,23]. Therefore, there is increased availability of leptin for binding to the long leptin receptor signaling isoform in the prostate tumor cell membrane. Cumulatively, the aminoacid change in this SNP may influence the signal for receptor intracellular recycling or degradation [24], modulating the availability of membrane-bound leptin receptor in tumor cells.

Osteopontin is a cytokine-like extracellular matrix molecule, that influences cell migration and anti-apoptosis in cancer [25]. This molecule has been implicated in aggressive and metastatic disease, and is one of a four-gene signature in prostate cancer that predicts metastasis and death [26,27]. The T-to-G substitution at position -66 in the human *OPN* gene modulates promoter activity [28]. The modified bioavailability of osteopontin may induce TH1-to-Th2 shift, modulating the microenvironment [28], and tumor development.

The IGF1-mediated activation of IGF1R has been demonstrated to contribute to tumor progression [29]. The IGF binding proteins modulate the effects of IGF1 and its biological function in different tissues. Recent evidence indicates increased risk of prostate cancer in individuals with high serum IGF1 levels, whereas risk was decreased in those with high levels of IGFBP-3 [30]. Furthermore, it was also found that the *IGFBP3*-202 A>C SNP was associated with prostate cancer and with low circulating levels of IGFBP3 [30]. The present study corroborates previous findings on the *IGFBP3*-202 A>C CC genotype risk for prostate cancer and high-grade disease [30,31]. Cumulatively, functional studies confirmed the underexpression of *IGFBP3* in C-allele carriers [32], resulting in increased IGF1 bioavailability. Signaling through the IGF1R is required for growth and survival [29]. The

synonymous *IGF1R* SNP at locus +3174 was described as a possible splicing regulator [33], thereby generating protein diversity [34] and serving as a mechanism for modulating gene expression [35]. Our findings showing that AA carriers remained independently associated with risk for all and for high-grade prostate cancer, suggest that this SNP may modulate *IGF1R* cell surface protein quantity, as well as *IGF1R*/*IGF1R* internalization and degradation, consequently influencing prostate tumor growth. Insulin receptor substrate – 1 (*IRS1*) is the primary docking protein of *IGF1R*, which mediates PI3K pathway activation within the *IGF1*/*IGF1R* system. Although the *IRS1* Gly972Arg SNP results in structural protein differences [36] in our study this SNP was not associated with prostate cancer risk, confirming previous findings [37].

FGF2 may have a role in tumorigenesis and cancer progression through induction of angiogenesis [38]. The *FGF*+223 variant in exon 1 is associated with FGF2 expression at the transcriptional and translational level [39]. Our findings show increased risk for all, high-grade, and high-metastasis risk prostate cancer among CC carriers, which are coherent with a functional upregulation of FGF2. This molecule interacts with a family of four distinct, high-affinity tyrosine kinase receptors, FGFR 1-4. Although increased availability of FGF2 and changes in FGFR2 receptor availability could play a role in the initiation and progression of prostate cancer, we did not find an association between the *FGFR2* rs2981582 in exon 2 and prostate cancer.

Initiation and progression of prostate cancer are stimulated by IL-6 [40]. Previous findings reported no association of the *IL6*-174 G>C SNP with prostate cancer [17,41], except for a small study of aggressive disease risk [42]. We did not find an association for the *IL6*-174 G>C SNP and prostate cancer. On the other hand, we found that carriers of the *IL6*-597 G-allele were at increased risk for high-grade prostate cancer. In fact, functional SNPs in the promoter region of *IL6* (-174, -572 and -597) do not act independently in the regulation of *IL6*

transcription [43]. The GG genotype in *IL6*-597 is linked to the GG genotype in *IL6*-174, which is associated with increased IL6 mRNA and protein levels. Therefore, the higher risk of high-grade prostate cancer associated with the *IL6*-597 G-allele may be due to increased IL6. IL6 signals are transmitted via a heterodimeric receptor complex consisting of a soluble interleukin-6 alpha subunit and a membrane-bound signal-transducing subunit, gp130. The common *IL6R* Asp385Ala variant is responsible for serum levels of soluble IL6R and IL6 and associates with IL6R membrane binding due to altered cleavage site [44], therefore, explaining our findings. The predominant activation of trans-signaling IL6/soluble IL6R pathway in aggressive prostate cancer [45], together with the functional *IL6R* Asp358Ala influence in this mechanism, supports the increased risk for high-grade prostate cancer we observed for C carriers (Ala carriers).

Several of the candidate SNPs in adipokine pathways known to affect oncogenesis, investigated here, were not associated with prostate cancer risk. Most of our null results for candidate SNPs in *APM1*+276, *VEGF*-460, *VEGF*+405, *VEGF*+936, *PPARG* Pro12Ala and *TNFA*-308, are in agreement with other studies [14,17,46,47]. To our knowledge, there have been no prior reports of null associations of *KDR*-604, *PPARD*-87, *PGC1A* Gly482Ser, *TNFR1*-329, *APM1*+45, *APM1*-11426, *GP130* Gly148Arg, *IL6*-6331, and *TNFA*-863 functional SNPs with prostate cancer.

We observed that some SNPs have a significant risk effect mainly in younger ages. The all-life exposure to increased levels of adipokines and pathway activation may influence early development of prostate cancer. Furthermore, *IL6R* Asp358Ala and *IGF1R* +3174 SNPs were significantly associated with early-onset prostate cancer, possibly due to accelerated tumor formation.

We tested each SNP for association with two clinically-relevant definitions of unfavorable outcomes: high-grade (combined Gleason score $\geq$ 7) and high -metastasis risk

(combined Gleason score ≥ 8 and/or PSA ≥ 20 ng/mL) prostate cancers. Combined Gleason score is a powerful predictor of disease progression and mortality [48], whereas Gleason score ≥ 8 is associated with aggressive biological behavior and increased risk of occult disseminated disease [49]. We found functional variants in genes from leptin, osteopontin, insulin growth factor, fibroblast growth factor 2 and interleukin 6 pathways to be related with high-grade prostate cancer, while SNPs in the leptin, osteopontin and fibroblast growth factor 2 axis associate with high-metastasis risk prostate cancer. These pathways are known to be involved in aggressive prostate cancer, lending support for these SNPs as clinical markers of aggressive disease. The SNPs in the risk score predict high grade/aggressive disease, but they also predict overall prostate cancer risk. The ability to predict overall as well as high grade cancers might be due to the significant proportion of high grade prostate cancer (Gleason ≥ 7) (83%) in our cancer population.

Although a wealth of evidence demonstrates the effects of individual adipokines on prostate carcinogenesis, it is unlikely that the overall pathophysiological impact is due to the influence of a single adipokine in vivo. We showed that consideration of the cumulative susceptibility contributed by SNPs from adipokine pathways helps in risk stratification. Our analyses indicate that the inclusive (age and PSA added to the multi-locus genetic set) risk score provides improvements in discrimination and prediction of all prostate cancer, and high-grade prostate cancer. We suggest that risk genotypes in the inclusive model may cooperate to influence the endocrine and paracrine activity of adipokine pathways that leads to tumor development and progression. However, the mechanisms underlying these high-order interactions among genetic polymorphisms in adipokine pathways genes in modulating prostate cancer risk remain to be fully elucidated.

In this cohort of men subjected to prostate biopsy due to abnormal clinical and/or PSA findings where an extensive biopsy scheme was used, we showed that by adding a genetic

score based on 7 SNPs significantly improved the discriminative ability of an established parsimonious model with only PSA and age. The AUC increased significantly from 0.65 to 0.68 for all prostate cancer and from 0.68 to 0.71 in high grade prostate cancer, when the genetic variants were added to the model. Furthermore, the improved discriminative ability and prostate cancer risk persisted with a four SNPs risk score (excluding SNPs deviated from Hardy-Weinberg equilibrium). Although we present the largest effort to date to study the association between adipokine genetic risk score and risk of prostate cancer, our results should be interpreted in the context of several potential limitations. We took a focused candidate gene approach to evaluate key SNPs in adipokine pathways but our SNP panel could be incomplete. Likewise, several newly reported prostate cancer risk-associated SNPs from genome-wide association studies were not included in the risk prediction model. Had we been able to include them, the overall risk prediction might have improved. We also estimated risk associations in the same study population, although the all-inclusive risk score was tested through bootstrap resampling with 10000 permutations; further studies in independent populations are required. Finally, despite our relatively large sample size, we had limited statistical power to examine genetic variants in relation to high-metastasis risk prostate cancer, because of the small number of cases in this group. However, our study has several strengths: i) it was prospective and large enough for key outcomes of interest, ii) most of the genes and SNPs selected were based on biological evidence of functional importance; iii) study design and statistical analyses accounted for relevant risk factors such as ethnicity and age [50], and although a large set of subjects missed heredity information < 2.2% were actually younger than 55 years of age [50]; iv) we used statistical strategies to assess the robustness of associations, such as bootstrap resampling and discrimination improvement measures; and v) all men were screened for prostate cancer based on both PSA level and

digital rectal exam during the recruitment period and diagnosis was determined by standard biopsy.

In summary, we identified SNPs in adipokine pathways that are associated with prostate cancer development and with a more aggressive phenotype. The inclusion of SNPs in the risk score model significantly improved, albeit modestly, the performance of PSA and age to predict overall prostate cancer and high-grade prostate cancer risk in men subjected to biopsy. The inclusion of further functional SNPs in a susceptibility model for prostate cancer is warranted, in order to determine a multi-locus model to accurately predict prostate cancer and disease aggressiveness. The use of improved risk models, such as the one described here, may impact public health if shown to have clinical utility when combined with individualized screening and risk reduction strategies.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the ethics committees of Porto Military Hospital and São João Hospital (Porto, Portugal). Patients were included after signing a written informed consent.

Subjects

Participants were enrolled between September 2007 and October 2010, after being referred to the urology departments of the participating hospitals for prostatic transrectal ultrasound guided biopsy (8-13 cores), on the basis of abnormal digital rectal examinations and/or single baseline PSA levels over 2.5 ng/mL. Our study population consists of 1099

consecutively-admitted Caucasian men who had histological evaluation and consented for genotyping.

In our study, from the prospectively enrolled men undergoing prostate biopsy, patients with non-prostate cancer (BPH or chronic prostatitis) were deemed controls for several orders of reasons: diagnosis was contemporary with cancers; advanced age at diagnosis allowed matching with elderly cancer patients; all undergone digital rectal examination, PSA estimate and prostate needle biopsy, making the possibility of crossover remote; most men have BPH or chronic prostatitis by the 7th-8th decades of life, making it normal in men of that age to carry benign prostatic disease, and corresponding to the median age at diagnosis in our prostate cancer group (age 69.0 years); men without prostatic disease would only be eligible from a much younger range of ages, which could introduce bias.

Prostate pathology and Gleason scores were determined via biopsy. In re-biopsed individuals only the last, most relevant pathological diagnosis was considered. Ninety-three men were excluded from the study due to a pathology report of high-grade prostatic intraepithelial neoplasia or a biopsy suspicious of cancer only. None of the participants had undergone prostate cancer treatment (hormonal castration, surgery, chemotherapy, or radiotherapy). All remaining 1006 eligible patients were included for molecular analysis.

Genetic variants and genotyping

Candidate SNPs were selected from the best evidence from published studies and through public databases that provide information on the phenotypic risks. Candidate genes involved in adipokine pathways known to affect oncogenesis were selected. SNPs with minor allele frequencies <0.05 were excluded. A total of 29 literature-defined putative functional SNPs in 19 different genes were selected, corresponding to 9 adipokine pathways (Table S1).

Genotyping for 22 SNPs (two in *ADIPOQ*, *IL6*, *IL6R*, *KDR*, three in *VEGF*, *LEP*, two in *LEPR*, *PPARG*, *PGC1A*, *PPARD*, *OPN*, *IGF1R*, *IGFBP3*, *IRS1*, *FGF2*, *FGFR2*, *TNFA*, *TNFRSF1A*) was performed using TaqMan allelic discrimination (Applied Biosystems), whereas 7 SNPs were genotyped through polymerase chain reaction - restriction fragment length polymorphism analysis (*IL6*-597/-572/-174, *APM1*+45, *GP130* Gly148Arg, *LEPR* Gln223Arg and *TNFA*-863), using previously described protocols. For quality control we used non-template controls in all runs and blind replicate genotype assessment in 5% of the samples. For the majority of SNPs, we observed almost complete concordance among duplicates.

Statistical analysis

We used means and medians as descriptive statistics for continuous variables and the Kolmogorov-Smirnov test to assess their departure from normality. The Mann-Whitney test was used to compare means between prostate cancer and non-cancer groups. The chi-square test was used to test for departures from Hardy-Weinberg equilibrium for each SNP based on the distribution among the non-prostate cancer group.

Unconditional logistic regression was used to estimate age-adjusted odds ratios (aORs) and 95% confidence intervals (95% CIs) for the associations between the polymorphisms and development of prostate cancer based on both recessive and dominant models. We examined the association of genetic markers with overall prostate cancer, restricted to high-grade prostate cancer (combined Gleason score ≥ 7), and restricted to high-risk prostate cancer for metastasis (PSA at diagnosis ≥ 20 ng/mL and/or combined Gleason score ≥ 8). Sensitivity analyses were conducted on the risk-associated SNPs that exhibited deviation from Hardy-Weinberg equilibrium. This was done by restricting the non-prostate cancer group to

normal/benign prostate hyperplasia histology, and with serum PSA < 4 ng/mL and then retesting the risk associations and departure from Hardy-Weinberg equilibrium.

To assess whether risk-associated SNPs affected time to clinical onset of disease we constructed Kaplan-Meier plots of the cumulative probabilities for having prostate cancer diagnosed at different ages according to each SNP. This analysis was conducted among prostate cancer cases only.

Stepwise multivariate logistic regression with backward elimination (P-value for retention=0.15) was conducted in SNPs with aOR $\leq$ 0.7 or aOR $\geq$ 1.3 (30% decrease or increase in odds of the outcome) plus age and PSA as continuous variables. Bootstrapping analyses were performed through MonteCarlo simulation (1000 replications).

We constructed an inclusive multi-locus genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyses. For each SNP, the risk genotypes were coded as 1 and the non-risk alleles as 0. The model was determined by multiplying the β coefficient by the SNPs, plus the γ coefficient by the PSA value and the α coefficient by the patient's age (Inclusive Risk Score = $\sum \beta_i \times X_i + \gamma \times \text{PSA} + \alpha \times \text{Age}$; where X_i =SNPs scaled for risk, β_i =coefficient for SNPs, γ =coefficient for PSA, α =coefficient for Age). A parsimonious risk score was calculated based on a model that included only PSA and age at diagnosis. These models were fitted independently using all prostate cancers and then restricted to high-grade prostate cancers as outcomes. A likelihood-ratio test was used to assess the goodness of fit between the logistic regression models.

We assessed the clinical value of the above two scores in correctly predicting disease status by receiver operating characteristic (ROC) curve analysis. We compared the areas under the ROC curves (AUC) constructed with both scores (with and without genetic information), both for all prostate cancers and high-grade cancers, using a non-parametric algorithm [51].

We evaluated the improvement in model performance (PSA and age risk score) introduced by the inclusion of the SNPs risk information, using the net reclassification improvement (NRI) and the integrated discrimination improvement (IDI) tests [52,53]. The NRI measures the reclassification of men from one risk category to another by addition of the genetic information to the PSA and age prediction model, and the extent of clinical utility can be evaluated by the magnitude of the NRI. The IDI does not consider risk thresholds; rather it is the mean of increments and decrements in estimated probabilities of prostate cancer for cases and non cases, comparing models. Since the NRI measurement is heavily dependent on the threshold levels used, we used a threshold probability between 15% and 45%, similar to those previously reported in such clinical context [54].

In order to validate the inclusive genetic risk score findings we performed logistic regression with 10 000 permutation (MonteCarlo simulation) in the full dataset (n=1006), and in a subset (the last 300 patients to be included in the study) from this full dataset, using the genetic risk score as continuous variable. All statistical analyses were conducted in STATA version 10.0 (StataCorp, College Station, Texas). For NRI and IDI calculations, we used the `nriidi`-package for Stata 11 [53].

420 REFERENCES

- 421 1. Gronberg H (2003) Prostate cancer epidemiology. *Lancet* 361: 859-864.
- 422 2. Andriole GL, Crawford ED, Grubb RL, 3rd, Buys SS, Chia D, et al. (2009) Mortality results from a
423 randomized prostate-cancer screening trial. *N Engl J Med* 360: 1310-1319.
- 424 3. Schroder FH, Hugosson J, Roobol MJ, Tammela TL, Ciatto S, et al. (2009) Screening and prostate-
425 cancer mortality in a randomized European study. *N Engl J Med* 360: 1320-1328.
- 426 4. Vickers AJ, Roobol MJ, Lilja H (2011) Screening for Prostate Cancer: Early Detection or
427 Overdetection? *Annu Rev Med*.
- 428 5. Morote J, Trilla E, Esquena S, Serrallach F, Abascal JM, et al. (2002) The percentage of free
429 prostatic-specific antigen is also useful in men with normal digital rectal examination and
430 serum prostatic-specific antigen between 10.1 and 20 ng/ml. *Eur Urol* 42: 333-337.
- 431 6. Damber JE, Aus G (2008) Prostate cancer. *Lancet* 371: 1710-1721.
- 432 7. Lindstrom S, Schumacher F, Siddiq A, Travis RC, Campa D, et al. (2011) Characterizing associations
433 and SNP-environment interactions for GWAS-identified prostate cancer risk markers--results
434 from BPC3. *PLoS One* 6: e17142.
- 435 8. Febbo PG (2009) Genomic approaches to outcome prediction in prostate cancer. *Cancer* 115:
436 3046-3057.
- 437 9. Aly M, Wiklund F, Xu J, Isaacs WB, Eklund M, et al. (2011) Polygenic risk score improves prostate
438 cancer risk prediction: results from the stockholm-1 cohort study. *Eur Urol* 60: 21-28.
- 439 10. Hsing AW, Sakoda LC, Chua S, Jr. (2007) Obesity, metabolic syndrome, and prostate cancer. *Am J*
440 *Clin Nutr* 86: s843-857.
- 441 11. von Hafe P, Pina F, Perez A, Tavares M, Barros H (2004) Visceral fat accumulation as a risk factor
442 for prostate cancer. *Obes Res* 12: 1930-1935.
- 443 12. Mistry T, Digby JE, Desai KM, Randeva HS (2007) Obesity and prostate cancer: a role for
444 adipokines. *Eur Urol* 52: 46-53.
- 445 13. Ribeiro R, Lopes C, Medeiros R (2006) The link between obesity and prostate cancer: the leptin
446 pathway and therapeutic perspectives. *Prostate Cancer Prostatic Dis* 9: 19-24.
- 447 14. Moore SC, Leitzmann MF, Albanes D, Weinstein SJ, Snyder K, et al. (2009) Adipokine genes and
448 prostate cancer risk. *Int J Cancer* 124: 869-876.
- 449 15. Ribeiro R, Vasconcelos A, Costa S, Pinto D, Morais A, et al. (2004) Overexpressing leptin genetic
450 polymorphism (-2548 G/A) is associated with susceptibility to prostate cancer and risk of
451 advanced disease. *Prostate* 59: 268-274.
- 452 16. Kote-Jarai Z, Singh R, Durocher F, Easton D, Edwards SM, et al. (2003) Association between leptin
453 receptor gene polymorphisms and early-onset prostate cancer. *BJU Int* 92: 109-112.
- 454 17. Wang MH, Helzlsouer KJ, Smith MW, Hoffman-Bolton JA, Clipp SL, et al. (2009) Association of IL10
455 and other immune response- and obesity-related genes with prostate cancer in CLUE II.
456 *Prostate* 69: 874-885.
- 457 18. Silha JV, Krsek M, Sucharda P, Murphy LJ (2005) Angiogenic factors are elevated in overweight
458 and obese individuals. *Int J Obes (Lond)* 29: 1308-1314.
- 459 19. Gomez-Ambrosi J, Catalan V, Ramirez B, Rodriguez A, Colina I, et al. (2007) Plasma osteopontin
460 levels and expression in adipose tissue are increased in obesity. *J Clin Endocrinol Metab* 92:
461 3719-3727.
- 462 20. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G (2001) Adipose tissue tumor necrosis
463 factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol*
464 *Endocrinol Metab* 280: E745-751.
- 465 21. Park J, Euhus DM, Scherer PE (2011) Paracrine and Endocrine Effects of Adipose Tissue on Cancer
466 Development and Progression. *Endocr Rev*.
- 467 22. Quinton ND, Lee AJ, Ross RJ, Eastell R, Blakemore AI (2001) A single nucleotide polymorphism
468 (SNP) in the leptin receptor is associated with BMI, fat mass and leptin levels in
469 postmenopausal Caucasian women. *Hum Genet* 108: 233-236.

23. Sun Q, Cornelis MC, Kraft P, Qi L, van Dam RM, et al. (2010) Genome-wide association study identifies polymorphisms in LEPR as determinants of plasma soluble leptin receptor levels. *Hum Mol Genet* 19: 1846-1855.
24. da Silva BA, Bjorbaek C, Uotani S, Flier JS (1998) Functional properties of leptin receptor isoforms containing the gln-->pro extracellular domain mutation of the fatty rat. *Endocrinology* 139: 3681-3690.
25. Chakraborty G, Jain S, Behera R, Ahmed M, Sharma P, et al. (2006) The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. *Curr Mol Med* 6: 819-830.
26. Weber GF, Lett GS, Haubein NC (2010) Osteopontin is a marker for cancer aggressiveness and patient survival. *Br J Cancer* 103: 861-869.
27. Ding Z, Wu CJ, Chu GC, Xiao Y, Ho D, et al. (2011) SMAD4-dependent barrier constrains prostate cancer growth and metastatic progression. *Nature* 470: 269-273.
28. Hummelshoj T, Ryder LP, Madsen HO, Odum N, Svejgaard A (2006) A functional polymorphism in the Eta-1 promoter is associated with allele specific binding to the transcription factor Sp1 and elevated gene expression. *Mol Immunol* 43: 980-986.
29. Pollak MN, Schernhammer ES, Hankinson SE (2004) Insulin-like growth factors and neoplasia. *Nat Rev Cancer* 4: 505-518.
30. Chen W, Wang S, Tian T, Bai J, Hu Z, et al. (2009) Phenotypes and genotypes of insulin-like growth factor 1, IGF-binding protein-3 and cancer risk: evidence from 96 studies. *Eur J Hum Genet* 17: 1668-1675.
31. Li L, Huang X, Huo K (2010) IGFBP3 polymorphisms and risk of cancer: a meta-analysis. *Mol Biol Rep* 37: 127-140.
32. Deal C, Ma J, Wilkin F, Paquette J, Rozen F, et al. (2001) Novel promoter polymorphism in insulin-like growth factor-binding protein-3: correlation with serum levels and interaction with known regulators. *J Clin Endocrinol Metab* 86: 1274-1280.
33. de Alencar SA, Lopes JC (2010) A comprehensive in silico analysis of the functional and structural impact of SNPs in the IGF1R gene. *J Biomed Biotechnol* 2010: 715139.
34. Modrek B, Lee C (2002) A genomic view of alternative splicing. *Nat Genet* 30: 13-19.
35. Chen M, Manley JL (2009) Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nat Rev Mol Cell Biol* 10: 741-754.
36. Almind K, Inoue G, Pedersen O, Kahn CR (1996) A common amino acid polymorphism in insulin receptor substrate-1 causes impaired insulin signaling. Evidence from transfection studies. *J Clin Invest* 97: 2569-2575.
37. Fall K, Stark JR, Mucci LA, Chan J, Stampfer MJ, et al. (2008) No association between a polymorphic variant of the IRS-1 gene and prostate cancer risk. *Prostate* 68: 1416-1420.
38. Cronauer MV, Schulz WA, Seifert HH, Ackermann R, Burchardt M (2003) Fibroblast growth factors and their receptors in urological cancers: basic research and clinical implications. *Eur Urol* 43: 309-319.
39. Schulz S, Kohler K, Schagdarsurengin U, Greiser P, Birkenmeier G, et al. (2005) The human FGF2 level is influenced by genetic predisposition. *Int J Cardiol* 101: 265-271.
40. Culig Z, Steiner H, Bartsch G, Hobisch A (2005) Interleukin-6 regulation of prostate cancer cell growth. *J Cell Biochem* 95: 497-505.
41. Dossus L, Kaaks R, Canzian F, Albanes D, Berndt SI, et al. (2010) PTGS2 and IL6 genetic variation and risk of breast and prostate cancer: results from the Breast and Prostate Cancer Cohort Consortium (BPC3). *Carcinogenesis* 31: 455-461.
42. Tan D, Wu X, Hou M, Lee SO, Lou W, et al. (2005) Interleukin-6 polymorphism is associated with more aggressive prostate cancer. *J Urol* 174: 753-756.
43. Terry CF, Loukaci V, Green FR (2000) Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. *J Biol Chem* 275: 18138-18144.
44. Mullberg J, Oberthur W, Lottspeich F, Mehl E, Dittrich E, et al. (1994) The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. *J Immunol* 152: 4958-4968.

45. Santer FR, Malinowska K, Culig Z, Cavarretta IT (2010) Interleukin-6 trans-signalling differentially regulates proliferation, migration, adhesion and maspin expression in human prostate cancer cells. *Endocr Relat Cancer* 17: 241-253.
46. Danforth KN, Rodriguez C, Hayes RB, Sakoda LC, Huang WY, et al. (2008) TNF polymorphisms and prostate cancer risk. *Prostate* 68: 400-407.
47. Jacobs EJ, Hsing AW, Bain EB, Stevens VL, Wang Y, et al. (2008) Polymorphisms in angiogenesis-related genes and prostate cancer. *Cancer Epidemiol Biomarkers Prev* 17: 972-977.
48. Albertsen PC, Hanley JA, Fine J (2005) 20-year outcomes following conservative management of clinically localized prostate cancer. *JAMA* 293: 2095-2101.
49. Harnden P, Shelley MD, Coles B, Staffurth J, Mason MD (2007) Should the Gleason grading system for prostate cancer be modified to account for high-grade tertiary components? A systematic review and meta-analysis. *Lancet Oncol* 8: 411-419.
50. Heidenreich A, Bellmunt J, Bolla M, Joniau S, Mason M, et al. (2011) EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. *Eur Urol* 59: 61-71.
51. DeLong ER, DeLong DM, Clarke-Pearson DL (1988) Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 44: 837-845.
52. Pencina MJ, D'Agostino RB, Sr., D'Agostino RB, Jr., Vasan RS (2008) Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Stat Med* 27: 157-172; discussion 207-112.
53. Sundstrom J, Byberg L, Gedeberg R, Michaelsson K, Berglund L (2011) Useful tests of usefulness of new risk factors: Tools for assessing reclassification and discrimination. *Scand J Public Health* 39: 439-441.
54. Cavadas V, Osorio L, Sabell F, Teves F, Branco F, et al. (2010) Prostate cancer prevention trial and European randomized study of screening for prostate cancer risk calculators: a performance comparison in a contemporary screened cohort. *Eur Urol* 58: 551-558.

FIGURE LEGENDS

Figure 1. Kaplan Meier analyses plots of significant genetic polymorphisms. (A) *IL6R* D358A A>C and (B) *IGF1R* +3174 G>A. Log Rank test.

Figure 2. ROC curves and AUC for the inclusive risk score and PSA plus age alone. (A) All prostate cancer and (B) restricted to high-grade prostate cancer. The Likelihood ratio test was used to estimate the difference between risk models for all prostate cancer and high-grade prostate cancer (P=0.0002 and P=0.0001, respectively). PSA, prostate specific antigen.

SUPPORTING INFORMATION

Table S1. Characteristics of candidate Single Nucleotide Polymorphisms (SNPs) involved in adipokine pathways potentially associated with cancer. HW-E, Hardy-Weinberg Equilibrium; *APM1*, adiponectin gene; *IL6*, interleukin-6 gene; *IL6R*, interleukin-6 receptor gene; *GP130*, interleukin-6 signal transducer gene; *KDR*, vascular endothelial growth factor receptor 2 gene; *VEGF*, vascular endothelial growth factor gene; *LEP*, leptin gene; *LEPR*, leptin receptor gene; *PPARGC1A*, Peroxisome proliferator-activated receptor gamma co-activator 1 alpha gene; *PPARD*, Peroxisome proliferator-activated receptor delta gene; *PPARG*, Peroxisome proliferator-activated receptor gamma gene; *OPN*, osteopontin gene; *IRS1*, insulin receptor substrate 1 gene; *IGFBP3*, insulin growth factor binding protein 3 gene; *IGF1R*, insulin growth factor 1 receptor gene; *FGF2*, fibroblast growth factor 2 gene; *FGFR2*, fibroblast growth factor receptor 2 gene; *TNFA*, tumoral necrosis factor alpha gene; *TNFRSF1A*,

582 tumoral necrosis factor receptor 1 gene. ^a The percentage of successfully genotyped DNA
583 samples from the 1006 participants.

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585 Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer (PCa) according to
586 adipokine pathways polymorphisms. N, number of evaluable patients; SNP, single nucleotide
587 polymorphism; OR (95%CI), age-adjusted odds-ratio and respective 95% confidence interval.

588 ^a HGPCa, High-grade Prostate Cancer (Gleason grade ≥ 7). ^b HRPCaM, High-risk Prostate
589 Cancer for metastasis (Gleason grade ≥ 8 and/or PSA ≥ 20 ng.mL⁻¹).

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591 Table S3. Age-adjusted Odds Ratios and 95%CI for prostate cancer (PCa) associated with
592 selected SNPs, after age stratification. ^a High-grade Prostate Cancer, Gleason grade ≥ 7 ; ^b
593 High-risk Prostate Cancer for metastasis, Gleason grade ≥ 8 and/or PSA ≥ 20 ng/mL; aOR
594 (95%CI), age-adjusted odds ratio and respective 95% Confidence Interval; PCa, Prostate
595 Cancer; Median age at diagnosis=67.5 years; * Evaluable individuals for analysis.

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597 Table S4. Sensitivity analysis in SNPs with deviation from Hardy-Weinberg equilibrium.
598 Risk for prostate cancer after restriction on the non-prostate cancer group to just benign
599 prostate hyperplasia and normal or to PSA below 4 ng/mL. * Hardy-Weinberg equilibrium,
600 Pearson chi-square analysis for differences between observed and expected genotype
601 frequencies; ** Age-adjusted odds ratios; BPH, Benign Prostate Hyperplasia; PSA, Prostate-
602 specific Antigen; PSA, prostate-specific antigen; SNP, single nucleotide polymorphism; aOR
603 (95%CI), age-adjusted odds ratio and respective 95% confidence interval. ^a Biopsy findings:
604 normal, 14.9%; BPH, 5.4%, chronic prostatitis, 74.7%; atrophy, 5%; ^b Biopsy findings:

605 normal, 73.5%; BPH, 26.5%; ^c Biopsy findings: normal, 22.2%; BPH, 6.0%, chronic
606 prostatitis, 65.8%; atrophy, 6.0%.

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Table 1. Age and hormonal variables by disease status

	Disease Status				
	Non-Prostate cancer		Prostate cancer		
	N ^a	Median (IQR)	N ^a	Median (IQR)	P ^b
Age, years	553	66.2 (61.9-71.4)	447	69.0 (63.0-74.0)	<0.0001
PSA, ng/mL	540	5.9 (4.2-8.7)	437	8.2 (5.3-14.3)	<0.0001
Free PSA, ng/mL	485	1.2 (0.7-1.9)	373	1.1 (0.7-1.9)	0.373
Free/Total PSA ratio	482	0.20 (0.14-0.27)	372	0.14 (0.08-0.22)	<0.0001
Serum Testosterone, ng/mL	494	444.5 (345.8-575.3)	381	443.0 (337.5-584.5)	0.690

^a Number of evaluable patients for each variable; IQR, Interquartile range; ^b Differences between groups, Mann-

Whitney test. PSA, prostate specific antigen

Table 2. Stepwise multivariate logistic regression and Bootstrap analyses

	All PCa			Restricted to high-grade PCa			Restricted to high-risk PCa for Metastasis		
	Multivariate model		Bootstrap	Multivariate model		Bootstrap	Multivariate model		Bootstrap
	Genotype	OR (95%CI) ^a	OR (95%CI) ^b	OR (95%CI) ^a	OR (95%CI) ^b	OR (95%CI) ^a	OR (95%CI) ^b	OR (95%CI) ^a	OR (95%CI) ^b
Age at diagnosis		1.03 (1.01-1.05)	1.02 (1.00-1.04)	1.03 (1.01-1.05)	1.03 (1.01-1.06)	1.07 (1.03-1.11)	1.07 (1.03-1.11)	1.07 (1.03-1.11)	1.07 (1.03-1.11)
PSA at diagnosis		1.07 (1.04-1.09)	1.06 (1.04-1.09)	1.07 (1.05-1.10)	1.07 (1.04-1.11)	1.14 (1.09-1.19)	1.07 (1.04-1.09)	1.14 (1.09-1.19)	1.14 (1.09-1.19)
LEPR Gln223Arg	G carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent
(A>G)	AA	1.52 (1.14-2.02)	1.53 (1.13-2.07)	1.56 (1.15-2.12)	1.57 (1.14-2.14)	1.55 (0.93-2.58)	1.50 (0.91-2.45)	1.55 (0.93-2.58)	1.55 (0.93-2.58)
OPN-66 T>G	T carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent
	GG	1.86 (1.07-3.23)	1.77 (1.00-3.13)	1.97 (1.10-3.52)	1.89 (1.03-3.49)	2.52 (1.12-5.64)	2.64 (1.16-6.01)	2.52 (1.12-5.64)	2.52 (1.12-5.64)
IGF1R+3174	G carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent
G>A	AA	1.33 (0.93-1.89)	1.34 (0.94-1.93)	1.40 (0.96-2.05)	1.39 (0.93-2.09)	---	---	---	---
IGFBP3-202 A>C	A carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent
	CC	1.40 (1.02-1.92)	1.38 (1.01-1.88)	1.40 (1.00-1.95)	1.39 (1.00-1.93)	---	---	---	---
FGF2 +223 C>T	T carriers	Referent	Referent	Referent	Referent	Referent	Referent	Referent	Referent
	CC	1.45 (0.98-2.14)	1.45 (0.98-2.16)	1.55 (1.00-2.38)	1.54 (1.00-2.38)	2.22 (1.02-4.85)	2.20 (1.01-4.78)	2.22 (1.02-4.85)	2.22 (1.02-4.85)
IL6-597 G>A	AA	Referent	Referent	Referent	Referent	---	---	---	---
	G carriers	1.42 (0.92-2.19)	1.37 (0.88-2.13)	1.61 (0.99-2.62)	1.58 (0.97-2.56)	---	---	---	---

Age and PSA analyzed as continuous variables. PCa, prostate cancer. ^a Stepwise multivariate logistic regression; ^b MonteCarlo simulation (1000 replications). Empirical confounding variables were independently analyzed in each model (overall prostate cancer and both restricted groups)

Table 3. Inclusive risk score risk (ORs) and bootstrap validation (ORs and AUC)

	Full dataset *	Full dataset **	Subset **
Odds ratios	(n=1006)	(n=1006)	(n=300)
Non-PCa vs. PCa	2.52 (1.99-3.19)	2.53(1.98-3.22)	2.24(1.50-3.34)
NonPCa vs. HGPCa	2.77 (2.17-3.54)	2.97(2.27-3.89)	2.73(1.68-4.46)
AUC			
Non-PCa vs. PCa	---	0.68(0.65-0.71)	0.70(0.64-0.76)
Non-PCa vs. HGPCa	---	0.71(0.68-0.75)	0.75(0.69-0.81)

* Logistic regression analysis to compute age-adjusted Odds Ratios (ORs) and respective 95% confidence intervals (95% CIs) for prostate cancer and high grade disease, using age and GRS as continuous variables. ** Odds ratios were calculated by MonteCarlo bootstrapping (10 000 replications), using inclusive genetic risk score as a continuous variable. PCa, prostate cancer; HGPCa, high grade prostate cancer.

Table S1. Characteristics of candidate Single Nucleotide Polymorphisms (SNPs) involved in adipokine pathways potentially associated with cancer

Pathway	Gene	Reference SNP ID	Nucleotide substitution	Genomic location	In controls		
					Variant allele (%)	Genotyped (%) ^a	HW-E (P-value)
Adiponectin	<i>APM1</i>	rs1501299	G>T	+ 276 intron 2	29.9	99.8	0.80
	<i>APM1</i>	rs2241766	T>G	+45 intron 2	13.9	100	0.91
	<i>APM1</i>	rs16861194	A>G	-11426 promoter	10.4	100	1.00
Interleukin - 6	<i>IL6</i>	rs1800795	G>C	-174 promoter	36.2	99.8	0.98
	<i>IL6</i>	rs1800796	G>C	-572 promoter	14.3	99.9	0.89
	<i>IL6</i>	rs1800797	G>A	-597 promoter	35.3	99.9	0.89
	<i>IL6</i>	rs10499563	T>C	-6331 promoter	27.5	100	0.91
	<i>IL6R</i>	rs2228145	A>C	Asp358Ala	36.9	99.9	0.62
	<i>gp130</i>	rs3729960	G>C	Gly148Arg	9.5	99.9	0.63
Vascular Endothelial	<i>KDR</i>	rs2071559	T>C	-604 promoter	47.1	100	0.96
Growth Factor	<i>VEGF</i>	rs2010963	G>C	+405 5'-UTR	32.3	99.8	0.72
	<i>VEGF</i>	rs833061	C>T	-460 promoter	48.2	99.8	0.95
	<i>VEGF</i>	rs3025039	C>T	+936 3'-UTR	13.1	99.8	0.85
Leptin	<i>LEP</i>	rs7799039	G>A	-2548 promoter	39.3	99.8	0.96
	<i>LEPR</i>	rs1137100	A>G	Lys109Arg	22.8	99.8	0.77
	<i>LEPR</i>	rs1137101	A>G	Gln223Arg	44.9	100	<0.01
	<i>LEPR</i>	rs8179183	G>C	Lys656Asn	20.3	99.8	0.30
Peroxisome proliferator-	<i>PPARGC1A</i>	rs8192678	A>G	Gly482Ser	37.3	99.7	0.83

activated receptor	<i>PPARD</i>	rs2016520	T>C	-87 5'-UTR	99.7	21.4	0.93
	<i>PPARG</i>	rs1801282	C>G	Pro12Ala	99.9	8.8	0.37
Osteopontin	<i>OPN</i>	rs28357094	T>G	-66 promoter	99.9	22.8	0.97
Insulin growth factor 1	<i>IRS1</i>	rs1801278	C>T	Gly972Arg	100	9.7	< 0.01
	<i>IGFBP3</i>	rs2854744	A>C	-202 promoter	100	47.3	0.63
	<i>IGF1R</i>	rs2229765	G>A	+3174 exon 16	100	43.7	< 0.01
Fibroblast growth factor 2	<i>FGF2</i>	rs1449683	C>T	+223 5'-UTR	99.7	9.5	< 0.01
	<i>FGFR2</i>	rs2981582	C>T	Intron 2	100	36.7	0.78
Tumor necrosis factor	<i>TNFA</i>	rs1800629	G>A	-308 promoter	100	14.1	0.36
alpha	<i>TNFA</i>	rs1800630	C>A	-863 promoter	99.8	21.1	0.19
	<i>TNFRSF1A</i>	rs4149570	G>T	-329 promoter	99.9	38.8	0.61

HW-E, Hardy-Weinberg Equilibrium; *APM1*, adiponectin gene; *IL6*, interleukin-6 gene; *IL6R*, interleukin-6 receptor gene; *GP130*, interleukin-6 signal transducer gene; *KDR*, vascular endothelial growth factor receptor 2 gene; *VEGF*, vascular endothelial growth factor gene; *LEP*, leptin gene; *LEPR*, leptin receptor gene; *PPARGC1A*, Peroxisome proliferator-activated receptor gamma co-activator 1 alpha gene; *PPARD*, Peroxisome proliferator-activated receptor delta gene; *PPARG*, Peroxisome proliferator-activated receptor gamma gene; *OPN*, osteopontin gene; *IRS1*, insulin receptor substrate 1 gene; *IGFBP3*, insulin growth factor binding protein 3 gene; *IGF1R*, insulin growth factor 1 receptor gene; *FGF2*, fibroblast growth factor 2 gene; *FGFR2*, fibroblast growth factor receptor 2 gene; *TNFA*, tumoral necrosis factor alpha gene; *TNFRSF1A*, tumoral necrosis factor receptor 1 gene. ^a The percentage of successfully genotyped DNA samples from the 1006 participants.

Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer (PCa) according to adipokine pathways polymorphisms

Pathway	SNP	Model	Genotypes	Non-PCa		All PCa		Age-adjusted Odds Ratios			
				N	N	Restricted to HGPCa ^a		Restricted to HRPCaM ^b			
						OR (95% CI)	N	OR (95% CI)	N	OR (95% CI)	
Adiponectin	APM1+45 T>G		TT	414	347	Referent	Referent	289	Referent	120	Referent
			TG	131	90	0.8 (0.3-1.8)	0.7 (0.3-1.7)	74	0.7 (0.3-1.7)	30	0.7 (0.2-2.1)
			GG	12	12	0.9 (0.4-1.9)	0.8 (0.3-1.8)	11	0.8 (0.3-1.8)	5	0.7 (0.2-2.2)
		Dominant	TT	414	347	Referent	Referent	289	Referent	120	Referent
			G carriers	143	102	0.9 (0.7-1.2)	0.9 (0.7-1.3)	85	0.9 (0.7-1.3)	35	1.0 (0.6-1.5)
			T carriers	545	437	Referent	Referent	363	Referent	150	Referent
		Recessive	GG	12	12	1.2 (0.5-2.7)	1.3 (0.6-3.0)	11	1.3 (0.6-3.0)	5	1.4 (0.5-4.2)
			GG	276	228	Referent	Referent	196	Referent	79	Referent
			GT	226	172	0.8 (0.5-1.3)	0.9 (0.5-1.4)	138	0.9 (0.5-1.4)	58	0.8 (0.4-1.6)
		Dominant	TT	53	49	0.9 (0.6-1.4)	1.0 (0.6-1.6)	40	1.0 (0.6-1.6)	18	0.9 (0.5-1.7)
			GG	276	228	Referent	Referent	196	Referent	79	Referent
			T carriers	279	221	1.0 (0.7-1.2)	0.9 (0.7-1.2)	178	0.9 (0.7-1.2)	76	1.0 (0.7-1.4)
		Recessive	G carriers	502	400	Referent	Referent	334	Referent	137	Referent
			TT	53	49	1.1 (0.7-1.7)	1.1 (0.7-1.7)	40	1.1 (0.7-1.7)	18	1.1 (0.6-2.0)
			AA	447	360	Referent	Referent	299	Referent	123	Referent
Interleukine 6	IL6-6331 T>C		AG	104	85	1.3 (0.4-4.8)	1.1 (0.3-4.1)	71	1.1 (0.3-4.1)	31	2.0 (0.2-18.0)
			GG	6	4	1.3 (0.4-4.6)	1.1 (0.3-3.9)	4	1.1 (0.3-3.9)	1	1.8 (0.2-15.6)
			AA	447	360	Referent	Referent	299	Referent	123	Referent
		Dominant	G carriers	110	89	1.0 (0.7-1.4)	1.0 (0.7-1.4)	75	1.0 (0.7-1.4)	32	1.1 (0.7-1.7)
			A carriers	551	445	Referent	Referent	370	Referent	154	Referent
			GG	6	4	0.6 (0.1-2.4)	0.7 (0.2-2.8)	4	0.7 (0.2-2.8)	1	0.5 (0.1-4.7)
		Dominant	TT	291	236	Referent	Referent	198	Referent	87	Referent
			TC	226	185	1.0 (0.8-1.3)	1.0 (0.8-1.3)	153	1.0 (0.8-1.3)	60	0.9 (0.6-1.4)
			CC	40	28	0.9 (0.5-1.5)	0.9 (0.5-1.5)	23	0.9 (0.5-1.5)	8	0.7 (0.3-1.6)
		Recessive	TT	291	236	Referent	Referent	198	Referent	87	Referent
			C carriers	266	213	1.0 (0.8-1.3)	1.0 (0.7-1.3)	176	1.0 (0.7-1.3)	68	0.9 (0.6-1.3)
			T carriers	517	421	Referent	Referent	351	Referent	147	Referent
		CC	40	28	0.9 (0.5-1.4)	0.9 (0.5-1.5)	23	0.9 (0.5-1.5)	8	0.7 (0.3-1.7)	

Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer according to adipokine pathways polymorphisms (cont'd)

Pathway	SNP	Model	Genotypes	Non-PCa		All PCa		Age-adjusted Odds Ratios			
				N	OR (95% CI)	N	OR (95% CI)	Restricted to HGPCa ^a		Restricted to HRPCaM ^b	
								N	OR (95% CI)	N	OR (95% CI)
Interleukine 6	IL6-597 G>A	Dominant	GG	235	Referent	201	Referent	167	Referent	69	Referent
			GA	249	1.0 (0.7-1.3)	204	1.0 (0.8-1.3)	173	1.0 (0.8-1.3)	69	1.0 (0.7-1.4)
			AA	72	0.7 (0.5-1.1)	44	0.7 (0.5-1.1)	34	0.7 (0.4-1.1)	17	0.8 (0.4-1.5)
			GG	235	Referent	201	Referent	167	Referent	69	Referent
		Recessive	A carriers	321	0.9 (0.7-1.2)	248	0.9 (0.7-1.2)	207	0.9 (0.7-1.2)	86	0.9 (0.6-1.4)
			G carriers	484	Referent	405	Referent	340	Referent	138	Referent
			AA	72	0.7 (0.5-1.1)	44	0.7 (0.5-1.1)	34	0.7 (0.4-1.0)	17	0.8 (0.5-1.5)
		IL6-572 G>C	GG	407	Referent	314	Referent	259	Referent	109	Referent
			GC	139	1.2 (0.9-1.6)	125	1.2 (0.9-1.6)	106	1.2 (0.9-1.7)	45	1.3 (0.9-2.0)
			CC	10	1.3 (0.5-3.2)	10	1.3 (0.5-3.2)	9	1.5 (0.6-3.7)	1	0.4 (0.0-3.0)
	Dominant	GG	407	Referent	314	Referent	259	Referent	109	Referent	
		C carriers	149	1.2 (0.9-1.6)	135	1.2 (0.9-1.6)	115	1.2 (0.9-1.7)	46	1.2 (0.8-1.8)	
		G carriers	546	Referent	439	Referent	365	Referent	154	Referent	
	IL6-174 G>C	CC	10	1.2 (0.5-3.0)	10	1.2 (0.5-3.0)	9	1.4 (0.6-3.5)	1	0.3 (0.0-2.8)	
		GG	225	Referent	194	Referent	159	Referent	63	Referent	
		GC	259	0.9 (0.7-1.2)	209	0.9 (0.7-1.2)	177	1.0 (0.7-1.3)	74	1.0 (0.7-1.5)	
	Dominant	CC	72	0.7 (0.5-1.1)	45	0.7 (0.5-1.1)	37	0.7 (0.5-1.2)	17	0.8 (0.5-1.6)	
		GG	225	Referent	194	Referent	159	Referent	63	Referent	
		C carriers	331	0.9 (0.7-1.2)	254	0.9 (0.7-1.2)	214	0.9 (0.7-1.2)	91	1.0 (0.7-1.4)	
	Recessive	G carriers	484	Referent	403	Referent	336	Referent	137	Referent	
CC		72	0.8 (0.5-1.1)	45	0.8 (0.5-1.1)	37	0.7 (0.5-1.1)	17	0.8 (0.5-1.5)		
IL6R Asp358Ala (A>C)		Dominant	AA	227	Referent	164	Referent	132	Referent	54	Referent
	AC		248	1.2 (0.9-1.6)	209	1.2 (0.9-1.6)	176	1.2 (0.9-1.7)	70	1.3 (0.8-1.9)	
	CC		81	1.3 (0.9-1.9)	76	1.3 (0.9-1.9)	66	1.4 (1.0-2.1)	31	1.6 (0.9-2.6)	
	Recessive	AA	227	Referent	164	Referent	132	Referent	54	Referent	
		C carriers	329	1.2 (0.9-1.6)	285	1.2 (0.9-1.6)	242	1.3 (1.0-1.7)	101	1.3 (0.9-2.0)	
		A carriers	475	Referent	373	Referent	308	Referent	124	Referent	
	CC	81	1.2 (0.8-1.7)	76	1.2 (0.8-1.7)	66	1.3 (0.9-1.8)	31	1.4 (0.9-2.2)		

Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer according to adipokine pathways polymorphisms (cont'd)

Pathway	SNP	Model	Genotypes	Non-PCa		All PCa		Age-adjusted Odds Ratios			
				N	OR (95% CI)	N	OR (95% CI)	Restricted to HGPCa ^a		Restricted to HRPCaM ^b	
								N	OR (95% CI)	N	OR (95% CI)
Interleukine 6	gp130 Gly140Arg (G>C)		GG	457	Referent	381	Referent	316	Referent	127	Referent
			GC	92	0.8 (0.6-1.2)	60	0.8 (0.6-1.2)	52	0.9 (0.6-1.3)	25	1.1 (0.7-1.9)
			CC	7	1.2 (0.4-3.5)	8	1.2 (0.4-3.5)	6	1.1 (0.4-3.4)	3	1.2 (0.3-4.9)
		Dominant	GG	457	Referent	381	Referent	316	Referent	127	Referent
		Recessive	C carriers	99	0.8 (0.6-1.2)	68	0.8 (0.6-1.2)	58	0.9 (0.6-1.3)	28	1.1 (0.7-1.8)
			G carriers	549	Referent	441	Referent	368	Referent	152	Referent
Vascular endothelial growth factor	KDR-604 T>C		CC	7	1.3 (0.5-3.6)	8	1.3 (0.5-3.6)	6	1.2 (0.4-3.5)	3	1.2 (0.3-4.8)
			TT	154	Referent	127	Referent	105	Referent	48	Referent
			CT	281	0.9 (0.7-1.2)	215	0.9 (0.7-1.2)	177	0.9 (0.7-1.3)	72	0.8 (0.5-1.3)
		Dominant	CC	122	1.1 (0.7-1.5)	107	1.1 (0.7-1.5)	92	1.1 (0.8-1.6)	35	1.0 (0.6-1.6)
			TT	154	Referent	127	Referent	105	Referent	48	Referent
		Recessive	C carriers	403	1.0 (0.7-1.3)	322	1.0 (0.7-1.3)	269	1.0 (0.7-1.3)	107	0.9 (0.6-1.3)
	VEGF-460 C>T		T carriers	435	Referent	342	Referent	282	Referent	120	Referent
			CC	122	1.1 (0.8-1.5)	107	1.1 (0.8-1.5)	92	1.2 (0.9-1.6)	35	1.1 (0.7-1.7)
			CC	131	Referent	114	Referent	99	Referent	46	Referent
		Dominant	CT	274	0.9 (0.6-1.2)	201	0.9 (0.6-1.2)	166	0.8 (0.6-1.1)	74	0.8 (0.5-1.2)
			TT	151	1.1 (0.8-1.5)	133	1.1 (0.8-1.5)	108	1.0 (0.7-1.5)	34	0.7 (0.4-1.1)
		Recessive	TT	151	Referent	133	Referent	108	Referent	34	Referent
VEGF+405 G>C	VEGF-460 C>T		C carriers	405	0.9 (0.6-1.1)	315	0.9 (0.6-1.1)	265	0.9 (0.6-1.2)	120	1.2 (0.8-1.9)
			T carriers	425	Referent	334	Referent	274	Referent	108	Referent
			CC	131	1.1 (0.8-1.4)	114	1.1 (0.8-1.4)	99	1.1 (0.8-1.5)	46	1.3 (0.9-2.0)
		Dominant	GG	251	Referent	200	Referent	169	Referent	77	Referent
			GC	252	1.0 (0.8-1.3)	197	1.0 (0.8-1.3)	162	1.0 (0.7-1.3)	66	0.8 (0.6-1.2)
		Recessive	CC	54	1.2 (0.8-1.9)	50	1.2 (0.8-1.9)	41	1.2 (0.8-1.9)	10	0.7 (0.3-1.4)
	VEGF+405 G>C		GG	251	Referent	200	Referent	169	Referent	77	Referent
			C carriers	306	1.0 (0.8-1.3)	247	1.0 (0.8-1.3)	203	1.0 (0.8-1.3)	76	0.8 (0.6-1.2)
			G carriers	503	Referent	397	Referent	331	Referent	143	Referent
		Dominant	CC	54	1.2 (0.8-1.9)	50	1.2 (0.8-1.9)	41	1.2 (0.8-1.9)	10	0.7 (0.4-1.5)
			CC	54	1.2 (0.8-1.9)	50	1.2 (0.8-1.9)	41	1.2 (0.8-1.9)	10	0.7 (0.4-1.5)
		Recessive	CC	54	1.2 (0.8-1.9)	50	1.2 (0.8-1.9)	41	1.2 (0.8-1.9)	10	0.7 (0.4-1.5)

Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer according to adipokine pathways polymorphisms (cont'd)

Pathway	SNP	Model	Genotypes	Non-PCa		All PCa		Age-adjusted Odds Ratios			
				N	OR (95% CI)	N	OR (95% CI)	Restricted to HGPCa ^a		Restricted to HRPcCaM ^b	
								N	OR (95% CI)	N	OR (95% CI)
Vascular endothelial growth factor	VEGF+936 C>T		CC	421	Referent	341	Referent	282	Referent	114	Referent
			CT	123	1.0 (0.7-1.3)	100	1.0 (0.7-1.3)	87	1.0 (0.7-1.4)	39	1.1 (0.7-1.6)
			TT	11	0.9 (0.3-2.2)	8	0.9 (0.3-2.2)	5	0.7 (0.2-2.0)	2	0.7 (0.1-3.2)
		Dominant	CC	421	Referent	341	Referent	282	Referent	114	Referent
		Recessive	T carriers	134	1.0 (0.7-1.3)	108	1.0 (0.7-1.3)	92	1.0 (0.7-1.3)	41	1.0 (0.7-1.6)
			C carriers	544	Referent	441	Referent	369	Referent	153	Referent
Leptin	LEP-2548 G>A		TT	11	0.9 (0.4-2.2)	8	0.9 (0.4-2.2)	5	0.7 (0.2-2.0)	2	0.7 (0.1-3.1)
			GG	203	Referent	164	Referent	135	Referent	58	Referent
			GA	268	1.0 (0.8-1.3)	212	1.0 (0.8-1.3)	178	1.0 (0.8-1.4)	75	1.0 (0.7-1.6)
		Dominant	AA	84	1.1 (0.7-1.5)	73	1.1 (0.7-1.5)	61	1.1 (0.7-1.6)	22	0.9 (0.5-1.7)
			GG	203	Referent	164	Referent	135	Referent	58	Referent
			A carriers	352	1.0 (0.8-1.3)	285	1.0 (0.8-1.3)	239	1.0 (0.8-1.4)	97	1.0 (0.7-1.5)
		Recessive	G carriers	471	Referent	376	Referent	313	Referent	133	Referent
			AA	84	1.1 (0.8-1.5)	73	1.1 (0.8-1.5)	61	1.1 (0.7-1.5)	22	0.9 (0.5-1.6)
			AA	334	Referent	264	Referent	214	Referent	91	Referent
		Dominant	AG	190	1.0 (0.8-1.4)	157	1.0 (0.8-1.4)	138	1.1 (0.9-1.5)	57	1.1 (0.7-1.6)
			GG	32	1.1 (0.6-1.9)	28	1.1 (0.6-1.9)	22	1.1 (0.6-1.9)	7	0.8 (0.3-1.9)
			AA	334	Referent	264	Referent	214	Referent	91	Referent
LEPR Lys109Arg (A>G)	LEPR Lys109Arg (A>G)		G carriers	222	1.0 (0.8-1.4)	185	1.0 (0.8-1.4)	160	1.1 (0.9-1.5)	64	1.0 (0.7-1.5)
			A carriers	524	Referent	421	Referent	352	Referent	148	Referent
			GG	32	1.0 (0.6-1.8)	28	1.0 (0.6-1.8)	22	1.0 (0.5-1.7)	7	0.8 (0.3-1.8)
		Dominant	AA	151	Referent	166	Referent	141	Referent	63	Referent
			AG	312	0.6 (0.5-0.8)	206	0.6 (0.5-0.8)	173	0.6 (0.4-0.8)	72	0.6 (0.4-0.8)
			GG	94	0.7 (0.5-1.1)	77	0.7 (0.5-1.1)	60	0.7 (0.5-1.0)	20	0.5 (0.3-0.8)
		Recessive	AA	151	Referent	166	Referent	141	Referent	63	Referent
			G carriers	406	0.6 (0.5-0.8)	283	0.6 (0.5-0.8)	233	0.6 (0.5-0.8)	92	0.5 (0.4-0.8)
			A carriers	463	Referent	372	Referent	314	Referent	135	Referent
			GG	94	1.0 (0.7-1.4)	77	1.0 (0.7-1.4)	60	0.9 (0.6-1.3)	20	0.7 (0.4-1.1)

Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer according to adipokine pathways polymorphisms (cont'd)

Pathway	SNP	Model	Genotypes	Non-PCa		All PCa		Age-adjusted Odds Ratios			
				N	N	OR (95% CI)	N	Restricted to HGPCa ^a		Restricted to HRPCaM ^b	
								N	OR (95% CI)	N	OR (95% CI)
Leptin	LEPR Lys656Asn (G>C)		GG	347	298	Referent	245	Referent	99	Referent	Referent
			GC	192	133	0.8 (0.6-1.1)	113	0.8 (0.6-1.1)	48	0.9 (0.6-1.3)	0.9 (0.6-1.3)
			CC	17	17	1.1 (0.5-2.2)	15	1.1 (0.6-2.3)	7	1.4 (0.5-3.5)	1.4 (0.5-3.5)
		Dominant	GG	347	298	Referent	245	Referent	99	Referent	Referent
		Recessive	C carriers	209	150	0.8 (0.6-1.1)	128	0.9 (0.7-1.1)	55	0.9 (0.6-1.4)	0.9 (0.6-1.4)
			G carriers	539	431	Referent	358	Referent	147	Referent	Referent
Peroxisome proliferator-activated receptors	PPARG Pro12Ala (C>G)		CC	17	17	1.2 (0.6-2.3)	15	1.2 (0.6-2.5)	7	1.4 (0.6-3.6)	1.4 (0.6-3.6)
			CC	466	384	Referent	319	Referent	129	Referent	Referent
			CG	84	61	0.9 (0.6-1.3)	53	0.9 (0.6-1.3)	26	1.1 (0.7-1.8)	1.1 (0.7-1.8)
		Dominant	GG	7	3	0.5 (0.1-2.0)	2	0.4 (0.1-2.1)	0	----	----
			CC	466	384	Referent	319	Referent	129	Referent	Referent
			G carriers	91	64	0.9 (0.6-1.2)	55	0.9 (0.6-1.3)	26	1.0 (0.6-1.6)	1.0 (0.6-1.6)
		Recessive	C carriers	550	445	Referent	372	Referent	155	Referent	Referent
			GG	7	3	0.5 (0.1-2.1)	2	0.4 (0.1-2.1)	0	----	----
			AA	222	166	Referent	135	Referent	55	Referent	Referent
		Dominant	AG	254	217	1.2 (0.9-1.5)	185	1.3 (0.9-1.7)	74	1.2 (0.8-1.8)	1.2 (0.8-1.8)
			GG	81	63	1.1 (0.7-1.6)	53	1.1 (0.8-1.7)	25	1.3 (0.8-2.3)	1.3 (0.8-2.3)
			AA	222	166	Referent	135	Referent	55	Referent	Referent
PPARD -87 T>C	Gly482Ser (A>G)		G carriers	335	280	1.1 (0.9-1.5)	238	1.2 (0.9-1.6)	99	1.2 (0.8-1.8)	1.2 (0.8-1.8)
			A carriers	476	383	Referent	320	Referent	129	Referent	Referent
			GG	81	63	1.0 (0.7-1.4)	53	1.0 (0.7-1.5)	25	1.2 (0.7-2.0)	1.2 (0.7-2.0)
		Dominant	TT	342	260	Referent	219	Referent	94	Referent	Referent
			TC	190	159	1.1 (0.8-1.4)	131	1.1 (0.8-1.4)	51	1.0 (0.7-1.4)	1.0 (0.7-1.4)
			CC	24	28	1.5 (0.8-2.6)	24	1.5 (0.8-2.7)	10	1.4 (0.6-3.2)	1.4 (0.6-3.2)
		Recessive	TT	342	260	Referent	219	Referent	94	Referent	Referent
			C carriers	214	187	1.1 (0.9-1.5)	155	1.1 (0.8-1.5)	61	1.0 (0.7-1.5)	1.0 (0.7-1.5)
			T carriers	532	419	Referent	350	Referent	145	Referent	Referent
			CC	24	28	1.4 (0.8-2.5)	24	1.5 (0.8-2.7)	10	1.5 (0.7-3.2)	1.5 (0.7-3.2)

Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer according to adipokine pathways polymorphisms (cont'd)

Pathway	SNP	Model	Genotypes	Non-PCa		All PCa		Age-adjusted Odds Ratios			
				N	OR (95% CI)	N	OR (95% CI)	Restricted to HGPCa ^a		Restricted to HRPCaM ^b	
								N	OR (95% CI)	N	OR (95% CI)
Osteopontin	OPN -66 T>G		TT	330	268	Referent	Referent	224	Referent	94	Referent
			TG	198	143	0.9 (0.7-1.2)	0.9 (0.6-1.2)	118	0.9 (0.6-1.2)	45	0.8 (0.5-1.2)
			GG	28	38	1.7(1.0-2.9)	1.8 (1.0-3.1)	32	1.8 (1.0-3.1)	16	2.2 (1.1-4.5)
		Dominant	TT	330	268	Referent	Referent	224	Referent	94	Referent
			G carriers	226	181	1.0 (0.8-1.3)	1.0 (0.7-1.3)	150	1.0 (0.7-1.3)	61	0.9 (0.7-1.4)
		Recessive	T carriers	528	411	Referent	Referent	342	Referent	139	Referent
Insulin growth factor	IGF1R+3174 G>A		GG	28	38	1.8 (1.1-3.0)	1.9 (1.1-3.2)	32	1.9 (1.1-3.2)	16	2.4 (1.2-4.8)
			GG	158	133	Referent	Referent	111	Referent	49	Referent
			GA	311	232	0.9 (0.7-1.2)	0.9 (0.6-1.2)	193	0.9 (0.6-1.2)	80	0.9 (0.6-1.3)
		Dominant	AA	88	84	1.2 (0.8-1.8)	1.2 (0.8-1.8)	70	1.2 (0.8-1.8)	26	1.1 (0.6-2.0)
			GG	158	133	Referent	Referent	111	Referent	49	Referent
		Recessive	A carriers	399	316	1.0 (0.7-1.3)	0.9 (0.7-1.3)	263	0.9 (0.7-1.3)	106	0.9 (0.6-1.4)
	IGFBP3-202 A>C		G carriers	469	365	Referent	Referent	304	Referent	129	Referent
			AA	88	84	1.3 (1.0-1.9)	1.3 (0.9-1.9)	70	1.3 (0.9-1.9)	26	1.2 (0.8-2.0)
			AA	149	117	Referent	Referent	96	Referent	44	Referent
		Dominant	AC	289	212	0.9 (0.7-1.3)	1.0 (0.7-1.3)	176	1.0 (0.7-1.3)	74	0.9 (0.6-1.4)
			CC	119	120	1.3 (0.9-1.8)	1.3 (0.9-1.9)	102	1.3 (0.9-1.9)	37	1.0 (0.6-1.7)
		Recessive	AA	149	117	Referent	Referent	96	Referent	44	Referent
IRS1 Gly972Arg (C>T)	IGFBP3-202 A>C		C carriers	408	332	1.0 (0.8-1.4)	1.1 (0.8-1.4)	278	1.1 (0.8-1.4)	111	0.9 (0.6-1.4)
			A carriers	438	329	Referent	Referent	172	Referent	118	Referent
			CC	119	120	1.3 (1.0-1.8)	1.3 (1.0-1.8)	102	1.3 (1.0-1.8)	37	1.1 (0.7-1.7)
		Dominant	CC	461	374	Referent	Referent	314	Referent	130	Referent
			CT	84	68	1.1 (0.7-1.4)	0.9 (0.6-1.4)	54	0.9 (0.6-1.4)	21	0.8 (0.5-1.4)
		Recessive	TT	12	7	0.8 (0.3-2.0)	0.8 (0.3-2.2)	6	0.8 (0.3-2.2)	4	1.4 (0.4-4.7)
	IGFBP3-202 A>C		CC	461	374	Referent	Referent	314	Referent	130	Referent
			T carriers	96	75	1.0 (0.7-1.3)	0.9 (0.6-1.3)	60	0.9 (0.6-1.3)	25	0.9 (0.5-1.5)
			C carriers	545	442	Referent	Referent	368	Referent	151	Referent
		Dominant	TT	12	7	0.8 (0.3-2.0)	0.8 (0.3-2.2)	6	0.8 (0.3-2.2)	4	1.5 (0.4-4.8)
		Recessive									

Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer according to adipokine pathways polymorphisms (cont'd)

Age-adjusted Odds Ratios											
Pathway	SNP	Model	Genotypes	Non-PCa		All PCa		Restricted to HGPCa ^a		Restricted to HRPcCaM ^b	
				N		N	OR (95% CI)	N	OR (95% CI)	N	OR (95% CI)
Fibroblast growth factor 2	FGF2+223 C>T		CC	463		391	Referent	328	Referent	138	Referent
			CT	80		52	0.8 (0.5-1.2)	41	0.8 (0.5-1.1)	15	0.7 (0.5-1.3)
			TT	13		4	0.4 (0.1-1.2)	3	0.3 (0.1-1.2)	1	0.3 (0.0-2.3)
		Dominant	CC	463		391	Referent	328	Referent	138	Referent
			T carriers	93		56	0.7 (0.5-1.1)	44	0.7 (0.5-1.0)	16	0.6 (0.4-1.2)
		Recessive	C carriers	543		443	Referent	369	Referent	153	Referent
			TT	13		4	0.4 (0.1-1.2)	3	0.4 (0.1-1.3)	1	0.3 (0.0-2.4)
				CC	227		185	Referent	153	Referent	60
			CT	251		207	1.0 (0.8-1.3)	170	1.0 (0.8-1.4)	69	1.0 (0.7-1.5)
			TT	79		57	0.9 (0.6-1.3)	51	1.0 (0.6-1.5)	26	1.3 (0.8-2.3)
Tumoral necrosis factor alpha	TNFA-308 G>A	Dominant	CC	227		185	Referent	153	Referent	60	Referent
			T carriers	330		264	1.0 (0.8-1.3)	221	1.0 (0.8-1.3)	95	1.1 (0.7-1.6)
			C carriers	478		392	Referent	323	Referent	129	Referent
		Recessive	TT	79		57	0.9 (0.6-1.3)	51	1.0 (0.7-1.4)	26	1.3 (0.8-2.1)
				GG	407		326	Referent	277	Referent	110
			GA	143		115	1.0 (0.7-1.3)	89	1.0 (0.7-1.2)	41	1.0 (0.6-1.5)
			AA	7		8	1.3 (0.5-3.8)	8	1.6 (0.6-4.4)	4	1.9 (0.5-6.6)
		Dominant	GG	407		326	Referent	277	Referent	110	Referent
			A carriers	150		123	1.0 (0.8-1.3)	97	1.0 (0.7-1.3)	45	1.0 (0.7-1.6)
		Recessive	G carriers	550		441	Referent	366	Referent	151	Referent
AA	7			8	1.3 (0.5-3.7)	8	1.6 (0.6-4.5)	4	1.9 (0.5-6.6)		
	TNFA-863 C>A	Dominant	CC	353		289	Referent	238	Referent	93	Referent
			CA	171		141	1.0 (0.8-1.3)	119	1.0 (0.8-1.4)	55	1.2 (0.8-1.8)
			AA	32		18	0.7 (0.4-1.2)	16	0.7 (0.4-1.4)	6	0.7 (0.3-1.7)
		Dominant	CC	353		289	Referent	238	Referent	93	Referent
			A carriers	203		159	1.0 (0.7-1.2)	135	1.0 (0.7-1.3)	61	1.1 (0.8-1.6)
		Recessive	C carriers	524		430	Referent	357	Referent	148	Referent
			AA	32		18	0.7 (0.4-1.2)	16	0.7 (0.4-1.4)	6	0.6 (0.3-1.6)

Table S2. Age-adjusted Odds Ratios and 95%CI of prostate cancer according to adipokine pathways polymorphisms (cont'd)

Pathway	SNP	Model	Genotypes	Non-PCa		All PCa		Age-adjusted Odds Ratios			
				N	OR (95% CI)	N	OR (95% CI)	Restricted to HGPCa ^a		Restricted to HRPCaM ^b	
								N	OR (95% CI)	N	OR (95% CI)
Tumoral necrosis factor alpha	TNFRSF1A-329		GG	203	Referent	155	Referent	130	Referent	58	Referent
	G>T		GT	275	1.1 (0.8-1.4)	228	1.1 (0.8-1.4)	191	1.1 (0.8-1.4)	73	0.9 (0.6-1.3)
			TT	78	1.1 (0.8-1.7)	66	1.1 (0.8-1.7)	53	1.1 (0.7-1.7)	24	1.1 (0.6-2.0)
		Dominant	GG	203	Referent	155	Referent	130	Referent	58	Referent
			T carriers	353	1.1 (0.8-1.4)	294	1.1 (0.8-1.4)	244	1.1 (0.8-1.4)	97	0.9 (0.6-1.3)
		Recessive	G carriers	478	Referent	383	Referent	321	Referent	131	Referent
			TT	78	1.1 (0.8-1.6)	66	1.1 (0.8-1.6)	53	1.1 (0.7-1.6)	24	1.2 (0.7-2.0)

N, number of evaluable patients; SNP, single nucleotide polymorphism; OR (95%CI), age-adjusted odds-ratio and respective 95% confidence interval

^a HGPCa, High-grade Prostate Cancer (Gleason grade ≥ 7)

^b HRPCaM, High-risk Prostate Cancer for metastasis (Gleason grade ≥ 8 and/or PSA ≥ 20 ng.mL⁻¹)

Table S3. Age-adjusted Odds Ratios and 95%CI for prostate cancer (PCa) associated with selected SNPs, after age stratification

SNPs	Age Group	Non-PCa		All PCa		Restricted to High-grade PCa ^a		Restricted to High-risk PCa for metastasis ^b	
		N *		N	aOR (95%CI)	N *	aOR (95%CI)	N *	aOR (95%CI)
<i>LEPR</i> Gln223Arg AA	< Median	276		163	1.7(1.1-2.6)	126	1.8(1.1-2.8)	42	2.3(1.2-4.4)
	≥ Median	277		284	1.5(1.1-2.2)	247	1.6(1.1-2.3)	112	1.7(1.0-2.7)
<i>IL6R</i> Asp358Ala C carriers	< Median	276		163	1.8(1.2-2.7)	126	1.9(1.2-2.9)	42	3.4(1.5-7.5)
	≥ Median	277		284	0.9(0.6-1.3)	247	1.0(0.7-1.4)	112	0.9(0.6-1.5)
<i>IL6</i> -597 G carriers	< Median	276		163	1.2(0.7-2.2)	126	1.3(0.7-2.6)	42	2.0(0.6-6.6)
	≥ Median	277		284	1.5(0.9-2.5)	247	1.6(0.9-2.9)	112	1.0(0.5-1.9)
<i>OPN</i> -66 GG	< Median	276		163	2.1(1.0-4.4)	126	2.3 (1.1-4.9)	42	3.5(1.3-9.1)
	≥ Median	277		284	1.5(0.7-3.2)	247	1.5(0.7-3.2)	112	1.8(0.7-4.5)
<i>IGFBP3</i> -202 CC	< Median	276		163	1.6(1.0-2.6)	126	1.6(1.0-2.6)	42	1.1(0.5-2.4)
	≥ Median	277		284	1.1(0.8-1.6)	247	1.2(0.8-1.7)	112	1.1(0.6-1.8)
<i>IGF1R</i> +3174 AA	< Median	276		163	1.6(1.0-2.6)	126	1.7(1.0-2.7)	42	1.7(0.8-3.6)
	≥ Median	277		284	1.1(0.7-1.8)	247	1.1(0.7-1.9)	112	1.0(0.5-2.0)
<i>FGF2</i> +223 CC	< Median	276		163	1.4(0.8-2.3)	126	1.3(0.8-2.4)	42	1.4(0.5-3.4)
	≥ Median	277		283	1.3(0.8-2.1)	246	1.4(0.8-2.4)	111	1.6(0.8-3.4)

^a High-grade Prostate Cancer, Gleason grade ≥7; ^b High-risk Prostate Cancer for metastasis, Gleason grade ≥ 8 and/or PSA ≥ 20 ng/mL; aOR (95%CI), age-adjusted odds ratio and respective 95% Confidence Interval; PCa, Prostate Cancer; Median age at diagnosis=67.5 years; * Evaluable individuals for analysis

Table S4. Sensitivity analysis in SNPs with deviation from Hardy-Weinberg equilibrium. Risk for prostate cancer after restriction on the non-prostate cancer group to just benign prostate hyperplasia and normal or to PSA below 4 ng/mL

Risk genotypes	Non-Prostate Cancer Groups							
	All Non-Prostate Cancer ^a				Restricted to BPH and Normal ^b			
	N	aOR (95%CI) **	P *		N	aOR (95%CI) **	P *	Restricted to PSA < 4 ng.mL ^{-1c}
<i>LEPR</i> Gln223Arg AA	557	1.6 (1.2-2.1)	0.008	113	1.6 (1.0-2.5)	0.664	117	1.5 (0.9-2.3) 0.755
<i>IGF1R</i> +3174 AA	557	1.3 (1.0-1.9)	0.006	113	1.4 (0.8-2.6)	0.770	117	1.4 (0.8-2.5) 0.306
<i>FGF2</i> +223 CC	556	1.4 (1.0-2.0)	0.0005	113	1.2 (0.7-2.2)	0.936	117	1.6 (0.9-2.8) 0.036

* Hardy-Weinberg equilibrium, Pearson chi-square analysis for differences between observed and expected genotype frequencies; ** Age-adjusted odds ratios; BPH, Benign Prostate Hyperplasia; PSA, Prostate-specific Antigen; PSA, prostate-specific antigen; SNP, single nucleotide polymorphism; aOR (95%CI), age-adjusted odds ratio and respective 95% confidence interval. ^a Biopsy findings: normal, 14.9%; BPH, 5.4%, chronic prostatitis, 74.7%; atrophy, 5%; ^b Biopsy findings: normal, 73.5%; BPH, 26.5%; ^c Biopsy findings: normal, 22.2%; BPH, 6.0%, chronic prostatitis, 65.8%; atrophy, 6.0%

PAPER VII

Body adiposity and prostate cancer: relevance of adipokine pathways genes-environment interaction in risk prediction

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ABSTRACT

Obesity, determined by body mass index (BMI), has been associated with prostate cancer (PCa) aggressiveness and survival. In this study, we sought to investigate BMI diagnostic accuracy to detect excess body adiposity in older men at the time of prostatic biopsy. Additionally, we determined if body fat percentage (BFP) was associated with PCa and synergizes with a previously established adipokine pathways-based multilocus genetic risk score.

We examined BMI and BFP (using bioelectrical impedance) in a consecutive series of 380 patients scheduled for prostate biopsy at a single institution. Total and free serum PSA, testosterone, prolactin and insulin-like growth factor 1 were measured in patient's blood samples. Genomic DNA was used to genotype *LEPR* Gln223Arg (G>A), *OPN-66* T>G, *IGF1R*+3174 G>A, *IGFBP3*-202 A>C, *FGF2*+223 C>T and *IL6*-597 G>A polymorphisms through Real-Time PCR and PCR-RFLP to determine the risk score.

In this population of elderly men submitted to prostate biopsy, BMI has limited diagnostic performance to appropriately identify subjects with excess body fatness, particularly in intermediate ranges of body weight. Individuals classified by BFP as overweight or obese are at increased risk for developing PCa overall (OR=2.5, 95%CI: 1.1-5.5 and OR=8.2, 95%CI: 2.0-33.0, respectively) and high-grade PCa (OR=2.6, 95%CI: 1.0-6.7 and OR=8.2, 95%CI: 1.7-40.9, respectively), hitherto they were within the group of subjects misclassified as normal through BMI. The adipokine-based genetic risk score performed well independently, still following bootstrap with 10000

permutations (all PCa: OR=2.1, 95%CI: 1.4-3.3 and high-grade PCa: OR=2.4, 95%CI: 1.4-4.0, respectively). The risk score odds ratios for PCa and high-grade disease were higher in the group of BFP-determined obese men, also after bootstrapping (OR=3.2, 95%CI: 1.8-6.0 and OR=3.5, 95%CI: 1.7-7.5, respectively).

Our study suggests that using BMI as the measure of obesity in association studies with PCa might be missing subjects misclassified as normal by BMI. Adding adiposity measures to the genetic risk score identifies a group of obese patients at higher risk for PCa.

INTRODUCTION

Obesity is currently considered epidemic, mostly in Western countries, with a trend for increasing prevalence in the near future [1, 2]. It has long been hypothesized to influence the risk of prostate cancer (PCa), albeit studies assessing the association between obesity and prostate cancer yielded apparently conflicting results [3-6]. Most studies of PCa and obesity relied on weight, height and body mass index (BMI) measurements to define obesity [4, 5]. However, BMI and other classical anthropometric measures are imperfect estimates of adiposity, particularly in men because of greater lean mass. Body fat quantity, as opposed to BMI that fails to appropriately discriminate fat mass [7-10], has been seldom used to evaluate the association between obesity and PCa [11, 12]. Nevertheless, the predictive value of body composition for detecting PCa and aggressiveness, as evaluated at the time of prostatic biopsy has not been the subject of study.

Body fat represents an influential and responsive endocrine and paracrine tissue, adipose tissue, which has been proposed to produce mediators and cells that may affect prostate cancer progression and partially explain the causally invoked association between obesity and PCa [13-15]. Therefore, the use of more precise methods to evaluate body fat content may be more appropriate to analyze the impact of obesity in prostate cancer.

The effort to identify groups at risk for having PCa and a more aggressive disease is still ongoing. Apart from the established factors, age and ethnic background, other variables are now under consideration that includes obesity and germline genetic markers. Recently, selected SNPs from obesity-associated adipokine pathways were

analyzed and converted into a genetic risk score, which was shown to predict PCa risk and aggressiveness better than PSA and age (Ribeiro, unpublished results). Therefore, we hypothesized that the application of the adipokine pathways genetic risk score according to body composition phenotype may add information to determine high-risk groups for developing PCa and aggressive disease. This is the first study to assess the diagnostic performance of BMI in relation with obesity and its correlation with body fat measurements in a population of men eligible for prostate biopsy.

PATIENTS AND METHODS

Patients

Men referred to the urology department of São João Hospital, on the basis of abnormal digital rectal examination and/or single baseline PSA levels over 2.5 ng/mL, starting October 2009 until October 2010, were scheduled for guided transrectal ultrasound prostatic biopsy (8-13 cores). Patients with previous history of PCa or prostate surgery were not included. Three-hundred ninety six consecutively-admitted Caucasian men were enrolled after signing an informed consent. Approval was obtained from research ethics committees.

Pathological information on biopsies included the diagnosis of normal prostate, benign prostatic hyperplasia (BPH), chronic prostatitis, prostate inflammatory atrophy, high grade intraepithelial neoplasia and PCa with Gleason score. In re-biopsied individuals only the last, most relevant pathological diagnosis was considered. Only the benign conditions and PCa were deemed for analysis. Therefore, 16 patients were excluded from the study with diagnosis of high-grade prostatic intraepithelial neoplasia

(n=12) or a biopsy suspicious of cancer only (n=4). High grade disease was defined as a biopsy Gleason score ≥ 7 .

Anthropometric measurements and genetic risk score (GRS)

Patients were instructed to come for examination and biopsy in the morning after an overnight fast and to refrain from strenuous exercise on the day preceding biopsy. Before biopsy, patients underwent height measurement on a wall-mounted stadiometer, and weight (to 0.1 Kg) and body fat percent (BFP) using a bipolar bioimpedance analyzer (TBF-300, Body Composition Analyzer, Tanita). Percent body fat was calculated with the manufacturer's software.

Selection of single nucleotide polymorphisms (SNPs), genotyping and calculations to obtain the genetic risk score were described in detail in a previous work (Ribeiro, unpublished results). This risk score includes 6 SNPs (*LEPR* Gln223Arg (G>A), *OPN-66* T>G, *IGF1R*+3174 G>A, *IGFBP3*-202 A>C, *FGF2*+223 C>T, *IL6*-597 G>A), total PSA and age at diagnosis. For each participant, the coefficients were summed for each of these variables.

Statistical analysis

We used means and medians as descriptive statistics for continuous variables and the Shapiro-Francia test to assess their departure from normality. As appropriate, the Mann-Whitney test and Student t-test were used to compare means (age, anthropometric and hormonal variables) between prostatic disease groups. The Kruskal Wallis followed by Mann-Whitney two samples tests were used for analyses of non parametric variables.

The chi-square was used to test for departures from Hardy-Weinberg equilibrium for each SNP based on the distribution among the non-prostate cancer group.

Unconditional logistic regression was used to estimate age-adjusted odds ratios (aORs) and 95% confidence intervals (95% CIs) for the associations between anthropometric variables and circulating hormones levels with risk for developing prostate cancer, high-grade prostate cancer (combined Gleason score ≥ 7), and high -risk prostate cancer for metastasis (PSA at diagnosis ≥ 20 ng/mL and/or combined Gleason score ≥ 8). To estimate the strength of the linear association between continuous variables, we calculated the respective regression equations. Non-parametric Spearman's correlations were computed to assess the statistical dependence between variables.

As previously described, we calculated the genetic risk score for each participant by summing the coefficients of relevant variables (genetic polymorphisms in the genes *LEPR* Gln223Arg (G>A), *OPN-66* T>G, *IGF1R*+3174 G>A, *IGFBP3*-202 A>C, *FGF2*+223 C>T, and *IL6*-597 G>A, plus total PSA and age at diagnosis). The genetic risk score was validated with adjustment to adiposity measures (BMI and BFP), age at diagnosis and testosterone levels. Bootstrapping using Monte Carlo 10000 permutations test was then performed as an internal validation measure. Stratified analyses by BFP-defined obesity (cutoff 25%) were equally made. The software STATA version 10.0 (StataCorp, College Station, Texas) was used for statistical analyses.

RESULTS

This study included 380 men submitted to prostatic biopsy, comprising 154 diagnosed with PCa and 226 without histological evidence of cancer. Mean age at

diagnosis, BMI, body fat percent and total PSA were significantly higher in PCa compared with non-PCa ($P<0.0001$, $P=0.011$, $P=0.008$ and $P<0.0001$, respectively), whereas mean height and free/total PSA ratio were lower in cancer patients ($P=0.045$ and $P<0.0001$, respectively) (Table 1).

Diagnostic performance of BMI to identify excess body fatness

In our study 24 non-cancer (11%) and 33 PCa (21%) men were classified as obese according to BMI (>30). Using the BFP classification of obesity ($>25\%$) there was 97 non-cancer (43%) and 82 prostate cancer (53%) obese subjects.

Table 2 shows the classification of subjects as normal, overweight or obese according to BMI or BFP. Eighth percent of the men with BMI defined as normal were actually obese given their BFP, while only 2% considered obese by BMI were in reality normal according to BFP. Additionally, 65% of the BMI overweight-classified subjects were after all obese considering their BFP. This misclassification was higher for PCa patients (16% obese by BFP) than for non-PCa (4% obese by BFP) in BMI-determined normal subjects.

We observed high specificities and positive predictive values (PPV) for BMI at cut-off $\geq 30 \text{ Kg/m}^2$ (98 and 93%, respectively), despite the observed low sensitivities and negative predictive values (NPV) (30 and 61%, respectively) to determine BFP-defined obesity. For BMI at cut-off $\geq 25 \text{ Kg/m}^2$ there was good sensitivities and NPV (93 and 92%, respectively), although specificity and PPV (67 and 72%, respectively) were low. Stratification by disease status showed lower values for PCa compared with non-PCa.

Analyses of ROC curves revealed overall an area under the curve (AUC) of 0.895 for BMI to detect obesity evaluated by BFP $\geq 25\%$. After stratification, the AUC was lower for PCa (0.851) than for non-PCa (0.923) groups. Figure 1 shows plots and estimated linear fit regression to assess the association between BMI and body fat percentage in non-PCa and PCa groups. In non-PCa group BFP explained 64% of the variability in BMI ($R^2=0.642$, $P<0.0001$), whereas in PCa group it only explained 45% ($R^2=0.446$, $P<0.0001$). We observed a strong association between these anthropometric variables in both PCa and non-PCa groups, with an overall Spearman's rank correlation coefficient of 0.787 ($P<0.0001$).

Body composition, hormones and PCa risk

We found a higher percent of subjects with PCa, compared with non-PCa, in the obese category according to BFP (53% versus 43%), when the population was globally analyzed (Table 2). In Table 3 the ORs and respective 95% CIs are presented according to normoponderal, overweight and obesity WHO categories of BFP and BMI. Age-adjusted unconditional regression analyses showed a significantly higher risk for overall and high-grade PCa at time of biopsy in BFP-defined overweight and obese men, and increased risk for overall PCa, high-grade and metastatic PCa for obese men defined by BMI ≥ 30 Kg/m²) (Table 3). In normal men (according to BMI) the mean BFP was significantly higher for PCa compared with non-PCa group ($P=0.001$) (Figure 2). Concordantly, within the BMI-defined normal group, we found that men with a BFP corresponding to overweight and obesity are at increased risk for developing PCa (OR, 2.5, 95%CI, 1.1-5.5 and OR, 8.2, 95%CI, 2.0-33.0, respectively) and high-grade PCa (OR, 2.6, 95%CI, 1.0-

6.7 and OR, 8.2, 95%CI, 1.7-40.9, respectively), compared with BFP-defined normal (data not shown).

In this study, men in the top tertile of serum testosterone levels had decreased risk for having PCa with possibility of developing metastasis compared to the bottom tertile, while serum IGF-1 did not impact the risk of PCa (Table 4). Furthermore, we observed there is a significant proportion of variation in the observed serum testosterone values that are explained by BMI and body fat percent (Figure 3A and 3B). An inverse significant association was found between both body fat percent and BMI with testosterone levels ($r=-0.210$, $P<0.0001$ and $r=-0.204$, $P<0.0001$, respectively).

Adipokine pathways-based genetic profile, obesity and PCa risk

We used the genetic variables derived from our large Molecular Epidemiology study to obtain the risk score, which was used as a continuous variable. In this population, all except one SNP (*FGF2* +223 C>T, $P=0.009$) were in Hardy-Weinberg equilibrium among the control population. In the present study, BMI-, BFP-, testosterone-, prolactin- and age-adjusted odds ratios in increased genetic risk score were significantly associated with all and high-grade PCa risk (Table 5). Stratification according to BFP-defined obesity cut-off (25%), showed that the GRS risk for overall and high-grade PCa was increased in the obese group (Table 5), but not in BMI-determined obese (data not shown). Moreover, in the misclassified group as non-obese by BMI ($< 30 \text{ Kg/m}^2$) but BFP-defined as obese (BFP $> 25\%$), the GRS was associated with risk for PCa and high-grade PCa on multivariate analysis (OR, 3.8, 95%CI, 1.8-8.1 and OR, 4.0, 95%CI, 1.7-9.5, respectively).

DISCUSSION

Obesity is a risk factor for PCa and represents excess body fatness, which is correlated with comorbidities [16]. In our study, BMI presented low sensitivity for detecting body fatness, with more than half (70%) of obese subjects (by BFP measurement) being labeled as normal or overweight by BMI. These findings in men eligible for prostatic biopsy, suggest limited diagnostic performance of BMI to adequately identify elderly men with excess body fatness, particularly in subjects with prostate cancer. Although BMI and BFP are well correlated, BMI fails to discriminate BFP appropriately. Studies in distinct populations reported similar findings [8-10, 17]. The limited diagnostic performance of BMI in intermediate ranges of body weight can be explained by the inability of BMI to discriminate between BFP and lean mass [8], since the majority of human body weight comes from lean mass, particularly in men. In addition, our study indicates that obesity misclassification in men eligible to prostate biopsy, results in underdiagnosis of obesity and eventually of patients at risk for PCa. Furthermore, normal BMI-classified subjects ($\text{BMI} < 25$) presented increased risk for PCa and high-grade disease (for both overweight and obese) after stratification by BFP. Noteworthy, a previous report found a stronger association of central adiposity measures with advanced PCa in the group of individuals with normal BMI [18]. Conversely, we found good specificity and positive predictive value for diagnosing BFP-defined obesity with $\text{BMI} \geq 30 \text{ kg/m}^2$, which is associated with increased risk for overall, high-grade PCa and high-risk for metastasis. Only 7% of the subjects with BFP-defined normal and overweight were misclassified as BMI-defined obese. These findings may explain why

the risk for PCa and aggressive disease is higher when BMI is $\geq 30 \text{ Kg/m}^2$, suggesting that the suboptimal diagnostic performance of BMI to detect excess fat might be limited to intermediate ranges of BMI. In fact, among others, recent studies identified obesity (with BMI $\geq 30 \text{ Kg/m}^2$) as a risk factor for upstaged disease in low-risk PCa patients eligible for active surveillance and with potential for being included in prostate cancer nomogram scores [19-21]. PPV and NPV are directly related to the prevalence of disease (obesity) in the population, partially explaining the more consistent findings pertaining obesity-PCa association among USA series that have higher prevalence of obese subjects, in contrast with studies from other countries [22-26].

The free/total PSA ratio has been shown to increase the detection rate of PCa, differentiate cancer from benign disease and to predict pathologic features [27-29]. Our data shows lower free/total PSA values in subjects misclassified as normal by BMI although BFP-defined as obese, and in obese (BMI $\geq 30 \text{ kg/m}^2$ and BFP $\geq 25\%$ are concordant), compared with normal BMI- and BFP-classified men (P=0.003 and P=0.013, respectively) (data not shown). These findings lend support to the hypothesis that misclassified subjects as non-obese by BMI but obese through BFP may be at higher risk for PCa.

Present findings show the existence of risk for PCa already in subjects with a BMI $< 30 \text{ Kg/m}^2$ but with highly increased adiposity. Even though BMI is widely used as a proxy measure of body fat, it does not inform on body composition as evidenced herein. Considering the current understanding of adipose tissue effects on cancer [13-15, 30, 31], it would be expected that the association obesity-PCa would be more consistent. In our results, subjects with normal and mildly elevated BMI had a great variety of BFP, which

are likely to explain the inconsistent relationship between BMI and PCa. Noteworthy, it was shown that BMI has its worst diagnostic performance to detect obesity in the elderly [8], precisely the age group when most men with PCa are diagnosed.

Two previous studies reported about the effect of increased adiposity in PCa risk [11, 12]. In one study, authors found a modest association of fat mass with PCa risk [11], while in the other, body fat distribution was a stronger predictor than fatness [12]. In these cohorts researchers examined data from over 10000 men, with wide ranges of ages at recruitment (27-75 and 45-73 years), and approximately at the same time period (1990 to 1996). In these studies the anthropometric measures were not taken at the time of cancer diagnosis.

Adipose tissue deregulation has been proposed as a relevant mechanism underlying obesity-related cancer, due to inappropriate secretion of biologically active adipokines, modification of the hormonal environment and by releasing precursor cells to the tumor stroma (Ribeiro, unpublished results) [13, 31, 32]. One way excess adipose tissue may be linked to PCa risk and poorer disease pathology, is through its effect in hormone levels. Insulin and IGF-1 levels are elevated in obesity, and were associated with PCa due to its mitogenic properties [33, 34]. In the present study, IGF-1 levels were not associated with PCa. Furthermore, although other studies reported an association between height and PCa [35, 36], we found no such relationship. Height is associated with IGF-1 [37], and our population is mainly composed of short men (median height, 1.68 m; range, 1.40-1.85 m), which might explain the lack of association between height and IGF-1 with PCa. Moreover, the increased rate of peripheral conversion of testosterone to estradiol by aromatase in fat cells, results in decreased bioavailability of

testosterone [38], which has been associated with advanced and poorly differentiated PCa [39-42]. Indeed, in our study men in the top tertile of serum testosterone levels were at lower risk for having a cancer of the prostate at high-risk for developing metastasis. In agreement with previous findings [43, 44], the testosterone levels in our population were also inversely correlated with BMI and BFP. Modifications on IGF or adipokine axis, and in sex steroids, that come along with obesity, may influence PCa development and progression.

Our findings challenge the use of BMI to detect excess adiposity, particularly for men in the intermediate range of BMI, where it fall short to distinguish between excess in body fat or preserved lean mass. Therefore, in Portugal and other European countries, with a high percentage of overweight men (BMI 25-30 Kg/m²) [45, 46], it is desirable to include BFP measurements in the standard assessment of patients in the urological outpatient clinic both for diagnosis and therapeutic management, in order to implement obesity-related measures. In fact, several reports extend the need to make body composition assessment still after diagnosis, during follow-up of PCa-treated patients [47-50].

The gene-environment interaction that likely explains the obesity-PCa association should involve several genes and pathways from different cells (adipose tissue cells, tumor cells, immune cells) in specific environmental contexts. In a previous study, we developed an adipokine pathways-based genetic risk score and showed it was a better predictor of PCa than a parsimonious PSA plus age risk model. However, in that study, anthropometric or body composition data was not available, remaining untested the GRS-associated risk after adjustment for BMI and BFP. In the present study, using the same

protocol to calculate the GRS, we found that GRS was significantly associated with increased risk for developing overall and high-grade PCa, after adjustment for adiposity measures, age and testosterone levels. Furthermore, stratification according to BFP-defined obesity (cutoff 25%) we observed that GRS risk was increased only in obese subjects (for overall and high-grade PCa). Thus, the use of GRS could be more useful to predict PCa in the obese group of men ($BFP \geq 25\%$). Mechanistically, these genotypes of functional SNPs might impact the endocrine and paracrine activity of adipokine pathways that leads to tumor development and progression. This effect is stronger in men with excess fatness, revealing eventual synergies between adipokine SNPs and obesity status in association with overall and high-grade PCa.

The World Health Organization defines obesity as an excess of body fat, albeit it does not clearly state body fat cut-offs. To overcome this limitation we used the body fat cut-offs most frequently applied for whites [8, 51, 52]. Bioelectrical impedance was used to assess body fatness, although other methods are more precise (hydrostatic weighting, energy-dual X-ray absorptiometry or air displacement plethysmography). Nevertheless, bioelectrical impedance accuracy is acceptable, and its ease use, lack of radiation and relatively low cost suggest it is a feasible alternative for measuring body fatness, mostly on large populations in the clinical setting [53]. Measures of body fat distribution were not performed. Future studies should combine global BFP together with body fat distribution, in order to define a combined index for evaluating obesity-associated PCa risk.

In this study some major known PCa risk factors were considered, including age and ethnic background. Another strength of this study was the controlled assessment of

anthropometry and body composition prior to biopsy. We observed, as expected, that older patients with elevated total serum PSA and lower free/total PSA ratio presented increased risk of PCa. Furthermore, our PCa detection rate (41%) agrees with other recently published series, at the time of biopsy [54-56]. In addition, the non-PCa group includes exclusively men with histopathological confirmation of diagnosis.

In conclusion, despite the good correlation between BMI and BFP in this population of men eligible for prostatic biopsy, the diagnostic accuracy of BMI to diagnose obesity is limited, particularly for BMI-determined overweight individuals. Individuals classified by BFP as overweight or obese are at increased risk for developing PCa and high-grade disease, even within the group of subjects misclassified as normal through BMI. Direct and simple measures of body fatness together with measures of body fat distribution may be helpful in stratifying men at risk for PCa and aggressive disease; and by adding other predictive markers, such as DNA genetic variants, will certainly enhance the accuracy to predict PCa. Studies are warranted, in order to confirm and further explore the role of body composition and fat distribution in PCa etiology and progression, as well as to understand the biological mechanisms behind this association. In light of the rising incidence of obesity worldwide, identifying obesity as a risk factor for prostate malignancy is important, since it may be one of the few modifiable risk factors for PCa, therefore, with impact in the implementation of new adjuvant therapies, reinforcing public health guidelines and the definition of chemoprevention strategies.

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REFERENCES

- [1] Finucane MM, Stevens GA, Cowan MJ, et al. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. *Lancet*. 2011 Feb 12: **377**:557-67
- [2] Fesinmeyer MD, Gulati R, Zeliadt S, Weiss N, Kristal AR, Etzioni R. Effect of population trends in body mass index on prostate cancer incidence and mortality in the United States. *Cancer Epidemiol Biomarkers Prev*. 2009 Mar: **18**:808-15
- [3] Freedland SJ, Platz EA. Obesity and prostate cancer: making sense out of apparently conflicting data. *Epidemiol Rev*. 2007: **29**:88-97
- [4] Hsing AW, Sakoda LC, Chua S, Jr. Obesity, metabolic syndrome, and prostate cancer. *Am J Clin Nutr*. 2007 Sep: **86**:s843-57
- [5] MacInnis RJ, English DR. Body size and composition and prostate cancer risk: systematic review and meta-regression analysis. *Cancer Causes Control*. 2006 Oct: **17**:989-1003
- [6] Cao Y, Ma J. Body mass index, prostate cancer-specific mortality, and biochemical recurrence: a systematic review and meta-analysis. *Cancer Prev Res (Phila)*. 2011 Apr: **4**:486-501
- [7] Romero-Corral A, Lopez-Jimenez F, Sierra-Johnson J, Somers VK. Differentiating between body fat and lean mass-how should we measure obesity? *Nat Clin Pract Endocrinol Metab*. 2008 Jun: **4**:322-3
- [8] Romero-Corral A, Somers VK, Sierra-Johnson J, et al. Accuracy of body mass index in diagnosing obesity in the adult general population. *Int J Obes (Lond)*. 2008 Jun: **32**:959-66
- [9] Gomez-Ambrosi J, Silva C, Galofre JC, et al. Body adiposity and type 2 diabetes: increased risk with a high body fat percentage even having a normal BMI. *Obesity (Silver Spring)*. 2011 Jul: **19**:1439-44
- [10] Gomez-Ambrosi J, Silva C, Galofre JC, et al. Body mass index classification misses subjects with increased cardiometabolic risk factors related to elevated adiposity. *Int J Obes (Lond)*. 2011 May 17:
- [11] MacInnis RJ, English DR, Gertig DM, Hopper JL, Giles GG. Body size and composition and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev*. 2003 Dec: **12**:1417-21
- [12] Wallstrom P, Bjartell A, Gullberg B, Olsson H, Wirfalt E. A prospective Swedish study on body size, body composition, diabetes, and prostate cancer risk. *Br J Cancer*. 2009 Jun 2: **100**:1799-805
- [13] Mistry T, Digby JE, Desai KM, Randeva HS. Obesity and prostate cancer: a role for adipokines. *Eur Urol*. 2007 Jul: **52**:46-53
- [14] Ribeiro RJ, Monteiro CP, Cunha VF, et al. Tumor Cell-educated Periprostatic Adipose Tissue Acquires an Aggressive Cancer-promoting Secretory Profile. *Cell Physiol Biochem*. 2012: **29**:233-40
- [15] Ribeiro R, Monteiro C, Cunha V, et al. Human periprostatic adipose tissue promotes prostate cancer aggressiveness in vitro. *J Exp Clin Cancer Res*. 2012 Apr 2: **31**:32
- [16] Prentice AM, Jebb SA. Beyond body mass index. *Obes Rev*. 2001 Aug: **2**:141-7
- [17] Okorodudu DO, Jumeau MF, Montori VM, et al. Diagnostic performance of body mass index to identify obesity as defined by body adiposity: a systematic review and meta-analysis. *Int J Obes (Lond)*. 2010 May: **34**:791-9
- [18] Pischon T, Boeing H, Weikert S, et al. Body size and risk of prostate cancer in the European prospective investigation into cancer and nutrition. *Cancer Epidemiol Biomarkers Prev*. 2008 Nov: **17**:3252-61

- [19] Ploussard G, de la Taille A, Bayoud Y, et al. The Risk of Upstaged Disease Increases with Body Mass Index in Low-Risk Prostate Cancer Patients Eligible for Active Surveillance. *Eur Urol*. 2011 Jul 21:
- [20] Major JM, Klonoff-Cohen HS, Pierce JP, et al. Prostate cancer postoperative nomogram scores and obesity. *PLoS One*. 2011; **6**:e17382
- [21] Williams SB, Salami S, Regan MM, et al. Selective detection of histologically aggressive prostate cancer: An Early Detection Research Network Prediction model to reduce unnecessary prostate biopsies with validation in the Prostate Cancer Prevention Trial. *Cancer*. 2011 Oct 17:
- [22] Freedland SJ, Wen J, Wuerstle M, et al. Obesity is a significant risk factor for prostate cancer at the time of biopsy. *Urology*. 2008 Nov; **72**:1102-5
- [23] Freedland SJ, Banez LL, Sun LL, Fitzsimons NJ, Moul JW. Obese men have higher-grade and larger tumors: an analysis of the duke prostate center database. *Prostate Cancer Prostatic Dis*. 2009; **12**:259-63
- [24] Jayachandran J, Banez LL, Aronson WJ, et al. Obesity as a predictor of adverse outcome across black and white race: results from the Shared Equal Access Regional Cancer Hospital (SEARCH) Database. *Cancer*. 2009 Nov 15; **115**:5263-71
- [25] Gallina A, Karakiewicz PI, Hutterer GC, et al. Obesity does not predispose to more aggressive prostate cancer either at biopsy or radical prostatectomy in European men. *Int J Cancer*. 2007 Aug 15; **121**:791-5
- [26] Isbarn H, Jeldres C, Budaus L, et al. Effect of body mass index on histopathologic parameters: results of large European contemporary consecutive open radical prostatectomy series. *Urology*. 2009 Mar; **73**:615-9
- [27] Yokomizo Y, Miyoshi Y, Nakaigawa N, et al. Free PSA/total PSA ratio increases the detection rate of prostate cancer in twelve-core biopsy. *Urol Int*. 2009; **82**:280-5
- [28] Catalona WJ, Partin AW, Slawin KM, et al. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial. *JAMA*. 1998 May 20; **279**:1542-7
- [29] Arcangeli CG, Humphrey PA, Smith DS, et al. Percentage of free serum prostate-specific antigen as a predictor of pathologic features of prostate cancer in a screening population. *Urology*. 1998 Apr; **51**:558-64; discussion 64-5
- [30] Ribeiro R, Lopes C, Medeiros R. The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives. *Prostate Cancer Prostatic Dis*. 2006; **9**:19-24
- [31] Park J, Euhus DM, Scherer PE. Paracrine and Endocrine Effects of Adipose Tissue on Cancer Development and Progression. *Endocr Rev*. 2011 Jun 2:
- [32] Bellows CF, Zhang Y, Chen J, Frazier ML, Kolonin MG. Circulation of Progenitor Cells in Obese and Lean Colorectal Cancer Patients. *Cancer Epidemiol Biomarkers Prev*. 2011 Oct 18:
- [33] Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. *Nat Rev Cancer*. 2004 Jul; **4**:505-18
- [34] Rowlands MA, Gunnell D, Harris R, Vatten LJ, Holly JM, Martin RM. Circulating insulin-like growth factor peptides and prostate cancer risk: a systematic review and meta-analysis. *Int J Cancer*. 2009 May 15; **124**:2416-29
- [35] Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Willett WC. Height, body weight, and risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev*. 1997 Aug; **6**:557-63
- [36] Andersson SO, Wolk A, Bergstrom R, et al. Body size and prostate cancer: a 20-year follow-up study among 135006 Swedish construction workers. *J Natl Cancer Inst*. 1997 Mar 5; **89**:385-9
- [37] Signorello LB, Kuper H, Lagiou P, et al. Lifestyle factors and insulin-like growth factor 1 levels among elderly men. *Eur J Cancer Prev*. 2000 Jun; **9**:173-8

- [38] Rohrmann S, Shiels MS, Lopez DS, et al. Body fatness and sex steroid hormone concentrations in US men: results from NHANES III. *Cancer Causes Control*. 2011 Aug; **22**:1141-51
- [39] Kratzik C, Womastek I, Bieglmayer C, et al. Lower serum total testosterone is associated with lymph node metastases in a radical prostatectomy cohort study. *Anticancer Res*. 2011 Oct; **31**:3615-8
- [40] Massengill JC, Sun L, Moul JW, et al. Pretreatment total testosterone level predicts pathological stage in patients with localized prostate cancer treated with radical prostatectomy. *J Urol*. 2003 May; **169**:1670-5
- [41] Severi G, Morris HA, MacInnis RJ, et al. Circulating steroid hormones and the risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev*. 2006 Jan; **15**:86-91
- [42] Schatzl G, Madersbacher S, Thurnidl T, et al. High-grade prostate cancer is associated with low serum testosterone levels. *Prostate*. 2001 Apr; **47**:52-8
- [43] Garcia-Cruz E, Piqueras M, Huguet J, et al. Hypertension, dyslipidemia and overweight are related to lower testosterone levels in a cohort of men undergoing prostate biopsy. *Int J Impot Res*. 2012 Jan 19:
- [44] Frederiksen L, Hojlund K, Hougaard DM, et al. Testosterone therapy decreases subcutaneous fat and adiponectin in aging men. *Eur J Endocrinol*. 2012 Mar; **166**:469-76
- [45] Doak CM, Wijnhoven TM, Schokker DF, Visscher TL, Seidell JC. Age standardization in mapping adult overweight and obesity trends in the WHO European Region. *Obes Rev*. 2011 Nov 7:
- [46] do Carmo I, Dos Santos O, Camolas J, et al. Overweight and obesity in Portugal: national prevalence in 2003-2005. *Obes Rev*. 2008 Jan; **9**:11-9
- [47] Smith MR, Fuchs V, Anderson EJ, Fallon MA, Manola J. Measurement of body fat by dual-energy X-ray absorptiometry and bioimpedance analysis in men with prostate cancer. *Nutrition*. 2002 Jul-Aug; **18**:574-7
- [48] Galvao DA, Spry NA, Taaffe DR, et al. Changes in muscle, fat and bone mass after 36 weeks of maximal androgen blockade for prostate cancer. *BJU Int*. 2008 Jul; **102**:44-7
- [49] Smith MR, Finkelstein JS, McGovern FJ, et al. Changes in body composition during androgen deprivation therapy for prostate cancer. *J Clin Endocrinol Metab*. 2002 Feb; **87**:599-603
- [50] Berruti A, Dogliotti L, Terrone C, et al. Changes in bone mineral density, lean body mass and fat content as measured by dual energy x-ray absorptiometry in patients with prostate cancer without apparent bone metastases given androgen deprivation therapy. *J Urol*. 2002 Jun; **167**:2361-7; discussion 7
- [51] Deurenberg P, Andreoli A, Borg P, et al. The validity of predicted body fat percentage from body mass index and from impedance in samples of five European populations. *Eur J Clin Nutr*. 2001 Nov; **55**:973-9
- [52] De Lorenzo A, Deurenberg P, Pietrantuono M, Di Daniele N, Cervelli V, Andreoli A. How fat is obese? *Acta Diabetol*. 2003 Oct; **40 Suppl 1**:S254-7
- [53] Sun G, French CR, Martin GR, et al. Comparison of multifrequency bioelectrical impedance analysis with dual-energy X-ray absorptiometry for assessment of percentage body fat in a large, healthy population. *Am J Clin Nutr*. 2005 Jan; **81**:74-8
- [54] Presti JC, Jr., O'Dowd GJ, Miller MC, Mattu R, Veltri RW. Extended peripheral zone biopsy schemes increase cancer detection rates and minimize variance in prostate specific antigen and age related cancer rates: results of a community multi-practice study. *J Urol*. 2003 Jan; **169**:125-9

- [55] De Nunzio C, Freedland SJ, Miano L, Finazzi Agro E, Banez L, Tubaro A. The uncertain relationship between obesity and prostate cancer: An Italian biopsy cohort analysis. *Eur J Surg Oncol*. 2011 Dec: **37**:1025-9
- [56] Pruthi RS, Swords K, Schultz H, Carson CC, 3rd, Wallen EM. The impact of obesity on the diagnosis of prostate cancer using a modern extended biopsy scheme. *J Urol*. 2009 Feb: **181**:574-7; discussion 8

Table 1. Age, anthropometric and hormonal variables by disease status

	Disease Status					
	Non-PCa			PCa		
	N*	Median	P**	N*	Median	P**
Age, years	226	64.9	0.332	154	70.0	0.086
Height, cm	226	168.0	0.011	154	166.0	0.408
BMI, kg.m ⁻²	226	25.7	0.013	154	26.3	<0.0001
Body Fat, %	226	24.0	<0.0001	154	25.5	<0.0001
PSA, ng.mL ⁻¹	219	6.1	<0.0001	146	9.1	<0.0001
Free PSA, ng.mL ⁻¹	215	1.1	<0.0001	142	1.0	<0.0001
Free/Total PSA ratio	215	0.19	<0.0001	142	0.12	<0.0001
Serum Testosterone, ng.mL ⁻¹	205	466	0.016	137	434.0	<0.0001
Serum IGF-1, ng.mL ⁻¹	210	131.5	<0.0001	142	128.0	<0.0001

* Number of evaluable patients; ** P-value for departure from normality (Shapiro-Francia test); PCa, prostate cancer; BMI, body mass index; PSA, prostate-specific antigen; IGF-1, insulin growth factor 1.

Table 2. Distribution of subjects according to body mass index or body fat percent

	BMI (Kg/m ²)							
	All		Normal (<25)		Overweight (25 - 30)		Obese (≥ 30)	
	Non-PCa	PCa	Non-PCa	PCa	Non-PCa	PCa	Non-PCa	PCa
Body fat percent cutoffs (%)	n=380		n=147		n=176		n=57	
Normal (< 20)	58 (77)	17 (23)	51 (80)	13 (20)	6 (60)	4 (40)	1 (100)	0 (0)
Overweight (20 - 25)	71 (56)	55 (44)	41 (58)	30 (42)	30 (58)	22 (42)	0 (0)	3 (100)
Obese (≥ 25)	97 (54)	82 (46)	4 (33)	8 (67)	70 (61)	44 (39)	23 (43)	30 (57)

Results presented as number of subjects (frequencies); PCa, prostate cancer; BMI, body mass index.

Table 3. Age-adjusted odds ratios (aORs) and 95% CI of prostate cancer according to different categories of anthropometric measures

Cutoffs	Non-PCa	All Prostate Cancers		Restricted to High Grade PCa ^a		Restricted to HRPcCa for Metastasis ^b	
		Cases	aOR (95%CI)	Cases	aOR (95%CI)	Cases	aOR (95%CI)
Height tertiles, cm							
< 164.9	60	57	Referent	45	Referent	20	Referent
165-170.29	91	54	0.6 (0.4-1.1)	39	0.6 (0.3-1.0)	18	0.7 (0.3-1.4)
≥ 170.3	75	43	1.1 (0.4-1.1)	35	0.7 (0.4-1.3)	10	0.6 (0.2-1.4)
Body fat percentage, %							
Normal (< 20)	58	17	Referent	13	Referent	7	Referent
Overweight (20 - 25)	71	55	2.4 (1.2-4.7)	43	2.4 (1.1-5.0)	13	1.3 (0.5-3.7)
Obese (≥ 25)	97	82	2.4 (1.3-4.6)	63	2.4 (1.1-4.7)	28	1.8 (0.7-4.6)
Body mass index, Kg/m ²							
Normal (< 25)	96	51	Referent	37	Referent	12	Referent
Overweight (25 - 30)	106	70	1.1 (0.7-1.8)	57	1.2 (0.7-2.0)	25	1.4 (0.6-3.0)
Obese (≥ 30)	24	33	2.4 (1.2-4.5)	25	2.4 (1.2-5.1)	11	3.1 (1.1-8.6)

^a High-grade PCa (Gleason score ≥ 7); ^b HRPcCa for metastasis, high risk PCa for metastasis (PSA ≥ 20 ng/mL and/or Gleason score ≥ 8); aOR, age-adjusted odds ratio and 95%CI, 95% confidence interval; age was used as a continuous variable; PCa, prostate cancer.

Table 4. Age-adjusted odds ratios (aOR) and 95% CI of prostate cancer according to different categories of hormonal measures

Tertiles	All Prostate Cancers			Restricted to HGPCa ^a		Restricted to HRPcCa for Metastasis ^b	
	Non-PCa	aOR (95%CI)		Cases	aOR (95%CI)	Cases	aOR (95%CI)
		Cases					
Serum Testosterone, ng/mL							
< 393.59	68	54	Referent	39	Referent	18	Referent
393.6 – 540.2.9	69	43	0.7 (0.4-1.2)	36	0.8 (0.4-1.5)	17	0.9 (0.4-2.0)
≥ 540.3	68	40	0.6 (0.4-1.1)	32	0.7 (0.4-1.2)	9	0.3 (0.1-0.9)
Serum IGF-1, ng/mL							
< 111.29	70	54	Referent	45	Referent	18	Referent
111.3 – 154.59	70	47	0.8 (0.5-1.4)	38	0.7 (0.4-1.3)	13	0.6 (0.3-1.4)
≥ 154.6	70	42	0.8 (0.5-1.5)	29	0.7 (0.4-1.3)	13	0.9 (0.4-2.0)

^a High-grade PCa (Gleason score ≥ 7); ^b HRPcCa for metastasis, high risk PCa for metastasis (PSA ≥ 20 ng/mL and/or Gleason score ≥ 8); aOR, age-adjusted odds ratio and 95%CI, 95% confidence interval; age was used as a continuous variable; IGF-1, insulin growth factor 1; PCa, prostate cancer.

Table 5. Multivariate analysis of Genetic Risk Score (GRS) and risk for prostate cancer, overall and in obese or non obese men according to BFP-defined obesity cutoff

	All PCa				High grade PCa ^a			
	Multivariate		Bootstrap ^b		Multivariate		Bootstrap ^b	
	Non-PCa	Cases	aOR (95%CI)	aOR (95%CI)	Cases	aOR (95%CI)	aOR (95%CI)	aOR (95%CI)
All subjects	226	153	2.1 (1.5-3.1)	2.1 (1.4-3.3)	118	2.4 (1.6-3.6)	2.4 (1.4-4.0)	
Restricted to BFP < 25%	129	71	1.5 (1.0-2.3)	1.5 (0.9-2.7)	55	1.8 (1.1-2.9)	1.8 (0.9-3.6)	
Restricted to BFP > 25%	97	82	3.2 (1.7-6.1)	3.2 (1.8-6.0)	63	3.5 (1.7-7.2)	3.5 (1.7-7.5)	

^a High-grade PCa (Gleason score ≥ 7); aOR, age-, BMI-, testosterone- and BFP-adjusted odds ratio and 95%CI (95% confidence interval); GRS, age, BMI and testosterone analyzed as continuous variables; PCa, prostate cancer. ^b Monte Carlo simulation with 10000 permutations.

Figure 1. Estimated linear fit and plots between body mass index and body fat percent for prostate cancer and non-prostate cancer groups. PCa, prostate cancer; BMI, body mass index.

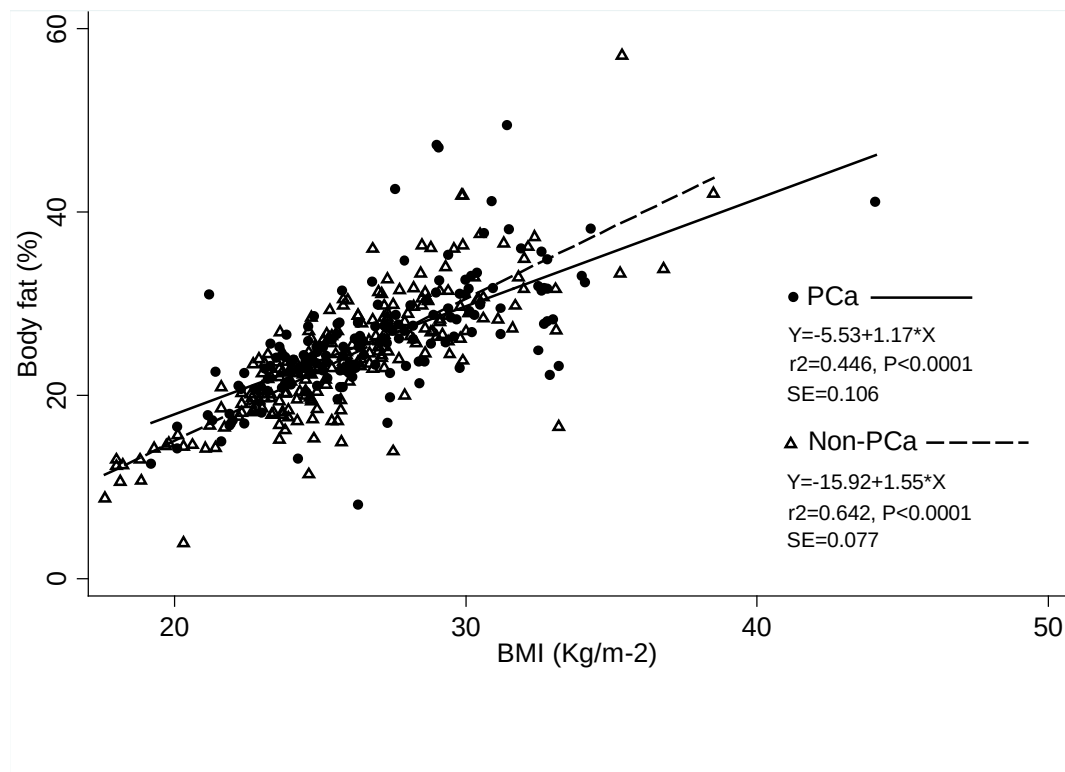


Figure 2. Body fat percent and BMI differences between PCa and non-PCa groups in BMI-defined groups. Bars represent means $\pm$ standard deviations. PCa, prostate cancer; BMI, body mass index; OB/OW, obese and overweight group; * $P=0.001$ (Mann Whitney test).

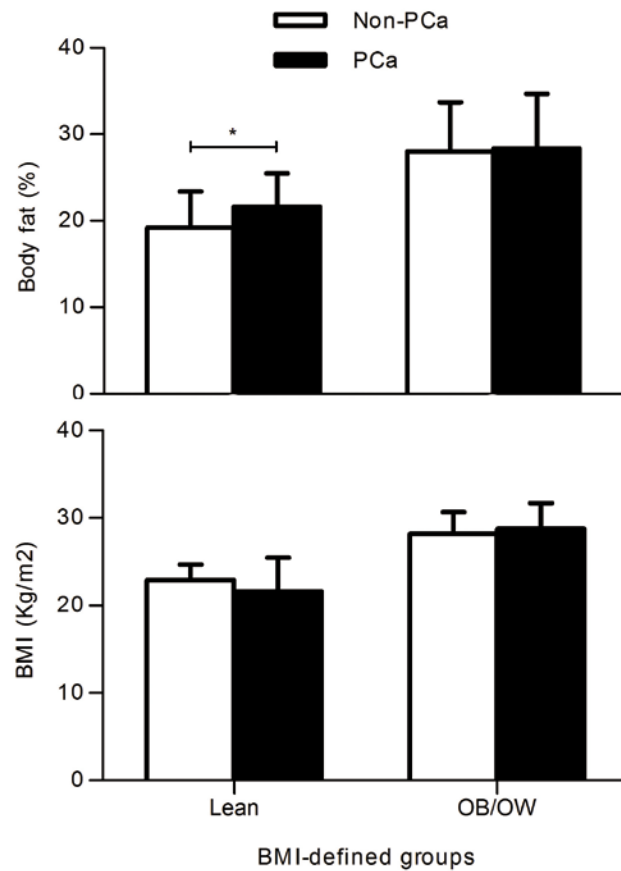
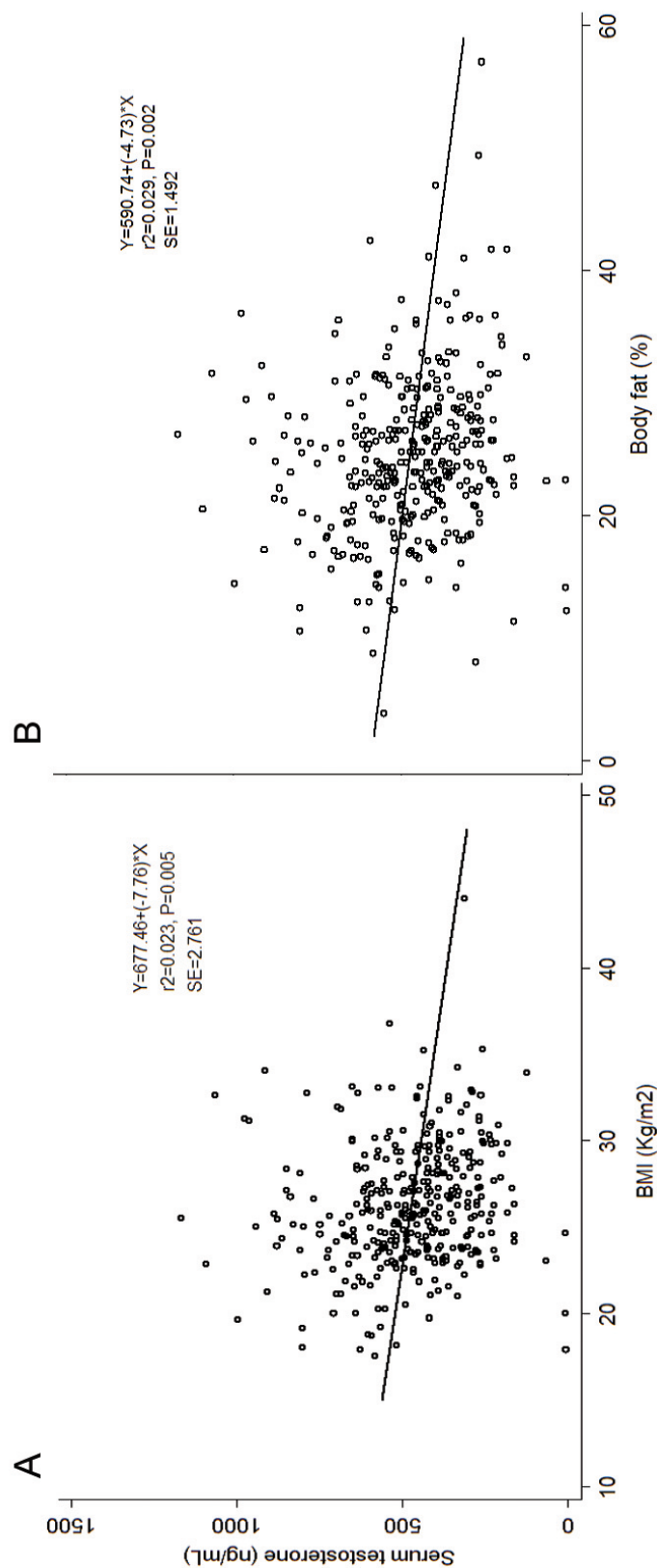


Figure 3. Linear regression analyses and equations to estimate the strength of association between body mass index (A) and body fat percent (B) with circulating testosterone levels. PCa, prostate cancer; Non-PCa, non prostate cancer; BMI, body mass index.



PAPER VIII

Inherited variation in adipokine pathway genes may determine prognosis for prostate cancer patients receiving androgen-deprivation therapy

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ABSTRACT

Purpose

Androgen deprivation therapy (ADT) is the foremost treatment in advanced and recurrent prostate cancer, although prognosis varies widely among individuals. We evaluated whether polymorphisms in genes of adipokine pathways are determinants of prostate cancer prognosis outcomes.

Patients and Methods

Four hundred eighty three patients treated with ADT were enrolled in this study and genotyped for 27 functional single nucleotide polymorphisms (SNPs) in 17 genes from 9 adipokine pathways. SNPs were also combined by pathway according to functional characteristics.

Results

The time to castration-resistant prostate cancer (CRPCa) was shorter in *ADIPOQ* +45 T>G GG, *ADIPOQ* +276 G>T T-allele and for low/intermediate tumor necrosis factor activation profile carriers ($P<0.05$), after adjusting for relevant clinical variables. Furthermore, the presence of *ADIPOQ* +45 T>G G homozygous, *PPARD* -87 T>C T-allele and *IL6R* Asp358Ala C homozygous genotypes showed independent association with survival following ADT initiation ($P<0.05$). The *LEPR* Gln223Arg G-allele variant was associated with a more than threefold increased risk of developing metastasis (OR=2.1, 95%CI: 1.2-3.6).

Conclusion

This report is the first to examine that among men treated with ADT, genetic polymorphisms in adipokine pathways might influence outcome prediction. In addition, combined targeting of identified adipokine pathways may represent a therapeutic strategy for CRPCa, metastasis development and ultimately survival.

INTRODUCTION

Depletion or blockage of androgen's action has been the standard of care for men with advanced prostate cancer during the last decade's ¹. Androgen deprivation therapy (ADT) results in PSA concentration decrease as well as waning of androgen receptor (AR)-dependent growth. The response to treatment is not durable as almost always patients become resistant to ADT, leading to castration-resistance an invariably fatal condition ². Clinical factors that may partially account for disease burden and some of the inherent tumor biology have been reported as useful prognostic predictors, including Gleason score, serum PSA and metastatic disease ³. Albeit mechanisms responsible for prostate cancer cells survive after ADT are not entirely understood, there is evidence that AR reactivation and AR-independent pathways may be implicated ^{4,5}. Recent studies associating DNA polymorphisms with response to ADT were performed with emphasis on SNPs of genes involved in biosynthesis and metabolism of steroids and androgens ⁶⁻⁹. In addition, recent findings showed that susceptibility SNPs might also improve outcome prediction following ADT ¹⁰⁻¹³.

While germline DNA polymorphisms in androgen pathways were shown to influence the response to ADT, no study has examined the predictive role of polymorphisms in genes of adipokine pathways on clinical outcomes after ADT initiation. Adipokines are adipose tissue-produced and obesity-related molecules known to be mechanistically involved in prostate tumor aggressiveness ^{14,15}. While some functional SNPs in genes encoding molecules of these pathways have been shown to be associated with prostate cancer risk ¹⁶⁻¹⁹, a recent study found that obese men were at increased risk of developing castration-resistant prostate cancer and development of metastasis ²⁰. In this study, we examined the prognostic significance of 27 putative functional SNPs in genes of adipokine pathways on metastasis, response to hormonal

castration and all-cause mortality in a cohort of prostate cancer patients treated with ADT.

MATERIALS AND METHODS

Patients

Patients with histopathologically confirmed prostate cancer, treated with androgen deprivation therapy between 1990 and 2009, were included in this study (n=483), after signing informed consent. Patients were recruited from 4 Hospitals in Portugal: Portuguese Institute of Oncology – Porto Centre, Porto Military Hospital, Porto Hospital Centre and Central Lisbon Hospital Centre. The research protocol and consent form were approved by the participating Institution's Ethics Committees.

All patients were treated with androgen deprivation therapy (ADT) (orchiectomy or LHRH agonist with or without anti-androgen), after diagnosis with advanced/metastatic prostate cancer or after relapsing disease from definitive local therapy, and followed up prospectively. Patients with adjuvant hormonal therapy for local disease were not considered to take part in this study. Hormonal treatment continued until castration-resistance on the basis of the PSA concentration, imaging, and clinical findings. The primary endpoint was resistance to hormonal castration, defined as the time from ADT initiation to two consecutive rises of PSA (1 week apart) greater than the PSA nadir, despite at least two consecutive hormonal manipulations or progression of osseous lesions (new or size increase soft tissue metastasis, or at least 2 new metastatic spots in bone scintigraphy) ^{21,22}. The secondary end points included overall survival, defined as the time from ADT initiation to death from any cause, and appearance of distant metastasis at any time during the course of the disease (identified by x-rays, computed tomography scans or bone scintigraphy.). Information concerning

clinical endpoints was collected on the basis of standardized extractions from patient files. Samples of peripheral blood for genotyping were collected independent of treatment initiation.

Genetic variants and genotyping

Candidate genes involved in adipokine pathways known to affect oncogenesis were selected. SNPs were preferred from the best evidence from published studies. A total of 27 literature-defined putative functional SNPs in 17 different genes were chosen, corresponding to 9 adipokine pathways (Supplementary table 1). In agreement with literature, we performed combinations of SNPs by adipokine pathway according to their functional repercussion (Supplementary table 2).

Allelic discrimination through Taqman genotyping (Applied Biosystems) was used for 20 SNPs (two in *ADIPOQ*, rs1501299 and rs16861194; *IL6*, rs10499563; *IL6R*, rs2228145; *KDR*, rs2071559; three in *VEGF*, rs2010963, rs833061 and rs3025039; *LEP*, rs7799039; two in *LEPR*, rs1137100 and rs8179183; *PPARG*, rs1801282; *PGC1A*, rs8192678; *PPARD*, rs2016520; *OPN*, rs28357094; *IGF1R*, rs2229765; *IRS1*, rs1801278; *FGFR2*, rs2981582; *TNFA*, rs1800629; *TNFRSF1A*, rs4149570), whereas the remaining (three in *IL6*, rs1800797, rs1800796 and rs1800795; *ADIPOQ*, rs2241766; *IL6ST*, rs3729960; *LEPR*, rs1137101; *TNFA*, rs1800630) were genotyped by polymerase chain reaction - restriction fragment length polymorphism. Quality control included non-template controls in all runs and genotype reliability assessment in 5% of the samples.

Statistical analysis

Clinicopathologic characteristics of patients were summarized as number and percentage of patients for categorical variables, or mean and standard deviation for continuous variables; PSA at diagnosis was dichotomized at 20 ng/mL because of its association with micrometastasis²³.

Multivariate analyses were performed after selecting the relevant confounding variables by empirical evaluation for each of clinical and genetic models. For time-to-event analyses, cox regression models were used to assess hazard ratios (HR) and 95% confidence intervals (CIs) for estimating the association of clinicopathological characteristics with the time to CRPCa and time to all-cause mortality. Logistic regression analyses were conducted to evaluate risk for metastasis. Since the optimal genetic model in association studies is not warranted, we tested based on the minor allele the dominant (aa + Aa genotype versus AA genotype) and recessive (aa genotype versus Aa + AA genotype) models and functional SNP combinations to evaluate the individual association of 27 SNPs with three outcome measurements using Cox regression and logistic regression analyses. The model with the highest likelihood ratio was presented as the best-fitting genetic model for each SNP. Likewise, the functional combinations of SNPs by pathway were dicotomized (low/intermediate versus high and low versus intermediate/high) and the one with highest likelihood ratio was used. For the subset of clinical factors, SNPs and functional combinations of SNPs selected on the basis of probability <0.15, a multivariate Cox proportional hazards model was derived by stepwise selection (P-value for retention < 0.05) to identify the independent predictive risks for CRPCa and ACM. A multivariate logistic regression model was performed to evaluate clinical and genetic predictive factors for prostate cancer metastasis. Statistical analyses were conducted in STATA version 10.0 (StataCorp, College Station, Texas).

RESULTS

Four hundred and eighty three patients treated with ADT in the course of their disease were analyzed in this study. Patient and disease characteristics of this cohort are presented in Table 1. Twenty-seven percent of patients had distant metastasis at diagnosis, 62% had advanced clinical stage and 51% of patients had a biopsy Gleason score ≥ 7 (4+3). The median duration of ADT until CRPCa was 47.8 months and the median follow-up in the overall study population from ADT initiation to death or last visit was 58.2 months. Several clinical factors have been identified to predict CRPCa and ACM in univariate analysis (Table 1). Advanced clinical stage, biopsy Gleason score above 7 (4+3), PSA higher than 20 ng/mL, presence of metastasis at ADT initiation and not being submitted to definitive therapy were associated with shorter time to CRPCa and ACM. Combined therapy with LHRH agonist plus antiandrogen, development of resistance to hormonal castration and age at diagnosis were significantly associated with shorter time to ACM, but not time to CRPCa. The clinical variables associated with risk for metastasis included PSA at diagnosis (OR=2.73, 95%CI: 1.82-4.10, $P<0.0001$), clinical stage (OR=12.14, 95%CI: 7.03-20.95, $P<0.0001$) and Gleason grade (OR=2.78, 95%CI: 1.83-4.22, $P<0.0001$).

Empirical analysis using Cox regression was then performed to evaluate the association between SNPs and functional combinations with CRPCa, ACM following ADT and risk for metastasis. As shown in Supplementary table 3, we found that *IL6R* Asp358Ala CC, *ADIPOQ*+45 GG, *ADIPOQ*+276 T carriers, low TNFa expression and low/intermediate TNFa activation were associated with shorter time to develop resistance to hormonal castration. Interestingly, besides the *IL6R* Asp358Ala CC and *ADIPOQ*+45 GG genetic variants that were further associated with ACM, also high VEGF activation 2SNPs (VEGF+405/VEGF-460 and KDR-604 SNPs combined) and

IL6-572 C carriers were associated with time to ACM following ADT (Supplementary table 3). Similar findings were obtained with Kaplan-Meier estimates of CRPCa (Figure 1) or ACM following ADT (Figure 2). In unadjusted logistic regression analysis, *IL6* -6331 T carriers, *LEPR* Gln223Arg G carriers, high/intermediate LEPR signaling and high/intermediate TNFa activation were significantly related to greater risk of metastasis (Supplementary table 4).

The predictive effects of SNPs with $P < 0.15$ (univariate analysis) on time to CRPCa and ACM following ADT were evaluated together with significant clinicopathologic predictors from empirical examination (boldface in Table 1) using Cox regression. The effect of *ADIPOQ* +45 and *ADIPOQ* +276 variants, and TNFa activation profile on CRPCa was robust after adjustment for clinical factors in a multivariate analysis (Table 2). Analysis of the secondary endpoint ACM after adjusting for other predictors, showed that *ADIPOQ* +45 T>G and *IL6R* Asp358Ala remained significant predictors for time to ACM, including the confirmed age at diagnosis, Gleason grade, metastasis at ADT initiation and resistance to hormonal castration (Table 3). *PPARD* CC carriers also were independently associated with increased overall survival after ADT (HR=0.25, 95%CI: 0.08-0.81). On multivariate analysis, carrying the *LEPR* 223 G-allele remained associated with greater risk of developing distant metastatic disease (Table 4). Noteworthy, men with higher biopsy Gleason grade (> 4+3) and particularly advanced clinical stage at diagnosis were almost twofold and more than tenfold likely to develop metastasis.

DISCUSSION

In this study aiming to understand if germline polymorphisms in adipokine pathways are determinants of the response to ADT, the time to CRPCa was influenced

by two SNPs in *ADIPOQ* and by combined SNPs in TNF α pathway activation. The predictive ability of *ADIPOQ* +45 extended towards the secondary end point ACM, together with *PPARD* and *IL6R* genetic polymorphisms. Additionally, results also suggest an association of *LEPR* Gln223Arg polymorphism with development of metastasis.

Androgen deprivation therapy remains the mainstay treatment for advanced and recurrent prostate cancer ^{21,24}. Factors that influence the response to ADT includes clinical stage, Gleason score, PSA and metastasis ³, although SNPs may also be useful for prognosticating the response to ADT. By analyzing 27 SNPs of 17 genes related with 9 adipokine pathways, we have identified two SNPs, *ADIPOQ* +45 and *ADIPOQ* +276 and a functional *TNFA/TNFRSF1A* combination that are associated with CRPCa after controlling for clinical prognostic factors. The mechanisms responsible for CRPCa development are not definitely established. Despite increasing interest in androgen receptor-dependent pathway, other independent pathways have been described ^{4,25}, in which CRPCa cells use alternative survival pathways to overcome the growth inhibition imposed by ADT ^{4,26}. ADT is known to induce changes in adiposity and adipokine levels in circulation ^{27,28}. Adipokine pathways, have been implicated in intracellular signals such as those activated in hormonal castration resistance ²⁹. Furthermore, mitogenic and anti-apoptotic effects of some adipokines (e.g. leptin, IL-6, IGF-1) seem to be limited to hormone-refractory prostate cancer cells ³⁰⁻³².

In our study, *ADIPOQ* +45 G homozygous and *ADIPOQ* +276 T carriers had a shorter time to CRPCa. The G-allele in locus +45 and T-allele in locus +276 are associated with higher circulating adiponectin plasma levels ^{33,34}. Generally, low concentrations have been associated with prostate cancer risk and survival, rendering adiponectin a protective role against cancer ³⁵⁻³⁷. Nevertheless, this effect could be

dependent on the metabolic environment and tumor cell characteristics. ADT induces profound changes in metabolic environment, modulating specifically plasma adiponectin levels²⁸, and eventually modulating the expression of adiponectin receptors (ADIPOR1 and ADIPOR2), which have been shown to be receptive to cytokines and steroid hormones stimulus according to tumor cells androgen refractoriness status³⁸. Additionally, adiponectin has the capacity to exert stimulatory growth and motile effects in some prostate tumor cells, depending on PTEN status^{39,40}. Despite advances in understanding the role of adiponectin in prostate cancer risk, studies on CRPCa are scarce. Although *ADIPOQ* polymorphisms have been inconsistently associated with prostate cancer risk^{16,41,42}, our study is the first to evaluate *ADIPOQ* SNPs in association with CRPCa. When we combined SNPs in TNF pathway [*TNFA* -863 C>A (rs1800630), *TNFA* -308 G>A (rs1800629) and *TNFRSF1A* -329 G>T (rs4149570)] according to functional characteristics, the carriers of low/intermediate TNFa activity profile had shorter time to CRPCa, notwithstanding case-control studies showed lack of association between *TNFA* variants at locus -863 or -308 and prostate cancer^{43,44}. The TNF and TNFR superfamily plays crucial roles in mediating the inflammatory response and regulating immune function, in addition to triggering apoptosis of certain tumor cells⁴⁵. TNFa-mediated activation of TNFR1 signaling is critical for activating tumor-reactive T cells and arresting multistage carcinogenesis^{46,47}. More specifically, the proapoptotic actions of estrogen receptor beta in castration-resistant prostate cancer cells required TNFa signaling⁴⁸. The very low levels of testosterone as result of ADT, together with increased inflammatory markers⁴⁹, create an inflammatory environment for tumor under ADT. We hypothesize that this scenario may be altered by polymorphisms in *ADIPOQ* and by low/intermediate TNFa activation, which likely result in decreased inflammatory magnitude, further depressing immunosurveillance.

The few studies that examined the role of germline polymorphisms in association with prostate cancer mortality yielded inconsistent results⁵⁰⁻⁵³. In our cohort, where all patients received ADT for treating prostate cancer while taking into consideration previous treatment modalities, *ADIPOQ* +45 T>G, *PPARD* -87 T>C and *IL6R* Asp358Ala, remained independently associated with decreased survival. IL-6 has been implicated with poor prognosis in prostate cancer^{54,55}. Activation of IL-6/IL6R pathway is linked with neuroendocrine differentiation of prostate cancer cells, promiscuous activation of the AR and regulation of prostate intracrine androgen production⁵⁶⁻⁵⁸, mechanisms related with resistance to hormonal castration and ultimately mortality. IL-6 signals are transmitted via a heterodimeric receptor complex consisting of a soluble IL-6 alpha subunit and a signal-transducing subunit⁵⁹. The common genetic variant of *IL6R* that results in Asp-by-Ala substitution at codon 385 (A > C nucleotide substitution) is associated with increased soluble IL6R and IL-6 serum levels and with altered IL6R membrane binding^{60,61}. We observed after multivariate analysis that *IL6R* C homozygous remained significantly associated with reduced overall survival following ADT, which might correspond to higher activation of trans-signaling pathway associated with C-allele. In our study, the *ADIPOQ* +45 G homozygous carriers, besides being associated with CRPCa, had shorter time to ACM after ADT. The G-allele is associated with higher circulating adiponectin plasma levels³⁴. *In vitro* findings suggest adiponectin amplifies the activation of PI3kinase/Akt/mTOR pathway in prostate cancer cells with PTEN loss, which are features of aggressive tumors from patients with advanced or recurrent disease^{62,63}, as in this study. If further confirmed, these findings suggest the implementation of targeted dual inhibition of PI3K and mTOR in the treatment of advanced or recurrent prostate cancer patients, as previously proposed²⁹.

Leptin's actions in tumor development and progression are mediated by leptin receptor ^{14,15}, which is strongly expressed in prostate tumors ⁶⁴, where pathway activation induces aggressive cell phenotypes ^{65,66}. We found that *LEPR* 223 G carriers were at increased risk for developing metastatic disease. Although this SNP yielded mixed results in case-control studies ^{16,67} (Ribeiro et al., unpublished results), G carriers have a stronger leptin-binding affinity ⁶⁸. In addition, this polymorphism is associated with plasma soluble LEPR concentration and may influence receptor recycling and degradation ^{69,70}, thereby influencing free leptin levels and receptor availability at cell surface. Moreover, recent work evidenced a central role for leptin signaling in tumor-initiating stem cells growth and survival ⁷¹. There has been particular interest in the *PPARD* -87 T>C polymorphism that alters the binding affinity of the SP1 transcription factor, with carriers of the minor C-allele having higher transcriptional activity ⁷². We observed in a cohort of prostate cancer patients treated with ADT that CC carriers had increased survival. *PPARD* role in cancer is controversial, although no studies to date were conducted on prostate cancer. Nevertheless, some studies in colon and skin cancer concluded that *PPARD* attenuates carcinogenesis ^{73,74} and that its expression is positive for the prognosis of a given cancer ⁷⁵.

Inherited genetic markers have been fairly explored as predictors of prostate cancer outcomes. Albeit we took a focused candidate gene approach to evaluate the association of key SNPs in adipokine pathways with relevant prostate cancer outcomes in a cohort of patients in ADT, some limitations persisted. Testosterone levels were not available in all men to confirm castration; therefore we relied on PSA measurements, clinical and imagiologic information to define CRPCa. We hasten to admit that despite we included only functional SNPs from genes in adipokine pathways, our SNP panel and SNP combinations could be incomplete. In our study, potential gene-environment

interactions were not explored, due to missing body mass index data, even though it might be important in ADT; further studies exploring eventual synergies with adiposity measures are required. Strengths of our study include the large size and homogeneous population. The long follow-up time allowed analysis of primary and secondary end points with large number of events (CRPCa, 46.4%; mortality, 32.2%; metastasis, 44.9%).

In a time where alternative therapeutic opportunities arise in advanced prostate cancer^{76,77}, poor prognostic germline polymorphisms may be used along with known clinical factors to foster the capacity to prognosticate clinical course after ADT initiation, thereby helping clinical reasoning. Present findings also encourage the need for clarifying the effectiveness of personalized therapies targeted towards adiponectin, tumoral necrosis factor and leptin pathways. If confirmed, our findings might help targeting patients with precocious ADT failure and mortality, and at risk for metastasis development in the course of disease.

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REFERENCES

1. Pagliarulo V, Bracarda S, Eisenberger MA, et al: Contemporary role of androgen deprivation therapy for prostate cancer. *Eur Urol* 61:11-25, 2012
2. Scher HI, Kelly WM, Zhang ZF, et al: Post-therapy serum prostate-specific antigen level and survival in patients with androgen-independent prostate cancer. *J Natl Cancer Inst* 91:244-51, 1999
3. Hussain M, Tangen CM, Higano C, et al: Absolute prostate-specific antigen value after androgen deprivation is a strong independent predictor of survival in new metastatic prostate cancer: data from Southwest Oncology Group Trial 9346 (INT-0162). *J Clin Oncol* 24:3984-90, 2006
4. Feldman BJ, Feldman D: The development of androgen-independent prostate cancer. *Nat Rev Cancer* 1:34-45, 2001
5. Attar RM, Takimoto CH, Gottardis MM: Castration-resistant prostate cancer: locking up the molecular escape routes. *Clin Cancer Res* 15:3251-5, 2009
6. Ross RW, Oh WK, Xie W, et al: Inherited variation in the androgen pathway is associated with the efficacy of androgen-deprivation therapy in men with prostate cancer. *J Clin Oncol* 26:842-7, 2008
7. Hamada A, Sissung T, Price DK, et al: Effect of SLCO1B3 haplotype on testosterone transport and clinical outcome in caucasian patients with androgen-independent prostatic cancer. *Clin Cancer Res* 14:3312-8, 2008
8. Yang M, Xie W, Mostaghel E, et al: SLCO2B1 and SLCO1B3 may determine time to progression for patients receiving androgen deprivation therapy for prostate cancer. *J Clin Oncol* 29:2565-73, 2011
9. Lindstrom S, Adami HO, Balter KA, et al: Inherited variation in hormone-regulating genes and prostate cancer survival. *Clin Cancer Res* 13:5156-61, 2007
10. Bao BY, Pao JB, Huang CN, et al: Significant associations of prostate cancer susceptibility variants with survival in patients treated with androgen-deprivation therapy. *Int J Cancer* 130:876-84, 2012
11. Tsuchiya N, Wang L, Suzuki H, et al: Impact of IGF-I and CYP19 gene polymorphisms on the survival of patients with metastatic prostate cancer. *J Clin Oncol* 24:1982-9, 2006
12. Teixeira AL, Ribeiro R, Cardoso D, et al: Genetic polymorphism in EGF is associated with prostate cancer aggressiveness and progression-free interval in androgen blockade-treated patients. *Clin Cancer Res* 14:3367-71, 2008
13. Teixeira AL, Ribeiro R, Morais A, et al: Combined analysis of EGF+61G>A and TGFB1+869T>C functional polymorphisms in the time to androgen independence and prostate cancer susceptibility. *Pharmacogenomics J* 9:341-6, 2009
14. Mistry T, Digby JE, Desai KM, et al: Obesity and prostate cancer: a role for adipokines. *Eur Urol* 52:46-53, 2007
15. Ribeiro R, Lopes C, Medeiros R: The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives. *Prostate Cancer Prostatic Dis* 9:19-24, 2006
16. Moore SC, Leitzmann MF, Albanes D, et al: Adipokine genes and prostate cancer risk. *Int J Cancer* 124:869-76, 2009
17. Ribeiro R, Vasconcelos A, Costa S, et al: Overexpressing leptin genetic polymorphism (-2548 G/A) is associated with susceptibility to prostate cancer and risk of advanced disease. *Prostate* 59:268-74, 2004
18. Wang MH, Helzlsouer KJ, Smith MW, et al: Association of IL10 and other immune response- and obesity-related genes with prostate cancer in CLUE II. *Prostate* 69:874-85, 2009

19. Kwon EM, Salinas CA, Kolb S, et al: Genetic polymorphisms in inflammation pathway genes and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev* 20:923-33, 2011
20. Keto CJ, Aronson WJ, Terris MK, et al: Obesity is associated with castration-resistant disease and metastasis in men treated with androgen deprivation therapy after radical prostatectomy: results from the SEARCH database. *BJU Int*, 2011
21. Mottet N, Bellmunt J, Bolla M, et al: EAU guidelines on prostate cancer. Part II: Treatment of advanced, relapsing, and castration-resistant prostate cancer. *Eur Urol* 59:572-83, 2011
22. Scher HI, Halabi S, Tannock I, et al: Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. *J Clin Oncol* 26:1148-59, 2008
23. Heidenreich A, Bellmunt J, Bolla M, et al: EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. *Eur Urol* 59:61-71, 2011
24. Sharifi N, Gulley JL, Dahut WL: Androgen deprivation therapy for prostate cancer. *JAMA* 294:238-44, 2005
25. Devlin HL, Mudryj M: Progression of prostate cancer: multiple pathways to androgen independence. *Cancer Lett* 274:177-86, 2009
26. Schroder FH: Progress in understanding androgen-independent prostate cancer (AIPC): a review of potential endocrine-mediated mechanisms. *Eur Urol* 53:1129-37, 2008
27. Saylor PJ, Kozak KR, Smith MR, et al: Changes in biomarkers of inflammation and angiogenesis during androgen deprivation therapy for prostate cancer. *Oncologist* 17:212-9, 2012
28. Smith MR, Lee H, Fallon MA, et al: Adipocytokines, obesity, and insulin resistance during combined androgen blockade for prostate cancer. *Urology* 71:318-22, 2008
29. Chen J: Multiple signal pathways in obesity-associated cancer. *Obes Rev* 12:1063-70, 2011
30. Onuma M, Bub JD, Rummel TL, et al: Prostate cancer cell-adipocyte interaction: leptin mediates androgen-independent prostate cancer cell proliferation through c-Jun NH2-terminal kinase. *J Biol Chem* 278:42660-7, 2003
31. Chung TD, Yu JJ, Spiotto MT, et al: Characterization of the role of IL-6 in the progression of prostate cancer. *Prostate* 38:199-207, 1999
32. Iwamura M, Sluss PM, Casamento JB, et al: Insulin-like growth factor I: action and receptor characterization in human prostate cancer cell lines. *Prostate* 22:243-52, 1993
33. Fredriksson J, Carlsson E, Orho-Melander M, et al: A polymorphism in the adiponectin gene influences adiponectin expression levels in visceral fat in obese subjects. *Int J Obes (Lond)* 30:226-32, 2006
34. Berthier MT, Houde A, Cote M, et al: Impact of adiponectin gene polymorphisms on plasma lipoprotein and adiponectin concentrations of viscerally obese men. *J Lipid Res* 46:237-44, 2005
35. Li H, Stampfer MJ, Mucci L, et al: A 25-year prospective study of plasma adiponectin and leptin concentrations and prostate cancer risk and survival. *Clin Chem* 56:34-43, 2010
36. Michalakis K, Williams CJ, Mitsiades N, et al: Serum adiponectin concentrations and tissue expression of adiponectin receptors are reduced in patients with prostate cancer: a case control study. *Cancer Epidemiol Biomarkers Prev* 16:308-13, 2007
37. Lu JP, Hou ZF, Duivenvoorden WC, et al: Adiponectin inhibits oxidative stress in human prostate carcinoma cells. *Prostate Cancer Prostatic Dis* 15:28-35, 2012
38. Mistry T, Digby JE, Chen J, et al: The regulation of adiponectin receptors in human prostate cancer cell lines. *Biochem Biophys Res Commun* 348:832-8, 2006

39. Tang CH, Lu ME: Adiponectin increases motility of human prostate cancer cells via adipoR, p38, AMPK, and NF-kappaB pathways. *Prostate* 69:1781-9, 2009
40. Barb D, Neuwirth A, Mantzoros CS, et al: Adiponectin signals in prostate cancer cells through Akt to activate the mammalian target of rapamycin pathway. *Endocr Relat Cancer* 14:995-1005, 2007
41. Kaklamani V, Yi N, Zhang K, et al: Polymorphisms of ADIPOQ and ADIPOR1 and prostate cancer risk. *Metabolism* 60:1234-43, 2011
42. Dhillon PK, Penney KL, Schumacher F, et al: Common polymorphisms in the adiponectin and its receptor genes, adiponectin levels and the risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev* 20:2618-27, 2011
43. Kesarwani P, Mandhani A, Mittal RD: Polymorphisms in tumor necrosis factor- α gene and prostate cancer risk in North Indian cohort. *J Urol* 182:2938-43, 2009
44. Danforth KN, Rodriguez C, Hayes RB, et al: TNF polymorphisms and prostate cancer risk. *Prostate* 68:400-7, 2008
45. Chen G, Goeddel DV: TNF-R1 signaling: a beautiful pathway. *Science* 296:1634-5, 2002
46. Muller-Hermelink N, Braumuller H, Pichler B, et al: TNFR1 signaling and IFN- γ signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis. *Cancer Cell* 13:507-18, 2008
47. Calzascia T, Pellegrini M, Hall H, et al: TNF- α is critical for antitumor but not antiviral T cell immunity in mice. *J Clin Invest* 117:3833-45, 2007
48. McPherson SJ, Hussain S, Balanathan P, et al: Estrogen receptor-beta activated apoptosis in benign hyperplasia and cancer of the prostate is androgen independent and TNF α mediated. *Proc Natl Acad Sci U S A* 107:3123-8, 2010
49. Maggio M, Basaria S, Ceda GP, et al: The relationship between testosterone and molecular markers of inflammation in older men. *J Endocrinol Invest* 28:116-9, 2005
50. Gallagher DJ, Vijai J, Cronin AM, et al: Susceptibility loci associated with prostate cancer progression and mortality. *Clin Cancer Res* 16:2819-32, 2010
51. Penney KL, Pyne S, Schumacher FR, et al: Genome-wide association study of prostate cancer mortality. *Cancer Epidemiol Biomarkers Prev* 19:2869-76, 2010
52. Wiklund FE, Adami HO, Zheng SL, et al: Established prostate cancer susceptibility variants are not associated with disease outcome. *Cancer Epidemiol Biomarkers Prev* 18:1659-62, 2009
53. Lin DW, FitzGerald LM, Fu R, et al: Genetic variants in the LEPR, CRY1, RNASEL, IL4, and ARVCF genes are prognostic markers of prostate cancer-specific mortality. *Cancer Epidemiol Biomarkers Prev* 20:1928-36, 2011
54. Nakashima J, Tachibana M, Horiguchi Y, et al: Serum interleukin 6 as a prognostic factor in patients with prostate cancer. *Clin Cancer Res* 6:2702-6, 2000
55. George DJ, Halabi S, Shepard TF, et al: The prognostic significance of plasma interleukin-6 levels in patients with metastatic hormone-refractory prostate cancer: results from cancer and leukemia group B 9480. *Clin Cancer Res* 11:1815-20, 2005
56. Chun JY, Nadiminty N, Dutt S, et al: Interleukin-6 regulates androgen synthesis in prostate cancer cells. *Clin Cancer Res* 15:4815-22, 2009
57. Lee GT, Kwon SJ, Lee JH, et al: Macrophages induce neuroendocrine differentiation of prostate cancer cells via BMP6-IL6 Loop. *Prostate*, 2011
58. Malinowska K, Neuwirth H, Cavarretta IT, et al: Interleukin-6 stimulation of growth of prostate cancer in vitro and in vivo through activation of the androgen receptor. *Endocr Relat Cancer* 16:155-69, 2009
59. Boulanger MJ, Chow DC, Brevnova EE, et al: Hexameric structure and assembly of the interleukin-6/IL-6 α -receptor/gp130 complex. *Science* 300:2101-4, 2003
60. Mullberg J, Oberthur W, Lottspeich F, et al: The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. *J Immunol* 152:4958-68, 1994

61. Galicia JC, Tai H, Komatsu Y, et al: Polymorphisms in the IL-6 receptor (IL-6R) gene: strong evidence that serum levels of soluble IL-6R are genetically influenced. *Genes Immun* 5:513-6, 2004
62. Mulholland DJ, Tran LM, Li Y, et al: Cell autonomous role of PTEN in regulating castration-resistant prostate cancer growth. *Cancer Cell* 19:792-804, 2011
63. Sircar K, Yoshimoto M, Monzon FA, et al: PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. *J Pathol* 218:505-13, 2009
64. Hoon Kim J, Lee SY, Myung SC, et al: Clinical significance of the leptin and leptin receptor expressions in prostate tissues. *Asian J Androl* 10:923-8, 2008
65. Somasundar P, Yu AK, Vona-Davis L, et al: Differential effects of leptin on cancer in vitro. *J Surg Res* 113:50-5, 2003
66. Huang CY, Yu HS, Lai TY, et al: Leptin increases motility and integrin up-regulation in human prostate cancer cells. *J Cell Physiol* 226:1274-82, 2011
67. Kote-Jarai Z, Singh R, Durocher F, et al: Association between leptin receptor gene polymorphisms and early-onset prostate cancer. *BJU Int* 92:109-12, 2003
68. Quinton ND, Lee AJ, Ross RJ, et al: A single nucleotide polymorphism (SNP) in the leptin receptor is associated with BMI, fat mass and leptin levels in postmenopausal Caucasian women. *Hum Genet* 108:233-6, 2001
69. Sun Q, Cornelis MC, Kraft P, et al: Genome-wide association study identifies polymorphisms in LEPR as determinants of plasma soluble leptin receptor levels. *Hum Mol Genet* 19:1846-55, 2010
70. da Silva BA, Bjorbaek C, Uotani S, et al: Functional properties of leptin receptor isoforms containing the gln-->pro extracellular domain mutation of the fatty rat. *Endocrinology* 139:3681-90, 1998
71. Park J, Scherer PE: Leptin and cancer: from cancer stem cells to metastasis. *Endocr Relat Cancer* 18:C25-9, 2011
72. Skogsberg J, Kannisto K, Cassel TN, et al: Evidence that peroxisome proliferator-activated receptor delta influences cholesterol metabolism in men. *Arterioscler Thromb Vasc Biol* 23:637-43, 2003
73. Kim DJ, Akiyama TE, Harman FS, et al: Peroxisome proliferator-activated receptor beta (delta)-dependent regulation of ubiquitin C expression contributes to attenuation of skin carcinogenesis. *J Biol Chem* 279:23719-27, 2004
74. Harman FS, Nicol CJ, Marin HE, et al: Peroxisome proliferator-activated receptor-delta attenuates colon carcinogenesis. *Nat Med* 10:481-3, 2004
75. Reed KR, Sansom OJ, Hayes AJ, et al: PPARdelta status and Apc-mediated tumorigenesis in the mouse intestine. *Oncogene* 23:8992-6, 2004
76. de Bono JS, Logothetis CJ, Molina A, et al: Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 364:1995-2005, 2011
77. Kantoff PW, Higano CS, Shore ND, et al: Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 363:411-22, 2010

Figure 1. Cumulative incidence of progression to CRPCa by SNPs and functional combinations. Unadjusted survival curve with P-values reflecting Log-Rank tests. ADT, androgen deprivation therapy; CRPCa, castration-resistant prostate cancer. *TNFA* expression reflects the functional combination (as described in supplementary table 2) of *TNFA* -308 and -863 SNPs, whereas TNFa activity includes the *TNFRSF1A* besides the two *TNFA* SNPs.

Figure 2. Kaplan-Meier estimates of overall survival following ADT, stratified by SNPs or functional combinations. P-values were calculated by Log-Rank tests. ADT, androgen deprivation therapy; CRPCa, castration-resistant prostate cancer. VEGF pathway activation 2 SNPs relates to the functional combination (as described in supplementary table 2) of two *VEGF* SNPs -460 and +405 plus the *KDR* -604 polymorphism.

Table 1. Clinicopathologic characteristics of prostate cancer patients included in this study and univariate analysis of factors that predicted castration resistance and all-cause mortality after ADT

	N (%) *	No. of Events	CRPCa		No. of Events	ACM	
			aHR (95%CI)	P		aHR (95%CI)	P
Age at diagnosis, years,							
Mean (SD)	69.5(7.7)		0.99 (0.97-1.01)	0.214		1.05 (1.02-1.07)	< 0.0001
PSA at diagnosis							
< 20 ng/mL	235 (51)	75	Referent		57	Referent	
≥ 20 ng/mL	224 (49)	134	1.94 (1.46-2.59)	< 0.0001	89	1.58 (1.13-2.22)	0.008
Clinical stage							
Localized (T1-2)	171 (38)	35	Referent		30	Referent	
Advanced (T3-4)	282 (62)	173	3.68 (2.53-5.33)	< 0.0001	118	2.76 (1.83-4.17)	< 0.0001
Biopsy Gleason score							
≤ 7 (3+4)	220 (49)	75	Referent		47	Referent	
≥ 7 (4+3)	229 (51)	124	2.51 (1.86-3.39)	< 0.0001	85	3.02 (2.08-4.39)	< 0.0001
Definitive therapy							
No	327 (68)	168	Referent		120	Referent	
RP/RT	156 (32)	56	0.57 (0.40-0.79)	0.001	34	0.77 (0.50-1.18)	0.228
Treatment modality							
aLHRH	102 (21)	42	Referent		16	Referent	
Combined ADT	381 (79)	182	1.10 (0.77-1.58)	0.598	138	2.57 (1.48-4.48)	0.001
Metastases at ADT initiation							
No	297 (69)	111	Referent		79	Referent	
Yes	131 (31)	94	3.52 (2.64-4.70)	< 0.0001	67	2.96 (2.13-4.12)	< 0.0001
Resistance to hormonal castration							
No	259 (54)	--	---		26	Referent	
Yes	224 (46)	--	---	---	126	5.55 (3.63-8.49)	< 0.0001

* Column subtotals do not sum to 483 due to missing data. ACM, all-cause mortality; CRPCa, castration-resistant prostate cancer; ADT, androgen deprivation therapy; RP, radical prostatectomy; RT, radiotherapy; PSA, prostate specific antigen; aLHRH, agonist luteinizing hormone releasing hormone. aHR, age-adjusted hazard ratio; 95%CI, 95% confidence interval. P<0.05 are in boldface.

Table 2. Association between adipokine pathway SNPs and time-to-CRPCa following ADT

	Adjusted HR (95%CI)	P
Metastasis at ADT initiation		
No	Referent	
Yes	2.61 (1.80-3.79)	< 0.0001
Clinical stage		
Localized	Referent	
Advanced	2.40 (1.52-3.78)	< 0.0001
<i>ADIPOQ</i> + 45		
T carrier	Referent	
GG	4.14 (1.63-10.56)	0.003
<i>ADIPOQ</i> +276		
T carrier	Referent	
GG	0.70 (0.50-0.97)	0.034
TNFA activation		
Low/intermediate	Referent	
High	0.62 (0.41-0.93)	0.022

ADT, androgen deprivation therapy; CRPCa, castration-resistant prostate cancer; SNP, single nucleotide polymorphism; TNFA, tumor necrosis factor alpha; *ADIPOQ*, adiponectin gene; HR(95%CI), hazard ratio and 95% confidence interval. Covariates included in multivariate model: PSA at diagnosis (<20 vs. ≥20 ng/mL); clinical stage (localized vs. advanced); Gleason (<3+4 vs. ≥4+3) ; definitive therapy (RP/RT vs. No); metastasis at ADT initiation (Yes vs. No); *VEGF*+405; *VEGF*-460; *IL6*-572; *IL6R*; *IL6ST*; *IL6* activation LI_H; *ADIPOQ* +45; *ADIPOQ* +276; *ADIPOQ* haplotype; *LEPR*223; *LEP* activation; *TNFA*-863; *TNFA* activation LI_H; *TNFA* expression L_IH.

Table 3. Association between adipokine pathways and time to all-cause mortality following ADT

	Adjusted HR (95%CI)	P
Age at diagnosis, years	1.06 (1.03-1.10)	< 0.0001
Gleason score		
≤ 7 (3+4)	Referent	
≥ 7 (4+3)	1.82 (1.20-2.78)	0.005
Metastasis at ADT initiation		
No	Referent	
Yes	1.86 (1.23-2.80)	0.003
Resistance to hormonal castration		
No	Referent	
Yes	4.70 (2.80-7.90)	< 0.0001
<i>ADIPOQ</i> + 45		
T carrier	Referent	
GG	5.20 (1.82-14.86)	0.002
<i>PPARD</i>		
T carrier	Referent	
CC	0.25 (0.08-0.81)	0.021
<i>IL6R</i>		
CC	Referent	
A carrier	0.61 (0.37-0.99)	0.047

ADT, androgen deprivation therapy; SNP, single nucleotide polymorphism; *PPARD*, peroxisome-proliferator activated receptor delta; *ADIPOQ*, adiponectin gene; *IL6R*, interleukin 6 receptor; HR(95%CI), hazard ratio and 95% confidence interval. Covariates included in multivariate model: Age at diagnosis (continuous); PSA at diagnosis (<20 vs. ≥20 ng/mL); clinical stage (localized vs. advanced); Gleason (<3+4 vs. ≥4+3); treatment modality (aLHRH vs. combined ADT); metastasis at ADT initiation (Yes vs. No); resistance to hormonal castration (Yes vs. No); *VEGF*+405/-460 activation LI_H; *IL6*-572; *IL6R*; *ADIPOQ* +45; *ADIPOQ* +276; *LEPR*223; *PPARD*.

Table 4. SNPs in adipokine pathways and risk for developing distant metastasis using logistic regression

	OR (95%CI)	P
Clinical stage		
Localized	Referent	
Advanced	10.13 (5.48-18.75)	< 0.0001
Gleason score		
≤ 7 (3+4)	Referent	
≥ 7 (4+3)	1.83 (1.08-3.11)	0.025
<i>LEPR</i> 223		
AA	Referent	
G carriers	2.09 (1.21-3.62)	0.008

SNP, single nucleotide polymorphism; *LEPR*, leptin receptor; OR (95%CI), odds ratio and 95% confidence interval. Covariates included in multivariate model: Age at diagnosis (continuous); PSA at diagnosis (<20 vs. ≥20 ng/mL); clinical stage (localized vs. advanced); Gleason (<3+4 vs. ≥4+3); *VEGF*-460; *VEGF* activation +405/-460 LI_H; *IL6*-6331; *LEP*; *LEPR* 109; *LEPR* 223; *PPARD*; *IRS1*; *TNFSFR1*; *LEPR* signaling L_IH; TNFA activation L_IH.

Figure 1

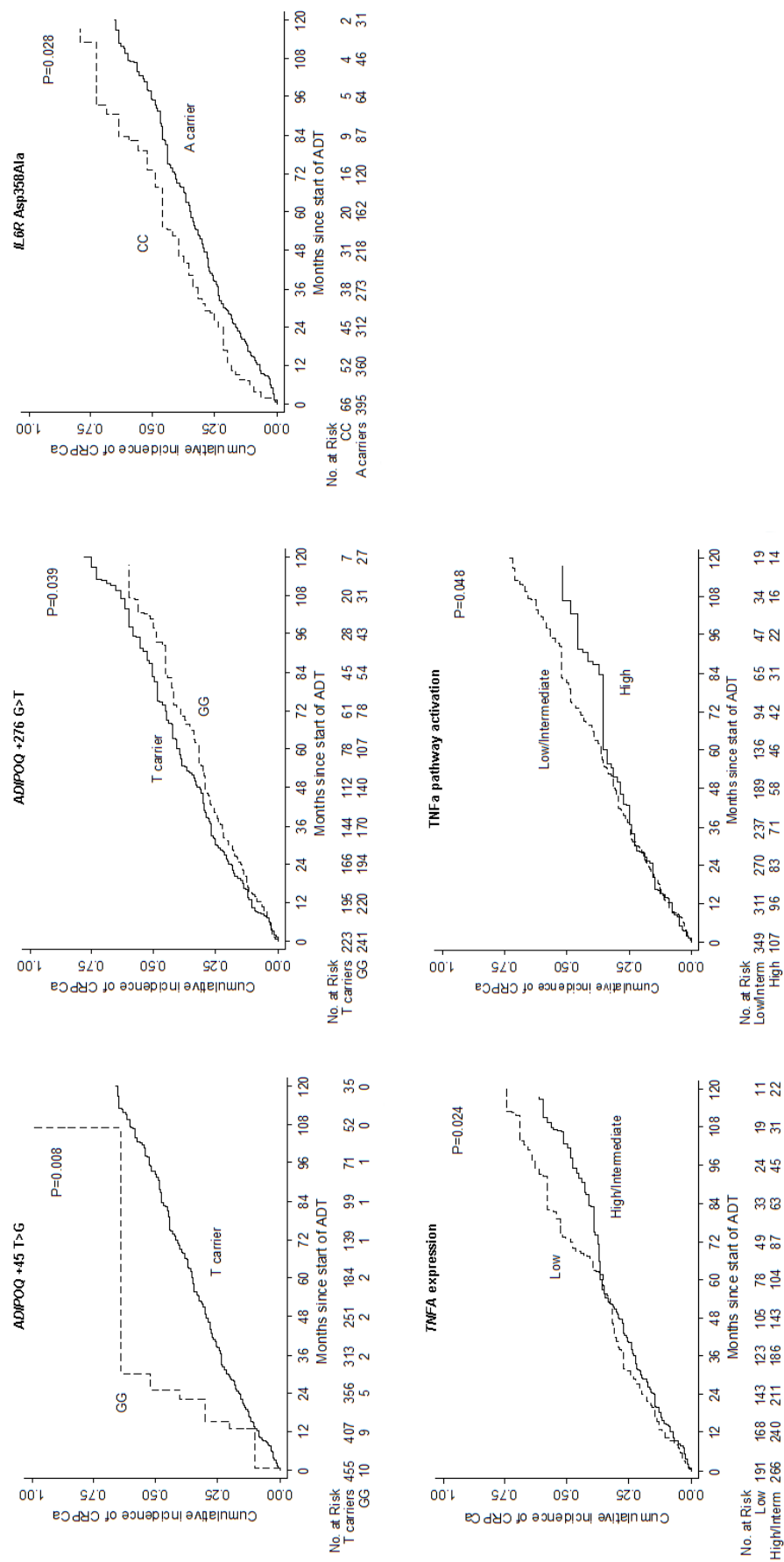
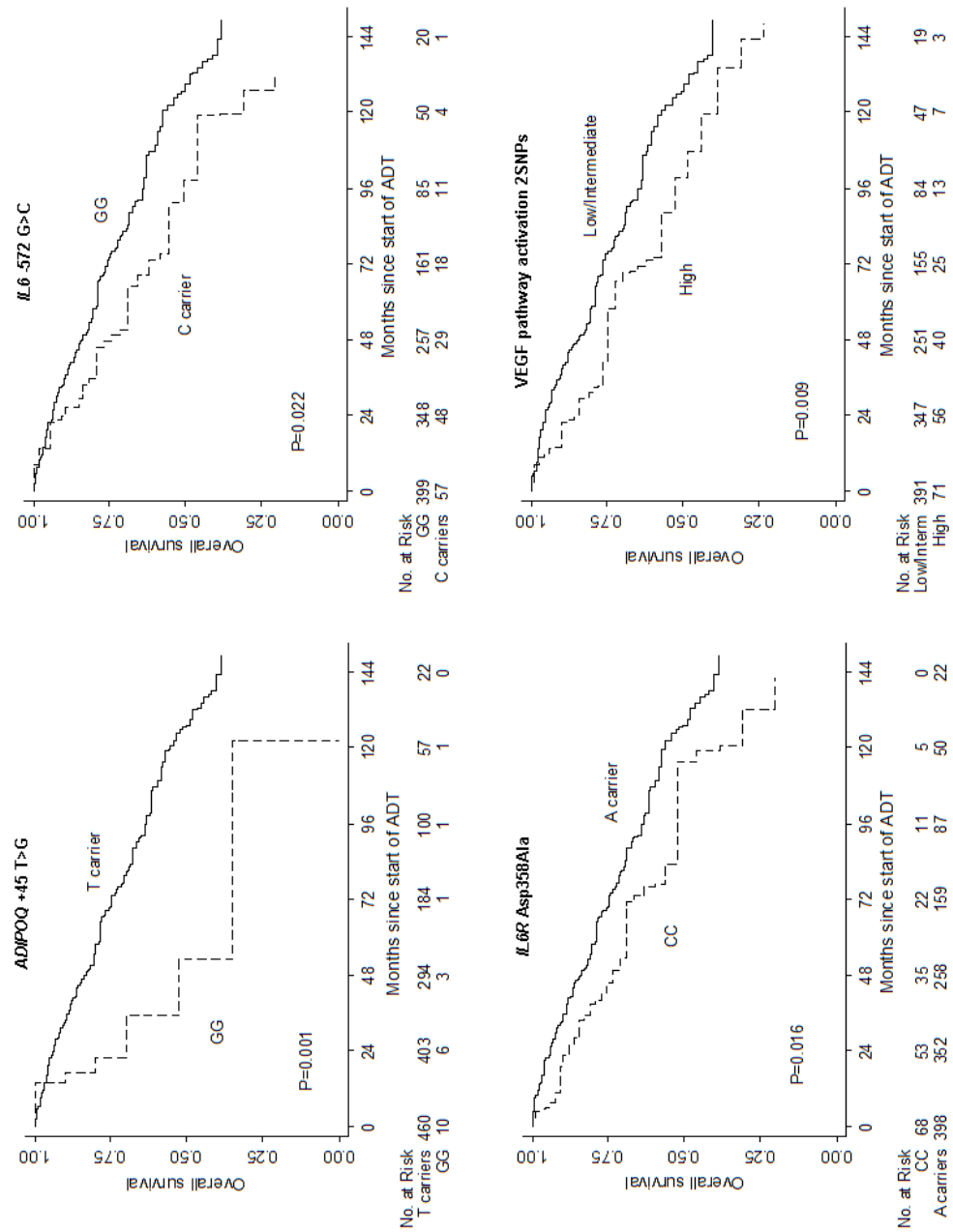


Figure 2



PAPER IX

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

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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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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 (TNF α), 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.

Material 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 μ M, panthotenate 18 μ M, ascorbate 100 μ M 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 μ m cell strainer (BD Falcon, BD Biosciences). After washing and erythrocyte lysis, SVFs were resuspended and seeded (500 μ L 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 37°C 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 TNF α , 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. ^aMean (95% confidence interval), ^bPercentage 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 TNF α , 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 μ g/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 CaCl₂). Gels were stained in a solution with Coomassie 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).

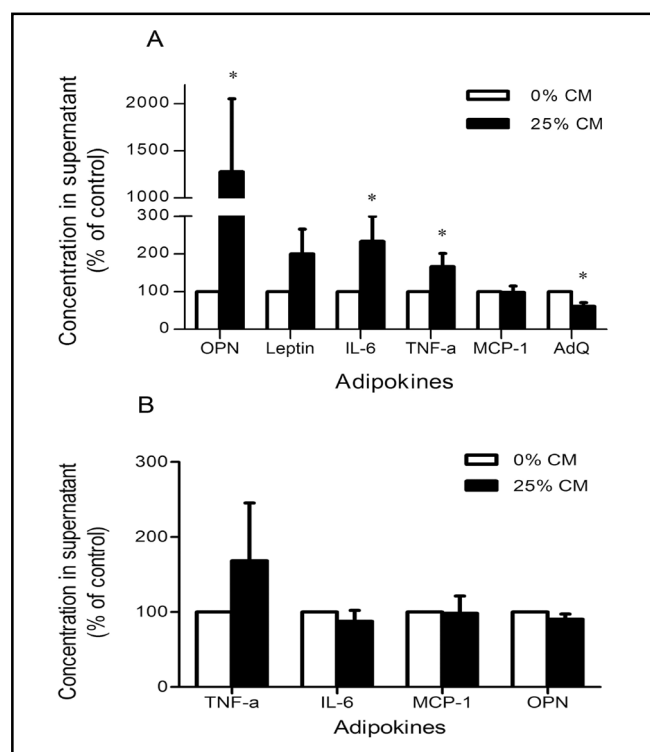


Fig. 1. Percent increase in adipokines concentration measured in supernatants of adipose tissue explants (A) and stromal vascular fraction (SVF) (B), after stimulation with conditioned medium (CM) from PC3 prostate tumor cells (mean $\pm$ SE). OPN, osteopontin; IL-6, interleukin-6; TNF α , tumoral necrosis 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 $\pm$ 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 log₁₀-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 TNF α 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

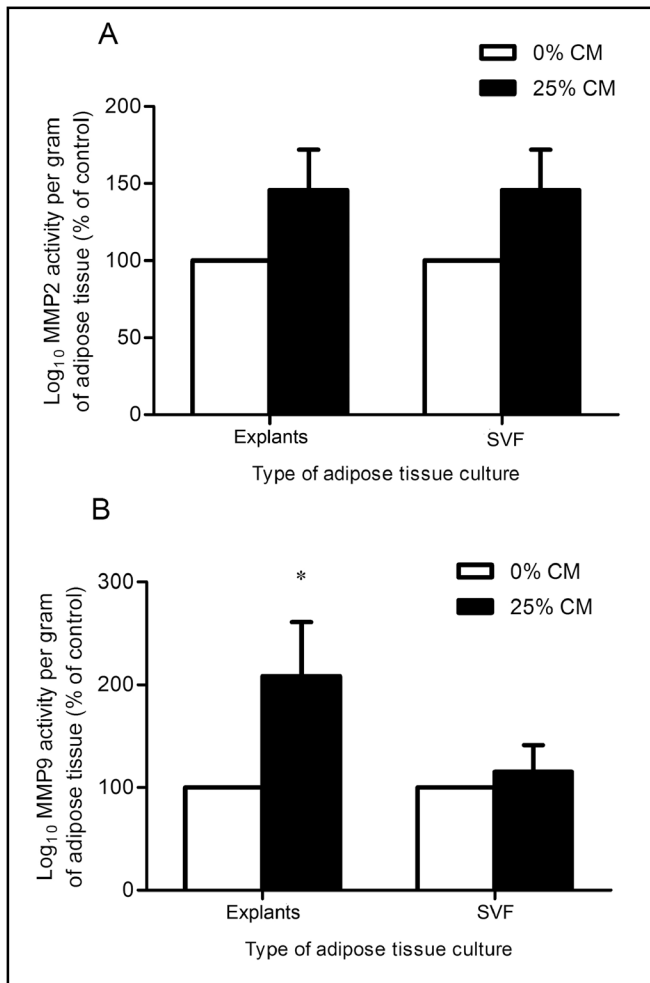


Fig. 2. Log-transformed (Log_{10}) matrix metallo-proteinase-2 (MMP-2) (A) and matrix metalloproteinase-9 (MMP-9) (B) activity per gram of tissue in adipose tissue explants and in stromal vascular fraction (SVF) after stimulation with PC-3 tumor cells conditioned medium (CM) (mean $\pm$ SE). * $p < 0.05$, 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 TNF α , IL-6 and OPN in PPAT in overweight/obese group (data not shown). Interestingly, following treatment with PC3 CM, the expression of TNF α , 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

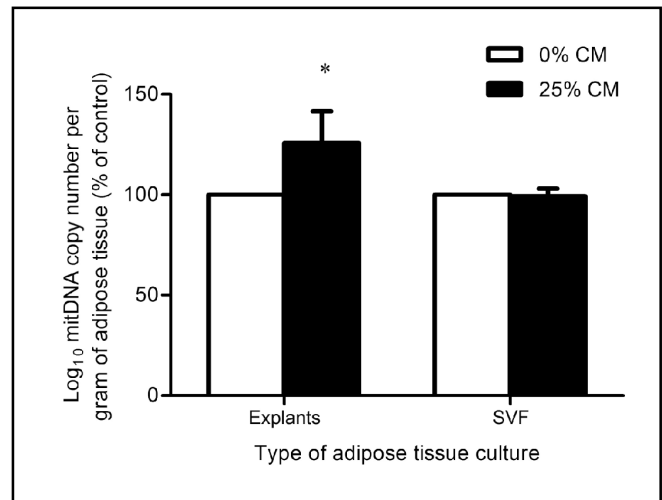


Fig. 3. Log-transformed mitochondrial DNA (Log_{10} mitDNA) copy number adjusted per gram of adipose tissue in adipose tissue explants and stromal vascular fraction (SVF) after stimulation with conditioned medium (CM) from PC3 prostate cancer cell line (mean $\pm$ SE). * $p < 0.05$, compared with control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

(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 TNF α , 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 TNF α 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 orchestrated 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 (TNF α , 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 hypertrophy), 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, TNF α , 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, TNF α , 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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References

- 1 Hsing AW, Sakoda LC, Chua S Jr: Obesity, metabolic syndrome, and prostate cancer. *Am J Clin Nutr* 2007;86:s843-857.
- 2 Cao Y, Ma J: Body mass index, prostate cancer-specific mortality, and biochemical recurrence: a systematic review and meta-analysis. *Cancer Prev Res (Phila)* 2011;4:486-501.
- 3 Baillargeon J, Rose DP: Obesity, adipokines, and prostate cancer (review). *Int J Oncol* 2006;28:737-745.
- 4 Mistry T, Digby JE, Desai KM, Randeve HS: Obesity and prostate cancer: a role for adipokines. *Eur Urol* 2007;52:46-53.
- 5 Hirano T, Morii H, Nakazawa K, Murakami D, Yamashita A, Asahi J, Orimo H, Tamae K, Tokura Y: Enhancement of adipogenesis induction by conditioned media obtained from cancer cells. *Cancer Lett* 2008;268:286-294.
- 6 Andarawewa KL, Motrescu ER, Chenard MP, Gansmuller A, Stoll I, Tomasetto C, Rio MC: Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. *Cancer Res* 2005;65:10862-10871.
- 7 Jotzu C, Alt E, Welte G, Li J, Hennessy BT, Devarajan E, Krishnappa S, Pinilla S, Droll L, Song YH: Adipose tissue derived stem cells differentiate into carcinoma-associated fibroblast-like cells under the influence of tumor derived factors. *Cell Oncol (Dordr)* 2011;34:55-67.
- 8 Dolinkova M, Dostalova I, Lacinova Z, Michalsky D, Haluzikova D, Mraz M, et al. The endocrine profile of subcutaneous and visceral adipose tissue of obese patients. *Mol Cell Endocrinol* 2008;291:63-70.
- 9 van Roermund JG, Hinnen KA, Tolman CJ, Bol GH, Witjes JA, Bosch JL, Kiemeny LA, van Vulpen M: Periprostatic fat correlates with tumour aggressiveness in prostate cancer patients. *BJU Int* 2011;107:1775-1779.
- 10 Sumitomo M, Asakuma J, Yoshii H, Sato A, Horiguchi A, Ito K, Nagakura K, Asano T: Anterior perirectal fat tissue thickness is a strong predictor of recurrence after high-intensity focused ultrasound for prostate cancer. *Int J Urol* 2010;17:776-782.
- 11 Finley DS, Calvert VS, Inokuchi J, Lau A, Narula N, Petricoin EF, Zaldivar F, Santos R, Tyson DR, Ornstein DK: Periprostatic adipose tissue as a modulator of prostate cancer aggressiveness. *J Urol* 2009;182:1621-1627.
- 12 Thalmann S, Juge-Aubry CE, Meier CA: Explant cultures of white adipose tissue. *Methods Mol Biol* 2008;456:195-199.
- 13 Song GJ, Lewis V: Mitochondrial DNA integrity and copy number in sperm from infertile men. *Fertil Steril* 2008;90:2238-2244.
- 14 Park J, Euhus DM, Scherer PE: Paracrine and Endocrine Effects of Adipose Tissue on Cancer Development and Progression. *Endocr Rev* 2011;in press.
- 15 George DJ, Halabi S, Shepard TF, Sanford B, Vogelzang NJ, Small EJ, Kantoff PW: The prognostic significance of plasma interleukin-6 levels in patients with metastatic hormone-refractory prostate cancer: results from cancer and leukemia group B 9480. *Clin Cancer Res* 2005;11:1815-1820.
- 16 Michalaki V, Syrigos K, Charles P, Waxman J: Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer. *Br J Cancer* 2004;90:2312-2316.
- 17 Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, Wang YY, Meulle A, Salles B, Le Gondec S, Garrido I, Escourrou G, Valet P, Muller C: Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res* 2011;71:2455-2465.
- 18 Culig Z: New insights into the role of interleukin-6 in human prostate cancer. *J Urol* 2009;182:1255-1256.
- 19 Chun JY, Nadiminty N, Dutt S, Lou W, Yang JC, Kung HJ, Evans CP, Gao AC: Interleukin-6 regulates androgen synthesis in prostate cancer cells. *Clin Cancer Res* 2009;15:4815-4822.
- 20 Weber GF, Lett GS, Haubein NC: Osteopontin is a marker for cancer aggressiveness and patient survival. *Br J Cancer* 2010;103:861-869.
- 21 Thalmann GN, Sikes RA, Devoll RE, Kiefer JA, Markwalder R, Klima I, Farach-Carson CM, Studer UE, Chung LW: Osteopontin: possible role in prostate cancer progression. *Clin Cancer Res* 1999;5:2271-2277.
- 22 McAllister SS, Gifford AM, Greiner AL, Kelleher SP, Saelzler MP, Ince TA, Reinhardt F, Harris LN, Hylander BL, Repasky EA, Weinberg RA: Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell* 2008;133:994-1005.
- 23 Balkwill F: TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev* 2006;25:409-416.
- 24 Yan B, Wang H, Rabbani ZN, Zhao Y, Li W, Yuan Y, Dewhirst MW, Li CY: Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. *Cancer Res* 2006;66:11565-11570.
- 25 Bates RC, Mercurio AM: Tumor necrosis factor-alpha stimulates the epithelial-to-mesenchymal transition of human colonic organoids. *Mol Biol Cell* 2003;14:1790-1800.
- 26 Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 2001;7:941-946.
- 27 Li H, Stampfer MJ, Mucci L, Rifai N, Qiu W, Kurth T, Ma J: A 25-year prospective study of plasma adiponectin and leptin concentrations and prostate cancer risk and survival. *Clin Chem* 2010;56:34-43.
- 28 Ribeiro R, Lopes C, Medeiros R: The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives. *Prostate Cancer Prostatic Dis* 2006;9:19-24.
- 29 Vaday GG, Peehl DM, Kadam PA, Lawrence DM: Expression of CCL5 (RANTES) and CCR5 in prostate cancer. *Prostate* 2006;66:124-134.
- 30 Kessenbrock K, Plaks V, Werb Z: Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 2010;141:52-67.
- 31 Bouloumie A, Sengenès C, Portolan G, Galitzky J, Lafontan M: Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. *Diabetes* 2001;50:2080-2086.
- 32 Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D: Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2000;2:737-744.
- 33 Lin CY, Tsai PH, Kandaswami CC, Lee PP, Huang CJ, Hwang JJ, Lee MT: Matrix metalloproteinase-9 cooperates with transcription factor Snail to induce epithelial-mesenchymal transition. *Cancer Sci* 2011;102:815-827.
- 34 Chakrabarti S, Patel KD: Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. *Exp Lung Res* 2005;31:599-621.
- 35 Sehgal G, Hua J, Bernhard EJ, Sehgal I, Thompson TC, Muschel RJ: Requirement for matrix metalloproteinase-9 (gelatinase B) expression in metastasis by murine prostate carcinoma. *Am J Pathol* 1998;152:591-596.

- 36 Johnson TR, Koul S, Kumar B, Khandrika L, Venezia S, Maroni PD, Meacham RB, Koul HK: Loss of PDEF, a prostate-derived Ets factor is associated with aggressive phenotype of prostate cancer: regulation of MMP 9 by PDEF. *Mol Cancer* 2010;9:148.
- 37 Trudel D, Fradet Y, Meyer F, Tetu B: Matrix metalloproteinase 9 is associated with Gleason score in prostate cancer but not with prognosis. *Hum Pathol* 2010;41:1694-1701.
- 38 Zhang L, Shi J, Feng J, Klocker H, Lee C, Zhang J: Type IV collagenase (matrix metalloproteinase-2 and -9) in prostate cancer. *Prostate Cancer Prostatic Dis* 2004;7:327-332.
- 39 Zhong WD, Han ZD, He HC, Bi XC, Dai QS, Zhu G, Ye YK, Liang YX, Qin WJ, Zhang Z, Zeng GH, Chen ZN: CD147, MMP-1, MMP-2 and MMP-9 protein expression as significant prognostic factors in human prostate cancer. *Oncology* 2008;75:230-236.
- 40 Sauer CG, Kappeler A, Spath M, Kaden JJ, Michel MS, Mayer D, Bleyl U, Grobholz R: Expression and activity of matrix metalloproteinases-2 and -9 in serum, core needle biopsies and tissue specimens of prostate cancer patients. *Virchows Arch* 2004;444:518-526.
- 41 Opdenakker G, Masure S, Grillet B, Van Damme J: Cytokine-mediated regulation of human leukocyte gelatinases and role in arthritis. *Lymphokine Cytokine Res* 1991;10:317-324.
- 42 Unal R, Yao-Borengasser A, Varma V, Rasouli N, Labbate C, Kern PA, Ranganathan G: Matrix metalloproteinase-9 is increased in obese subjects and decreases in response to pioglitazone. *J Clin Endocrinol Metab* 2010;95:2993-3001.
- 43 Catalan V, Gomez-Ambrosi J, Rodriguez A, Ramirez B, Silva C, Rotellar F, Gil MJ, Cienfuegos JA, Salvador J, Frühbeck G: Increased adipose tissue expression of lipocalin-2 in obesity is related to inflammation and matrix metalloproteinase-2 and metalloproteinase-9 activities in humans. *J Mol Med (Berl)* 2009;87:803-813.
- 44 Villarroja J, Giralt M, Villarroja F: Mitochondrial DNA: an up-and-coming actor in white adipose tissue pathophysiology. *Obesity (Silver Spring)* 2009;17:1814-1820.
- 45 Robin ED, Wong R: Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J Cell Physiol* 1988;136:507-513.
- 46 Kaaman M, Sparks LM, van Harmelen V, Smith SR, Sjolín E, Dahlman I, Arner P: Strong association between mitochondrial DNA copy number and lipogenesis in human white adipose tissue. *Diabetologia* 2007;50:2526-2533.
- 47 Yamauchi T, Kamon J, Waki H, Murakami K, Motojima K, Komeda K, Ide T, Kubota N, Terauchi Y, Tobe K, Miki H, Tsuchida A, Akanuma Y, Nagai R, Kimura S, Kadowaki T: The mechanisms by which both heterozygous peroxisome proliferator-activated receptor gamma (PPARgamma) deficiency and PPARgamma agonist improve insulin resistance. *J Biol Chem* 2001;276:41245-41254.
- 48 Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloso S, Czech M, Corvera S: Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* 2003;23:1085-1094.
- 49 Ducluzeau PH, Priou M, Weitheimer M, Flamment M, Duluc L, Iacobazi F, Soletti R, Simard G, Durand A, Rieusset J, Andriantsitohaina R, Malthiery Y: Dynamic regulation of mitochondrial network and oxidative functions during 3T3-L1 fat cell differentiation. *J Physiol Biochem* 2011;67:285-296.
- 50 Bogacka I, Ukropcova B, McNeil M, Gimble JM, Smith SR: Structural and functional consequences of mitochondrial biogenesis in human adipocytes in vitro. *J Clin Endocrinol Metab* 2005;90:6650-6656.
- 51 Toda S, Uchihashi K, Aoki S, Sonoda E, Yamasaki F, Piao M, Ootani A, Yonemitsu N, Sugihara H: Adipose tissue-organotypic culture system as a promising model for studying adipose tissue biology and regeneration. *Organogenesis* 2009;5:50-56.
- 52 Tuxhorn JA, Ayala GE, Rowley DR: Reactive stroma in prostate cancer progression. *J Urol* 2001;166:2472-2483.

PAPER X

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Human periprostatic adipose tissue promotes prostate cancer aggressiveness *in vitro*

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Abstract

Background

Obesity is associated with prostate cancer aggressiveness and mortality. The contribution of periprostatic adipose tissue, which is often infiltrated by malignant cells, to cancer progression is largely unknown. Thus, this study aimed to determine if periprostatic adipose tissue is linked with aggressive tumor biology in prostate cancer.

Methods

Supernatants of whole adipose tissue (explants) or stromal vascular fraction (SVF) from paired fat samples of periprostatic (PP) and pre-peritoneal visceral (VIS) anatomic origin from different donors were prepared and analyzed for matrix metalloproteinases (MMPs) 2 and 9 activity. The effects of those conditioned media (CM) on growth and migration of hormone-refractory (PC-3) and hormone-sensitive (LNCaP) prostate cancer cells were measured.

Results

We show here that PP adipose tissue of overweight men has higher MMP9 activity in comparison with normal subjects. The observed increased activities of both MMP2 and MMP9 in PP whole adipose tissue explants, likely reveal the contribution of adipocytes plus stromal-vascular fraction (SVF) as opposed to SVF alone. MMP2 activity was higher for PP when compared to VIS adipose tissue. When PC-3 cells were stimulated with CM from PP adipose tissue explants, increased proliferative and migratory capacities were observed, but not in the presence of SVF. Conversely, when LNCaP cells were stimulated with PP explants CM, we found enhanced motility despite the inhibition of proliferation, whereas CM derived from SVF increased both cell proliferation and motility. Explants culture and using adipose tissue of PP origin are most effective in promoting proliferation and migration of PC-3 cells, as respectively compared with SVF culture and using adipose tissue of VIS origin. In LNCaP cells, while explants CM cause increased migration compared to SVF, the use of PP adipose tissue to generate CM result in the increase of both cellular proliferation and migration.

Conclusions

Our findings suggest that the PP depot has the potential to modulate extra-prostatic tumor cells' microenvironment through increased MMPs activity and to promote prostate cancer cell survival and migration. Adipocyte-derived factors likely have a relevant proliferative and motile role.

Keywords

Adipose tissue, Cell line, Cell proliferation, Cell tracking, Obesity, Periprostatic, Prostate cancer

Background

In recent years substantial evidence has been provided for the linkage between adipose tissue dysfunction and cancer progression [1,2]. Excess accumulation of adipose tissue corresponds by definition to obesity, which has been associated with prostate cancer aggressiveness [3,4].

In prostate cancer, the extra-capsular extension of cancer cells into the periprostatic (PP) fat is a pathological factor related with worst prognosis [5]. It is now well established that the interactions between non-tumor cells in the microenvironment and the tumor cells are decisive of whether cancer cells progress towards metastasis or whether they remain dormant [6].

Prostate cancer cells generated within prostatic acini frequently infiltrate and even surpass the prostatic capsule, therefore interacting with the surrounding PP adipose tissue. Previous work showed that such adipose tissue has the potential to modulate prostate cancer aggressiveness, through the increased production of adipokines, namely interleukin 6 (IL-6) [7]. Moreover, a recent report showed an association of PP adipose tissue thickness with prostate cancer severity [8].

Different studies have demonstrated the critical influence of adipose tissue-derived factors in cancer cells [9-11], including prostate tumor cells [12-14]. Together, these reports indicate that factors produced by adipose tissue, particularly adipocytes may stimulate the progression of cancer cells. However, to our knowledge, the influence of PP adipose tissue-derived factors on prostate cancer cells has not been exploited. Noteworthy, we previously observed that prostate cancer induced the increase of PP adipose metabolic activity, promoting a favorable environment for aggressive tumor biology [15].

To address these issues, we first studied the gelatinolytic profile of PP whole adipose tissue and its respective stromal-vascular fraction. Next, we used PP adipose tissue-derived conditioned medium to analyze *in vitro* its influence in proliferation and migration of prostate cancer cells.

Methods

Patients and collection of human PP adipose tissue

Men diagnosed with clinically localized prostate cancer or nodular prostatic hyperplasia (BPH) and eligible for retropubic radical prostatectomy or prostate surgery of nodular hyperplasia, without other major co-morbidities, were included in this study after informed consent agreement. The project was approved by the ethics committees of the participating Hospitals. Human anterior-lateral PP and pre-peritoneal visceral (VIS) samples of adipose tissue were collected during surgery and immediately processed.

Adipose tissue primary cultures and preparation conditioned media (CM)

PP and VIS adipose tissue fragments were processed to primary whole adipose tissue (explants) cultures using a modified protocol from Thalmann et al. [16]. Briefly, after incubation of explants (0.3 g/mL) for 16 hours in DMEM/F12 (Gibco) medium, supplemented with biotin 16 μ M (Sigma Aldrich), panthotenate 18 μ M (Sigma Aldrich), ascorbate 100 μ M (Sigma Aldrich), and 1% penicillin-streptomycin (Sigma Aldrich) (sDMEM/F12), fresh medium was added, and was referred to as time zero for time-course experiments. Explant cultures were maintained at 37°C and 5% CO₂. After 48 hours, the supernatant was collected, centrifuged (20 000 g, 3 minutes), aliquoted and stored at -80°C as explant conditioned medium (CM).

Other pieces of VIS and PP adipose tissue were incubated with collagenase (2 mg/mL) (Collagenase A, Roche) for 60 minutes at 37°C with agitation (120 rpm). After removal of adipocytes layer, the supernatant was discarded and the stromal-vascular fraction (SVF) cell pellet resuspended in sDMEM/F-12 with 10% Newborn Calf Serum (NCS) (Sigma Aldrich) and filtered through a 40 μ m cell strainer (BD Falcon, BD Biosciences). Following erythrocyte lysis (Buffer EL, QIAgen), SVFs were resuspended and seeded (500 μ L of cell

suspension) in wells coated with 0.2% gelatin (Sigma Aldrich) in sDMEM/F-12 medium with 10% NCS. Stromal-vascular fraction cells were maintained at 37°C and 5% CO₂. After 48 hours, fresh medium free from NCS was added. Forty-eight hours after this time-point CM was collected, centrifuged at 20 000 g for 3 minutes and the supernatant stored at –80°C as SVF CM.

Human PC-3 and LNCaP cell lines

PC-3 and LNCaP cell lines were obtained from the European Collection of Cell Cultures (ECCAC) and from the American Type Cell Culture (ATCC), respectively. Both cell lines were maintained in RPMI 1640 medium, supplemented with (%) L-glutamine and (%) Hepes (Gibco), 10% FBS (Gibco) and 1% PS (Sigma Aldrich), at 37°C with 5% CO₂.

Cell proliferation

Cancer cells were seeded into 96-well plates (5×10^3 and 10×10^3 cells/well for PC-3 and LNCaP cells, respectively) and incubated for 24 hours in RPMI 1640 medium with 10% FBS. Next, supernatant was removed and new cell medium free from FBS, with (50% volume) or without (control) adipose tissue-derived conditioned medium was added to cancer cells.

Media was removed after 24 hours, and cells were stored at –80°C. Then, the pellet was solubilized in a lysis buffer supplemented with a DNA-binding dye (CyQUANT cell proliferation assay, Invitrogen). DNA content was evaluated in each well by fluorimetry at 480/535 nm using a standard curve previously generated for each cell type, after plotting measured fluorescence values in samples vs cell number, as determined from cell suspensions using a hemocytometer. Samples were performed in duplicate and the mean value used for analyses.

Zymography

Gelatinolytic activities of MMP2 and MMP9 of supernatants from adipose tissue primary cultures were determined on substrate impregnated gels. Briefly, total protein from supernatants of primary cultures of adipose tissue (12 µg/well), were separated on 10% SDS-PAGE gels containing 0.1% gelatin (Sigma-Aldrich). After electrophoresis a 30 minutes washing step (2% Triton X-100) was performed, and gels were incubated 16–18 h at 37°C in substrate buffer (50 mM Tris–HCl, pH7.5, 10 mM CaCl₂), to allow MMP reactivation. Next, gels were stained in a solution with Coomassie Brilliant Blue R-250 (Sigma-Aldrich), 40% methanol and 10% acetic acid for 30 minutes. The correspondent MMP2 and MMP9 clear lysed bands were identified based on their molecular weight and measured with a densitometer (Quantity One, BioRad).

Cell tracking and analysis of cellular motility

For the time-lapse microscopy analysis (Zeiss Axiovert inverted-fluorescence microscope), exponentially growing cancer cells were seeded into 96-well plates at a density of 5×10^3 and 10×10^3 cells/well, for PC-3 and LNCaP, respectively. After 24 hours incubation in RPMI 1640 media supplemented with 10% FBS, supernatant was removed and new medium with (50% volume) or without (control, 0% CM) adipose tissue-derived conditioned medium, were added to cancer cells. At this time point the time-lapse experiment was started. A digital

image of the field of interest was taken every 15 minutes for 24 hours, generating 85 frames that were arranged into sequences in .avi format (Zeiss Axiovert software). Two fields were selected in each well. The nucleus of each cell was followed using manual tracking from the first to the last frame and results recorded (Zeiss LSM Image Browser version 3.2.0.70).

We used mean speed (MS) and final relative distance to the origin (FRDO) as indicators to characterize cell trajectory and motility. Mean cell speed corresponds to the total distance covered during the experiment, divided by the duration of the experiment, which was considered to be representative of cell motility [17]. To assess the distance the cell migrated since its origin to the end of the observation, we analyzed the linear distance between the initial and final cell position that allows the identification of the statistical trend of cells that randomly explore a large area.

Statistical analysis

Results are presented as mean $\pm$ S.E.M. Adequate adjustment of results per gram of adipose tissue were performed when comparing between the fractions and depots of adipose tissue. Normality was assessed by Kolmogorov-Smirnov test. Data for adipose tissue gelatinase activity, prostate cancer cell count and motility (final relative distance to origin), were log₁₀-transformed to become normally distributed, whether adjusted or not to adipose tissue weight. One-way ANOVA with between groups' post-hoc Scheffe test or post-hoc Dunnett test, and the independent samples *t*-test, were used as appropriate. Whenever means for different groups wanted to be compared and normality conditions were not satisfied we used the Kruskal-Wallis test followed by Mann Whitney test once a significant P was obtained or only Mann Whitney test.

Statistical analyses were performed with SPSS 17.0. Significance was accepted at P less than 0.05. Details of the statistical analyses were included in each figure legend.

Results

Some clinicopathological variables, including the body mass index (mean, 26.5 and 95% CI, 24.6–28.5 Kg/m²), age at diagnosis (mean, 63.9 and 95% CI, 60.1–67.7 years of age) and prostate specific antigen at diagnosis (mean, 8.2 and 95% CI, 5.3–11.2 ng/dL) presented low dispersion of values between subjects. In order to investigate the proteolytic profile of PP adipose tissue, we evaluated gelatinase activity in conditioned medium from culture of PP adipose tissue explants, according to age at diagnosis, body mass index (BMI), pathologic status and Gleason grade of donors (Table 1). MMP9 was significantly elevated in obese/overweight compared to normoponderal subjects (P = 0.036).

Table 1 Gelatinase activity in conditioned medium from primary cultures of periprostatic (PP) adipose tissue explants, according to clinical and pathological characteristics

		MMPs activity in supernatant of PP adipose tissue explant cultures (A.U.)			
		Demographics	MMP2	MMP9	
		n (%)	mean ± S.E.M.	P	mean ± S.E.M.
Age at diagnosis, yrs ^a	13 (52.0)	982.9±154.8	0.591	498.9±71.6	0.624
	< median (65.1)	12 (48.0)	878.7±111.2	558.3±93.6	
	≥ median (65.1)				
BMI, Kg/m ² ^b	11 (44.0)	895.4±135.3	0.739	392.1±48.3	0.036
	< 25	14 (56.0)	960.3±134.4	635.8±87.5	
	≥ 25				
Pathologic status ^b	5 (20.0)	958.6±97.0	0.795	715.5±142.6	0.242
	BPH	14 (56.0)	873.8±150.2	461.9±68.1	
	PCa (< pT3)	6 (24.0)	1026.2±169.8	511.0±128.0	
	PCa (≥ pT3)				
Gleason grade ^a	8 (40.0)	930.7±189.5	0.967	477.0±94.9	0.987
	< 7	12 (60.0)	920.7±148.6	479.1±81.7	
	≥ 7				

Results from zymograms performed in supernatants of *in vitro* culture of PP adipose tissue explants (n = 25). ^a Independent samples *t*-test or ^b one-way ANOVA; A.U., arbitrary units; S.E.M., standard error of mean. MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9. BMI, body mass index. BPH, nodular prostatic hyperplasia; PCa, prostate cancer.

To understand which fraction of PP adipose tissue contributes to enhanced gelatinase activity, we analyzed paired explant and stromal-vascular fraction cultures from PP adipose tissue (Figure 1). Our results indicate that the proteolytic activity of both MMP2 and MMP9 is higher in cultures of adipose tissue explants than in the correspondent stromal-vascular fractions. A similar proteolytic pattern is present between explants and stromal-vascular fractions of VIS adipose tissue. Additionally, we observed that PP adipose tissues present higher MMP2 but not MMP9 activity, as compared with adipose tissue from a distinct anatomical fat depot (median pre-peritoneal visceral region) (Figure 1). Figure 2 depicts a representative image of zymogram findings.

Figure 1 Gelatinolytic activity of periprostatic (PP) adipose tissue and comparison with visceral pre-peritoneal fat depot. Analyses were performed in explants and stromal-vascular fraction primary culture of 21 samples of PP adipose tissue and 10 samples of VIS adipose tissue. Independent samples *t*-test was used. *** P < 0.0001 between explants and SVF fraction; * P < 0.05 in the comparison among fat depots. MMP, matrix metalloproteinase; VIS, visceral; PP, periprostatic; SVF, stromal-vascular fraction

Figure 2 MMP2 and MMP9 enzymatic activities in supernatants of whole adipose tissue and SVF fraction from VIS and PP depots. Representative bands corresponding to specific MMP2 and MMP9 are shown. Asterisks indicate active forms of MMP2 and MMP9 while arrows indicate the respective proforms. SVF, stromal-vascular fraction; PP, periprostatic; VIS, visceral; MMP, matrix metalloproteinase

Next, to examine whether soluble factors secreted by PP adipose tissue alter tumor cell behavior, its proliferative potential on an aggressive hormone-refractory prostate cancer cell line was investigated. We observed that factors secreted from explants of both PP and VIS adipose tissue increase proliferation of hormone-refractory prostate cancer cells, whereas only VIS SVF culture-derived factors stimulated proliferation (Figure 3A). The $\log_{10}$ -transformed PC-3 cell count per gram of adipose tissue, was significantly higher after stimulation with explants culture-derived CM compared with SVF, independently of the adipose tissue depot ($P < 0.0001$) (Figure 3B). Interestingly, the SVF-derived CM of PP adipose tissue had a stronger proliferative effect than SVFs of VIS origin ($P = 0.007$) (Figure 3B).

Figure 3 Influence of conditioned medium from distinct adipose tissue origins in the proliferation of PC-3 cells. Analyses were performed using conditioned medium of 21 samples of periprostatic (PP) and 10 samples of visceral (VIS) adipose tissue, after explants and stromal-vascular fraction primary cultures. A. Effect of adipose tissue-derived CM on PC-3 cell proliferation, in comparison with control (0% CM) (** $P < 0.01$ in relation with 0% CM, one-way ANOVA with two-sided post-hoc Dunnett test). B. PC-3 cell proliferation was normalized per gram of adipose tissue and compared according to fat depot and adipose tissue fraction (** $P < 0.01$ and *** $P < 0.0001$ between groups, independent samples t -test). CM, conditioned medium; PP, periprostatic; SVF, stromal-vascular fraction; VIS, visceral

The influence of PP adipose tissue secreted factors for cell proliferation of another less aggressive hormone-sensitive prostate cancer cell line was subsequently examined. Interestingly, while these cells also respond to the proliferative stimulus of CM from SVF fraction ($P < 0.0001$), an inhibitory effect in LNCaP cells was observed with explants CM ($P < 0.05$), independently of fat depot (Figure 4A). Comparisons between adipose tissue fractions, explants vs SVF-derived CM, in LNCaP cell proliferation were conducted using the logarithmically-transformed cell count per gram of adipose tissue (Figure 4B). For VIS but not PP adipose tissue, there was an increased influence of explants compared to SVF CM in LNCaP cell proliferation ($P < 0.0001$). Furthermore, when compared with VIS SVF CM, the SVF CM from PP adipose tissue increased LNCaP cell proliferation (Figure 4B).

Figure 4 Influence of conditioned medium from adipose tissue in the proliferation of LNCaP cells. Analyses were conducted using conditioned medium of periprostatic (PP) and visceral (VIS) adipose tissue from 10 subjects after explants and stromal-vascular fraction primary cultures. A. Influence of adipose tissue-derived CM in LNCaP cell proliferation, in comparison with control (0% CM) (* $P < 0.05$ and ** $P < 0.01$, relative to control, two-sided post-hoc Dunnett test). B. Comparison of the effect of CM from distinct adipose tissue depot and fractions in LNCaP proliferation after tissue weight normalization (** $P < 0.01$ and *** $P < 0.0001$ between groups, independent samples t -test). CM, conditioned medium. SVF, stromal-vascular fraction. PP, periprostatic; VIS, visceral

The enhanced proteolytic activity of PP and VIS adipose tissues led us to investigate their putative effect on prostate cancer cell motility. Therefore, the motile behavior of the PC-3 hormone-refractory and of the LNCaP hormone-sensitive prostate cancer cell lines were analyzed using adipose tissue samples from 4 additional subjects. In the first subject by subject analysis we observed that CM from any adipose tissue fraction or depot elicited, in comparison to untreated cells (control) increased motility, independently of donor's clinicopathological characteristics (data not shown). Figure 5 shows motile parameters of prostate cancer cells in response to adipose tissue CM. Comparing with control, LNCaP cells

stimulated with CM from any fraction or depot always resulted in higher mean speed and final relative distance to origin (FRDO) (Figure 5A). In PC-3 cells, while mean speed was higher for any CM condition compared with control, the FRDO was only increased after stimulation with CM from explants, both from PP and VIS depot (Figure 5B).

Figure 5 Motility of PC3 and LNCaP cells upon stimulation of adipose tissue-derived CM from explants and SVF. Influence of adipose tissue fractions in cell motility parameters. Data represent mean $\pm$ SE of at least 20 representative cell trajectories per each tested condition, with conditioned medium of primary adipose tissue cultures from four distinct subjects. Bars represent mean speed (MS) and plots the logarithmically transformed final relative distance to origin (FRDO). A. FRDO and MS of PC-3 cells (** $P < 0.0001$ relative to control). B. FRDO and MS of LNCaP cells (** $P < 0.01$ and *** $P < 0.0001$ relative to control). In the log-transformed FRDO we used one-way ANOVA with post-hoc Dunnett test (two-sided), whereas the mean speed was analyzed using Kruskal Wallis followed by Mann Whitney test. SVF, stromal-vascular fraction; PP, periprostatic; VIS, visceral

After adjustment of motility parameters to adipose tissue weight, in order to compare different culture types and depots, only the LNCaP cells mean speed was not statistically different between PP and VIS depot. Otherwise, motile parameters were higher after stimulation with CM from PP depot (Figure 6). For both PC-3 (Figure 6A) and LNCaP (Figure 6B) cells stimulated with explant-derived CM from PP and VIS adipose tissue, the mean speed and FRDO were significantly higher in comparison to SVF ($P < 0.0001$). Figure 7 shows a representative example of cell tracking in both cancer cell lines, using CM from PP adipose tissue.

Figure 6 Motility of PC-3 and LNCaP cells upon stimulation of adipose tissue-derived CM from explants and SVF. Data represent mean $\pm$ SE of at least 20 representative cell trajectories per each tested condition, from four distinct subjects. Bars represent mean speed (MS) per gram of adipose tissue and plots the logarithmically transformed final relative distance to origin per gram of adipose tissue (FRDO). A. FRDO and MS of PC-3 cells (* $P < 0.05$ and *** $P < 0.0001$ between treatment conditions). B. FRDO and MS of LNCaP cells (** $P < 0.01$ and *** $P < 0.0001$ between conditions). Analyses on MS were performed with Mann Whitney test, whereas FRDO was analyzed using independent samples *t*-test. SVF, stromal-vascular fraction; PP, periprostatic; VIS, visceral

Figure 7 Representative example of cell tracking and cancer cell trajectories after stimulation with periprostatic adipose tissue-derived CM. Sequential displacements of cells were captured by manual cell tracking and are represented as color lines. SVF, stromal-vascular fraction

Discussion

Prostate cancers frequently have a indolent course even if left without active treatment [18]. However, clinically relevant disease with significant morbidity and mortality also occurs in a significant number of patients [19]. The mechanisms responsible for this aggressive behavior remain elusive, albeit it is well established that the supporting tumor microenvironment has a decisive role in controlling prostate cancer growth, invasion and metastasis [20].

Cancer-implicated mammary and colonic fat pads [11,21] are physically close to epithelial cells, whereas in prostate there is initially a capsular-like structure separating the PP fat from tumor cells. Nevertheless, frequently prostate tumors infiltrate the PP fat pad by transposing or infiltrating the physical barriers, resulting in immediate proximity to adipose tissue. Once extension beyond the capsule occurs, the PP adipose tissue-secreted factors, extracellular matrix components or direct cell-cell contact may influence the phenotypic behavior of malignant cells. Recent studies observed that PP adipose tissue thickness was linked to prostate cancer severity [8], while its secretory profile associated with advanced disease [7]. In the present study, we found that PP adipose tissue-derived conditioned media may potentiate prostate cancer aggressiveness through modulation of metalloproteinases activity, and by promoting cancer cell proliferation and migration.

In tumors, cancer cells are not the only source of MMPs. In our study, MMP9 activity was significantly elevated in the PP adipose tissue of overweight/obese men ($\text{BMI} \geq 25 \text{ Kg/m}^2$), implying excess body fat and the PP fat depot in the modulation of extra-capsular cancer cells' microenvironment. Concordantly, other studies found MMP9 to be positively correlated with BMI [22]. Further research is warranted to uncover the effects of MMPs in association with distinct obesity grades. In our sample only two subjects presented $\text{BMI} > 30 \text{ Kg/m}^2$, limiting such approach.

Matrix metalloproteinases are proteolytic enzymes that regulate many cell mechanisms with prominence in cancer biology [23]. Their expression in prostate tumors is related with disease progression and metastasis [24], whereas MMP9 was shown to increase growth factors bioavailability and to elicit epithelial-to-mesenchymal transition in tumor cells [25,26], therefore promoting an aggressive phenotype. A recent report indicated that oesophageal tumors from obese patients express more MMP9 and that co-culture of VIS adipose tissue explants with tumor cells up-regulated MMP2 and MMP9 [27]. Remains undetermined the influence of PP adipose tissue in the expression of MMPs by prostate cancer cells, which might further contribute towards an aggressive phenotype. Noteworthy, cancer-derived factors stimulate other surrounding cells, including adipose tissue cells, to synthesize MMPs [15].

In an effort to understand if the effects of PP adipose tissue extend to other aggressiveness characteristics, we used adipose tissue-derived CM to perform cell proliferation assays in prostate cancer cell lines. We found that CM from *in vitro* culture of adipose tissue explants stimulated the proliferation of hormone-refractory prostate cancer cells. Conversely, this media inhibited growth in hormone-sensitive cells.

It is well-established that adipose tissue secretes a wide array of molecules [28]. These adipokines, exclusively or partially secreted by adipocytes or stromal-vascular fraction cells, are likely to have a role in modulating the risk of cancer progression [1,29,30]. Few studies examined the effect of adipocytes in prostate cancer cells growth [12,13]. While a proliferative effect was observed in hormone-refractory PC-3 cells, these findings didn't replicate in LNCaP cells [13]. In fact, the mitogenic and anti-apoptotic effects of several adipokines, alone and combined, in prostate cancer cell growth (e.g. leptin, IL-6, insulin-like growth factor 1, IGF-1), seems to be limited to hormone-refractory prostate cancer cells [12,31-34]. Previous studies also report on the suppression of LNCaP cell growth as response to adipokines (e.g. $\text{TNF-}\alpha$, decreased expression of vascular endothelial growth factor, VEGF), not observed in hormone-refractory cells [13,35-37].

Contrary to explants, CM from SVF cultures induces cancer cell proliferation, independently of cell line, except for the SVF from PP adipose tissue in PC-3 cells. Cells that constitute the SVF fraction of adipose tissue, where macrophages have a modulatory role, are known to secrete several angiogenic and antiapoptotic factors [38-40], which ultimately can impact prostate cancer cells growth. The lack of proliferative effect observed for the SVF fraction from PP adipose tissue may partially be due to the reported low number of macrophages in PP fat depot [7], diminishing the proliferative stimulus in prostate cancer cells.

Progression to an invasive and metastatic phenotype is responsible by prostate cancer mortality and morbidity. The increased cellular motility is another parameter associated with increased metastatic potential [41,42]. By employing time-lapsed imaging, we found that factors produced by whole adipose tissue cultures (explants) increased significantly the migration speed and the final relative distance to origin of both PC-3 and LNCaP cells compared with control. Only the SVF fraction-derived CM effect in the final relative distance to origin of PC-3 cells, was not increased compared with control.

The mechanisms involved in tumor cell movement are far from fully elucidated, although various biophysical processes are considered to be involved [41]: in order for a cell to move it must be polarized or have a sense of directionality; polarity is accompanied by 1) lamellipodia protrusion at the leading edge, followed by 2) detachment of the cell's rear end and subsequent 3) transcellular contractility. These mechanisms are modulated by the activation of several signaling pathways, such as PI3K, ERK/MAPK and c-Src tyrosine kinase [41], which are known downstream signals of adipokines [43]. In fact, many adipokines (e.g. IGF-1, osteopontin, leptin, adiponectin, VEGF, thrombospondin, interleukin-8 and IL-6) have been shown to modulate different steps of cell motile behavior [44-56]. The repetitive and coordinated cycling of these processes results in productive locomotion of the cell. Several key pathways and molecules involved in this process can be induced by factors secreted by adipose tissue, hence supporting the increased motility we found in stimulated prostate cancer cells. Nevertheless, besides the influence of extrinsic factors, migratory tumor cells also present autocrine growth factor signaling systems [57]. We disclose any potential bias from inadvertent selection using manual cell tracking analysis, urging careful interpretation of motility findings. Further studies using migration assays to extend and confirm our results are warranted.

Adipose tissue is a heterogeneous organ that consists of multiple cell types: adipocyte fraction, which contains lipid-loaded adipocytes, and stromal-vascular fraction, which includes preadipocytes, endothelial cells, fibroblasts, stem cells, macrophages and other immune cells [58]. The fractions of adipose tissue differ in that while explants reflect an organotypic cell culture system of whole adipose tissue, the major characteristic of stromal-vascular fraction culture is the depletion of adipocytes and absence of extracellular matrix. In order to investigate which fraction influenced tumor cells, we cultured paired explants and stromal-vascular fraction cells. To allow comparison between depots and adipose tissue fractions, the cell count was adjusted per gram of adipose tissue. Interestingly, our findings showed that media from explants and PP adipose tissue depot presented the higher gelatinolytic activity per gram of adipose tissue, compared with SVF cultures- and VIS adipose tissue-derived media. Although the amount of MMP9 has been described to be higher in stromal-vascular fraction of adipose tissue compared with adipocytes [22], the latter have greater plasticity to increase MMPs expression when interacting with other cells in adipose tissue [22,59]. The increased activity of metalloproteinases in CM from adipose tissue explants in culture compared with SVF, likely reflect the additive effect or interaction

between cells of the stromal-vascular fraction plus adipocytes. We found that MMP2 activity was increased in PP versus VIS adipose tissue supernatants. Although there is no evidence of MMP2 role in adipose tissue/cancer cells crosstalk, recent findings suggest MMP2 is up-regulated in tumor cells co-cultured with adipose tissue explants and that its expression and activation is modulated by several adipokines (e.g. Wdnl-like and visfatin) [27,60,61]. Additionally, other MMPs, notably MMP11, have been shown to be correlated with breast cancer-induced adipocyte's activated state [11,62]. If confirmed, our findings may reveal a novel specific proteinase expression and activity pattern in PP adipose tissue favorable to prostate cancer progression.

In this study, proliferation was increased with CM from PP and VIS explants versus SVF CM in PC-3 cells, whereas LNCaP cells only proliferated significantly more with VIS explants compared to VIS SVF. As the highest proliferation was seen following stimulation with CM from explants we speculate adipocytes may be the main effectors. Other studies also found a proliferative effect of adipocytes in prostate cancer cells [12,13]. Adipocytes add significantly to the proliferative effect in hormone-refractory prostate cancer cells, even though the adipokines responsible by these results have yet to be determined. Alternatively, since explants culture preserve the paracrine signals by maintaining the existing crosstalk among the different cell types [63], we hypothesize that the higher proliferative stimulus conferred by explants CM likely reflects a co-stimulatory and/or additive effect of adipokines produced by adipocytes and by the stromal vascular fraction cells.

Explants-derived CM, whether from VIS or PP origin exerted consistently, also across cell lines, an increased effect in migration speed and final relative distance to origin, when compared with SVF fraction. It is possible that explants CM, which reveal the secretory profile of adipocytes plus stromal-vascular cells, produce more motile factors and exclusive secretion of others (e.g. leptin and adiponectin), thereby resulting in increased total distance/mean speed and final relative distance to origin of prostate cancer cells.

The anatomical origin of adipose tissue accounts for increased gelatinolytic activity and different proliferative and migratory stimulus. CM from PP results in higher $\log_{10}$ -transformed PC-3 and LNCaP cell count per gram of adipose tissue, only when SVF CM was used. Furthermore, adipose tissue from PP origin exerted the stronger motile effect (of both analyzed parameters) in PC-3 cells compared to VIS depot, independently of the culture type. In LNCaP cells only the PP explants-derived CM didn't impact the mean speed more than CM from VIS explants. These findings suggest that VIS and PP fat pads may have distinct relative cellular composition or are differently programmed to secrete molecules involved in the regulation of cell proliferation and motility. We recently found increased amount of adipose stem cells ($CD34^+/CD45^-/CD31^-/CD146^-$) in PP compared with VIS adipose tissue (Ribeiro R, unpublished observations).

Tumor cell progression depends on itself as well as on the surrounding microenvironment, which is able to influence proliferation, migration and metastatic behavior of tumor cells by modulating the extracellular matrix and growth factor production [64]. If the tissues where tumor cells exist provide the missing extrinsic signals, then cells will proliferate and acquire an invasive phenotype, which may lead to metastasis. Whole periprostatic fat, not only stromal vascular fraction cells, seems to warrant the necessary factors to induce a specific microenvironment for prostate cancer tumor cells, which ultimately may result, as we found, in tumor cell survival, increased motility and availability of extracellular proteases. During cell migration, pericellular proteolysis of extracellular matrix is important for cell protrusion.

The increased production of MMPs found in PP adipose tissue can fuel invasive and metastatic behavior of PP fat-infiltrating prostate cancer cells.

Conclusions

In this study we found that PP adipose tissue-derived factors may potentiate prostate cancer aggressiveness through modulation of metalloproteinases activity, and by promoting cancer cell proliferation and motility. In addition, results indicate that factors secreted by whole periprostatic fat induce a favorable microenvironment for hormone-refractory prostate cancer tumor cells. These previously unrecognized findings suggest a role for PP adipose tissue in prostate cancer progression, and as a candidate explanatory mechanism to the causally invoked association between obesity and aggressive prostate cancer.

Abbreviations

BMI, Body mass index; BPH, Nodular prostatic hyperplasia; CM, Conditioned medium; FRDO, Final relative distance to origin; IL-6, Interleukin 6; LNCaP, Hormone-sensitive prostate cancer cell line; MMP, Matrix metalloproteinase; MS, Mean speed; PC-3, Hormone-refractory prostate cancer cell line; PP, Periprostatic; SVF, Stromal-vascular fraction; VIS, Visceral

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RR, VC and CM performed most of the experiments. MJO performed the zymography, assisted with the cell tracking experiment and edited the manuscript. MF assisted with some of the *in vitro* experiments and edited the manuscript. AF, PP, CL, FL, AM, VS, JSM, JO and FP collected adipose tissue and clinicopathologic patient information and edited the manuscript. RR and RM performed the statistical analysis. RR, CM, AMP, CL and RM designed the experiments and edited the manuscript. RR wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Park J, Euhus DM, Scherer PE: **Paracrine and Endocrine Effects of Adipose Tissue on Cancer Development and Progression.** *Endocr Rev* 2011, **32**: 550–570.
2. van Kruijsdijk RC, van der Wall E, Visseren FL: **Obesity and cancer: the role of dysfunctional adipose tissue.** *Cancer Epidemiol Biomarkers Prev* 2009, **18**:2569–2578.
3. Capitanio U, Suardi N, Briganti A, Gallina A, Abdollah F, Lughezzani G, Salonia A, Freschi M, Montorsi F: **Influence of obesity on tumour volume in patients with prostate cancer.** *BJU Int* 2011, **109**: 678–684.
4. Freedland SJ, Banez LL, Sun LL, Fitzsimons NJ, Moul JW: **Obese men have higher-grade and larger tumors: an analysis of the duke prostate center database.** *Prostate Cancer Prostatic Dis* 2009, **12**:259–263.
5. Cheng L, Darson MF, Bergstralh EJ, Slezak J, Myers RP, Bostwick DG: **Correlation of margin status and extraprostatic extension with progression of prostate carcinoma.** *Cancer* 1999, **86**:1775–1782.
6. Valastyan S, Weinberg RA: **Tumor metastasis: molecular insights and evolving paradigms.** *Cell* 2011, **147**:275–292.
7. Finley DS, Calvert VS, Inokuchi J, Lau A, Narula N, Petricoin EF, Zaldivar F, Santos R, Tyson DR, Ornstein DK: **Periprostatic adipose tissue as a modulator of prostate cancer aggressiveness.** *J Urol* 2009, **182**:1621–1627.
8. van Roermund JG, Hinnen KA, Tolman CJ, Bol GH, Witjes JA, Bosch JL, Kiemeny LA, van Vulpen M: **Periprostatic fat correlates with tumour aggressiveness in prostate cancer patients.** *BJU Int* 2011, **107**:1775–1779.
9. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, Romero IL, Carey MS, Mills GB, Hotamisligil GS, *et al*: **Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth.** *Nat Med* 2011, **17**:1498–1503.
10. Schnabele K, Roser S, Rechkemmer G, Hauner H, Skurk T: **Effects of adipocyte-secreted factors on cell cycle progression in HT29 cells.** *Eur J Nutr* 2009, **48**:154–161.
11. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, Wang YY, Meulle A, Salles B, Le Gonidec S, *et al*: **Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion.** *Cancer Res* 2011, **71**:2455–2465.
12. Onuma M, Bub JD, Rummel TL, Iwamoto Y: **Prostate cancer cell-adipocyte interaction: leptin mediates androgen-independent prostate cancer cell proliferation through c-Jun NH2-terminal kinase.** *J Biol Chem* 2003, **278**:42660–42667.
13. Tokuda Y, Satoh Y, Fujiyama C, Toda S, Sugihara H, Masaki Z: **Prostate cancer cell growth is modulated by adipocyte-cancer cell interaction.** *BJU Int* 2003, **91**:716–720.

14. Somasundar P, Yu AK, Vona-Davis L, McFadden DW: **Differential effects of leptin on cancer in vitro.** *J Surg Res* 2003, **113**:50–55.
15. Ribeiro RJ, Monteiro CP, Cunha VF, Azevedo AS, Oliveira MJ, Monteiro R, Fraga AM, Principe P, Lobato C, Lobo F, *et al*: **Tumor Cell-educated Periprostatic Adipose Tissue Acquires an Aggressive Cancer-promoting Secretory Profile.** *Cell Physiol Biochem* 2012, **29**:233–240.
16. Thalmann S, Juge-Aubry CE, Meier CA: **Explant cultures of white adipose tissue.** *Methods Mol Biol* 2008, **456**:195–199.
17. Desbois D, Couturier E, Mackiewicz V, Graube A, Letort MJ, Dussaix E, Roque-Afonso AM: **Epidemiology and genetic characterization of hepatitis A virus genotype IIA.** *J Clin Microbiol* 2010, **48**:3306–3315.
18. Albertsen PC, Hanley JA, Fine J: **20-year outcomes following conservative management of clinically localized prostate cancer.** *JAMA* 2005, **293**:2095–2101.
19. Taichman RS, Loberg RD, Mehra R, Pienta KJ: **The evolving biology and treatment of prostate cancer.** *J Clin Invest* 2007, **117**:2351–2361.
20. Chung LW, Baseman A, Assikis V, Zhau HE: **Molecular insights into prostate cancer progression: the missing link of tumor microenvironment.** *J Urol* 2005, **173**:10–20.
21. Notarnicola M, Miccolis A, Tutino V, Lorusso D, Caruso MG: **Low levels of lipogenic enzymes in peritumoral adipose tissue of colorectal cancer patients.** *Lipids* 2012, **47**:59–63.
22. Unal R, Yao-Borengasser A, Varma V, Rasouli N, Labbate C, Kern PA, Ranganathan G: **Matrix metalloproteinase-9 is increased in obese subjects and decreases in response to pioglitazone.** *J Clin Endocrinol Metab* 2010, **95**:2993–3001.
23. Egeblad M, Werb Z: **New functions for the matrix metalloproteinases in cancer progression.** *Nat Rev Cancer* 2002, **2**:161–174.
24. Lichtinghagen R, Musholt PB, Stephan C, Lein M, Kristiansen G, Hauptmann S, Rudolph B, Schnorr D, Loening SA, Jung K: **mRNA expression profile of matrix metalloproteinases and their tissue inhibitors in malignant and non-malignant prostatic tissue.** *Anticancer Res* 2003, **23**:2617–2624.
25. Chakrabarti S, Patel KD: **Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology.** *Exp Lung Res* 2005, **31**:599–621.
26. Lin CY, Tsai PH, Kandaswami CC, Lee PP, Huang CJ, Hwang JJ, Lee MT: **Matrix metalloproteinase-9 cooperates with transcription factor Snail to induce epithelial-mesenchymal transition.** *Cancer Sci* 2011, **102**:815–827.
27. Allott EH, Lysaght J, Cathcart MC, Donohoe CL, Cummins R, McGarrigle SA, Kay E, Reynolds JV, Pidgeon GP: **MMP9 expression in oesophageal adenocarcinoma is**

upregulated with visceral obesity and is associated with poor tumour differentiation. *Mol Carcinog* 2011 Nov 28. doi: 10.1002/mc.21840. [Epub ahead of print].

28. Trayhurn P: **Endocrine and signalling role of adipose tissue: new perspectives on fat.** *Acta Physiol Scand* 2005, **184**:285–293.

29. Mistry T, Digby JE, Desai KM, Randeva HS: **Obesity and prostate cancer: a role for adipokines.** *Eur Urol* 2007, **52**:46–53.

30. Ribeiro R, Lopes C, Medeiros R: **The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives.** *Prostate Cancer Prostatic Dis* 2006, **9**:19–24.

31. Hoda MR, Popken G: **Mitogenic and anti-apoptotic actions of adipocyte-derived hormone leptin in prostate cancer cells.** *BJU Int* 2008, **102**:383–388.

32. Chung TD, Yu JJ, Spiotto MT, Bartkowski M, Simons JW: **Characterization of the role of IL-6 in the progression of prostate cancer.** *Prostate* 1999, **38**:199–207.

33. Mori S, Murakami-Mori K, Bonavida B: **Interleukin-6 induces G1 arrest through induction of p27(Kip1), a cyclin-dependent kinase inhibitor, and neuron-like morphology in LNCaP prostate tumor cells.** *Biochem Biophys Res Commun* 1999, **257**:609–614.

34. Iwamura M, Sluss PM, Casamento JB, Cockett AT: **Insulin-like growth factor I: action and receptor characterization in human prostate cancer cell lines.** *Prostate* 1993, **22**:243–252.

35. Mizokami A, Gotoh A, Yamada H, Keller ET, Matsumoto T: **Tumor necrosis factor- α represses androgen sensitivity in the LNCaP prostate cancer cell line.** *J Urol* 2000, **164**:800–805.

36. Chopra DP, Menard RE, Januszewski J, Mattingly RR: **TNF- α -mediated apoptosis in normal human prostate epithelial cells and tumor cell lines.** *Cancer Lett* 2004, **203**:145–154.

37. Mistry T, Digby JE, Chen J, Desai KM, Randeva HS: **The regulation of adiponectin receptors in human prostate cancer cell lines.** *Biochem Biophys Res Commun* 2006, **348**:832–838.

38. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL: **Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells.** *Circulation* 2004, **109**:1292–1298.

39. Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G, Zlabinger GJ, Stulnig TM: **Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production.** *Int J Obes (Lond)* 2007, **31**:1420–1428.

40. Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A, Brichard SM: **Adipokines oversecreted by omental adipose tissue in human obesity.** *Am J Physiol Endocrinol Metab* 2007, **293**:E656–665.
41. Kharait S, Dhir R, Lauffenburger D, Wells A: **Protein kinase Cdelta signaling downstream of the EGF receptor mediates migration and invasiveness of prostate cancer cells.** *Biochem Biophys Res Commun* 2006, **343**:848–856.
42. Mohler JL: **Cellular motility and prostatic carcinoma metastases.** *Cancer Metastasis Rev* 1993, **12**:53–67.
43. Chen J: **Multiple signal pathways in obesity-associated cancer.** *Obes Rev* 2011, **12**:1063–1070.
44. Wells A, Gupta K, Chang P, Swindle S, Glading A, Shiraha H: **Epidermal growth factor receptor-mediated motility in fibroblasts.** *Microsc Res Tech* 1998, **43**:395–411.
45. Desai B, Ma T, Chellaiah MA: **Invadopodia and matrix degradation, a new property of prostate cancer cells during migration and invasion.** *J Biol Chem* 2008, **283**:13856–13866.
46. Subramaniam V, Vincent IR, Jothy S: **Upregulation and dephosphorylation of cofilin: modulation by CD44 variant isoform in human colon cancer cells.** *Exp Mol Pathol* 2005, **79**:187–193.
47. Huang CY, Yu HS, Lai TY, Yeh YL, Su CC, Hsu HH, Tsai FJ, Tsai CH, Wu HC, Tang CH: **Leptin increases motility and integrin up-regulation in human prostate cancer cells.** *J Cell Physiol* 2011, **226**:1274–1282.
48. Tang CH, Lu ME: **Adiponectin increases motility of human prostate cancer cells via adipoR, p38, AMPK, and NF-kappaB pathways.** *Prostate* 2009, **69**:1781–1789.
49. Im E, Motiejunaite R, Aranda J, Park EY, Federico L, Kim TI, Clair T, Stracke ML, Smyth S, Kazlauskas A: **Phospholipase Cgamma activation drives increased production of autotaxin in endothelial cells and lysophosphatidic acid-dependent regression.** *Mol Cell Biol* 2010, **30**:2401–2410.
50. Takahashi M, Ota S, Hata Y, Ogura K, Kurita M, Terano A, Nakamura T, Omata M: **Constitutive expression of hepatocyte growth factor may maintain the sheet construction of gastric epithelial cells through facilitating actin-myosin contractile system.** *Biochem Biophys Res Commun* 1996, **219**:40–46.
51. Kinoshita M, Shimokado K: **Autocrine FGF-2 is responsible for the cell density-dependent susceptibility to apoptosis of HUVEC: A role of a calpain inhibitor-sensitive mechanism.** *Arterioscler Thromb Vasc Biol* 1999, **19**:2323–2329.
52. Hoang MV, Nagy JA, Fox JE, Senger DR: **Moderation of calpain activity promotes neovascular integration and lumen formation during VEGF-induced pathological angiogenesis.** *PLoS One* 2010, **5**:e13612.

53. Taraboletti G, Roberts DD, Liotta LA: **Thrombospondin-induced tumor cell migration: haptotaxis and chemotaxis are mediated by different molecular domains.** *J Cell Biol* 1987, **105**:2409–2415.
54. Wang JM, Taraboletti G, Matsushima K, Van Damme J, Mantovani A: **Induction of haptotactic migration of melanoma cells by neutrophil activating protein/interleukin-8.** *Biochem Biophys Res Commun* 1990, **169**:165–170.
55. Ferry G, Tellier E, Try A, Gres S, Naime I, Simon MF, Rodriguez M, Boucher J, Tack I, Gesta S, et al: **Autotaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation. Up-regulated expression with adipocyte differentiation and obesity.** *J Biol Chem* 2003, **278**:18162–18169.
56. Azare J, Doane A, Leslie K, Chang Q, Berishaj M, Nnoli J, Mark K, Al-Ahmadie H, Gerald W, Hassimi M, et al: **Stat3 mediates expression of autotaxin in breast cancer.** *PLoS One* 2011, **6**:e27851.
57. Geho DH, Bandle RW, Clair T, Liotta LA: **Physiological mechanisms of tumor-cell invasion and migration.** *Physiology (Bethesda)* 2005, **20**:194–200.
58. Khandekar MJ, Cohen P, Spiegelman BM: **Molecular mechanisms of cancer development in obesity.** *Nat Rev Cancer* 2011, **11**:886–895.
59. O'Hara A, Lim FL, Mazzatti DJ, Trayhurn P: **Microarray analysis identifies matrix metalloproteinases (MMPs) as key genes whose expression is up-regulated in human adipocytes by macrophage-conditioned medium.** *Pflugers Arch* 2009, **458**:1103–1114.
60. Wu Y, Smas CM: **Wdnm1-like, a new adipokine with a role in MMP-2 activation.** *Am J Physiol Endocrinol Metab* 2008, **295**:E205–215.
61. Patel ST, Mistry T, Brown JE, Digby JE, Adya R, Desai KM, Randeva HS: **A novel role for the adipokine visfatin/pre-B cell colony-enhancing factor 1 in prostate carcinogenesis.** *Peptides* 2010, **31**:51–57.
62. Andarawewa KL, Motrescu ER, Chenard MP, Gansmuller A, Stoll I, Tomasetto C, Rio MC: **Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front.** *Cancer Res* 2005, **65**:10862–10871.
63. Toda S, Uchihashi K, Aoki S, Sonoda E, Yamasaki F, Piao M, Ootani A, Yonemitsu N, Sugihara H: **Adipose tissue-organotypic culture system as a promising model for studying adipose tissue biology and regeneration.** *Organogenesis* 2009, **5**:50–56.
64. Sung SY, Chung LW: **Prostate tumor-stroma interaction: molecular mechanisms and opportunities for therapeutic targeting.** *Differentiation* 2002, **70**:506–521.

Figure 1

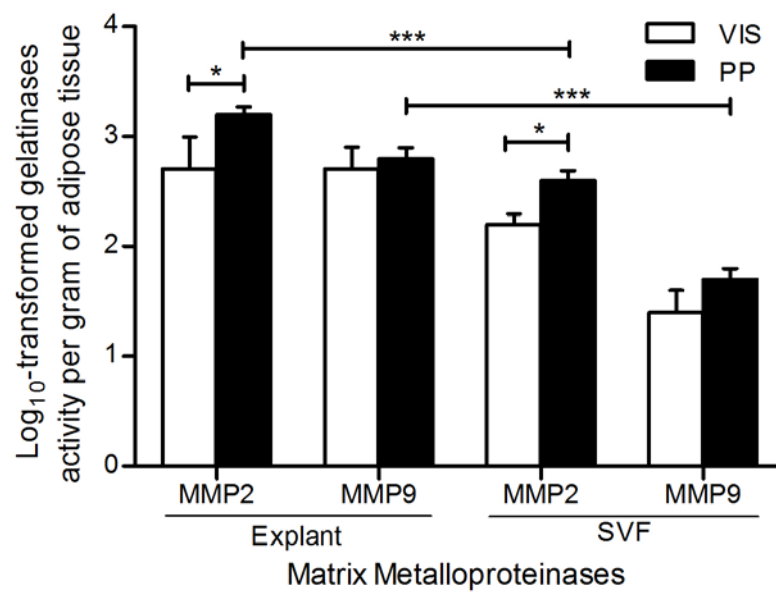


Figure 2

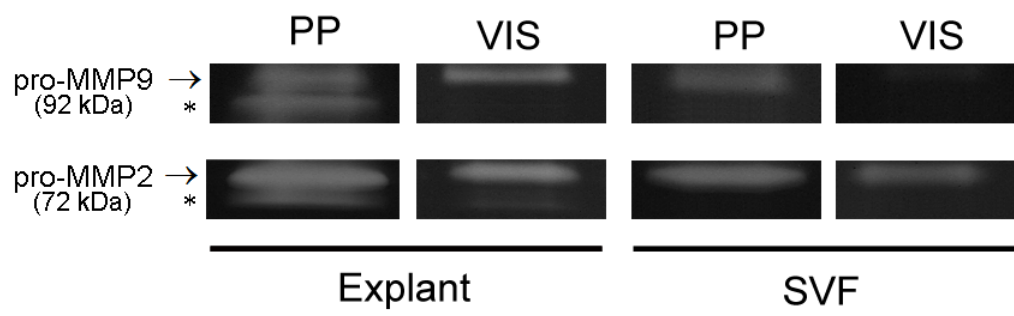


Figure 3

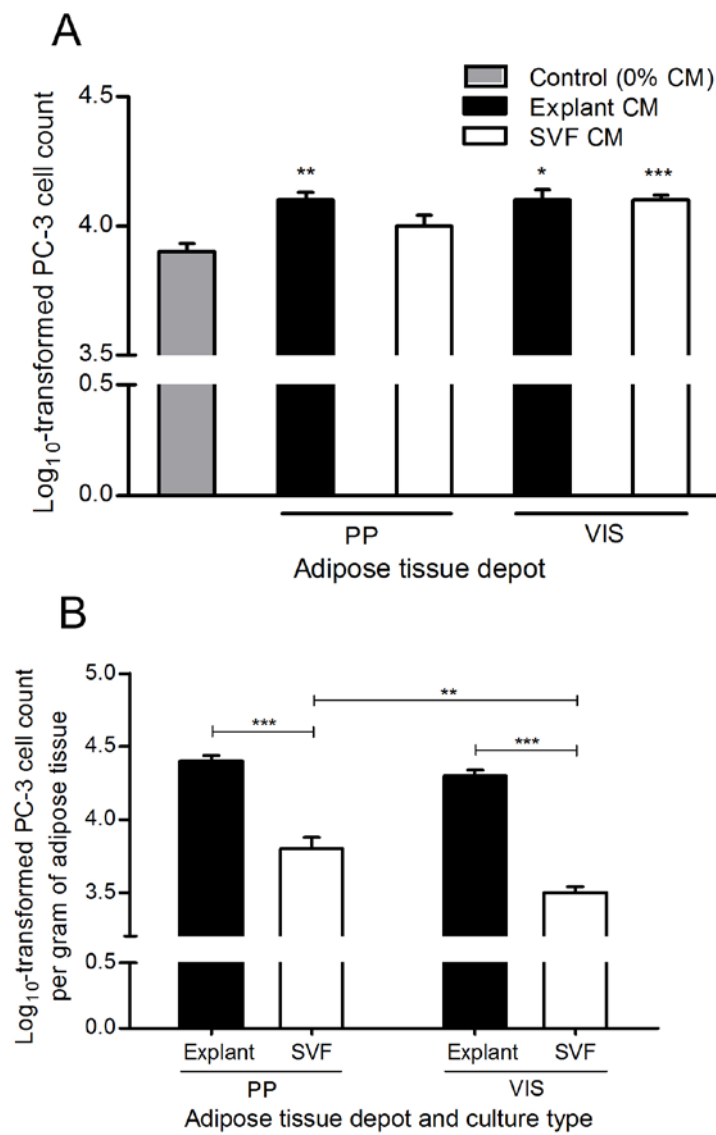


Figure 4

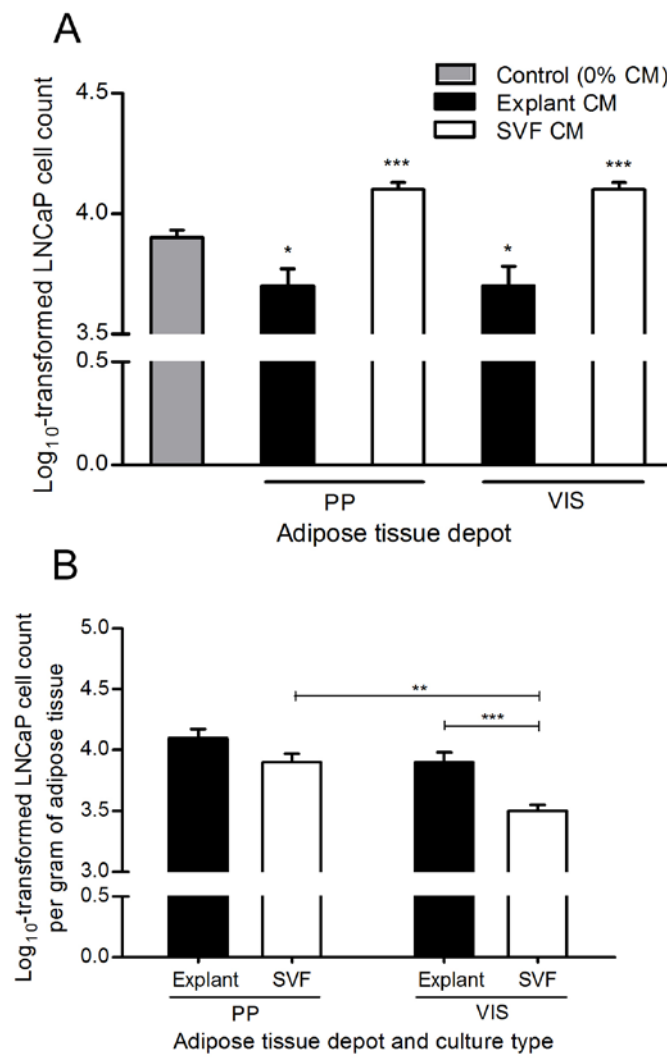


Figure 5

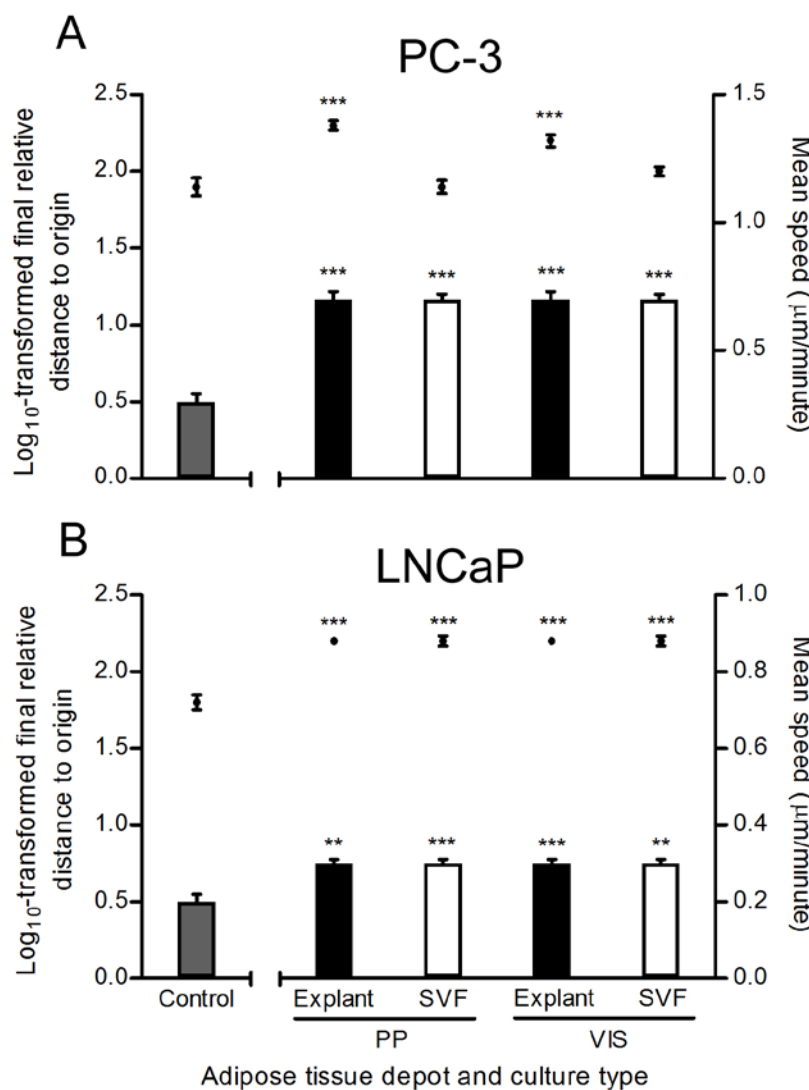


Figure 6

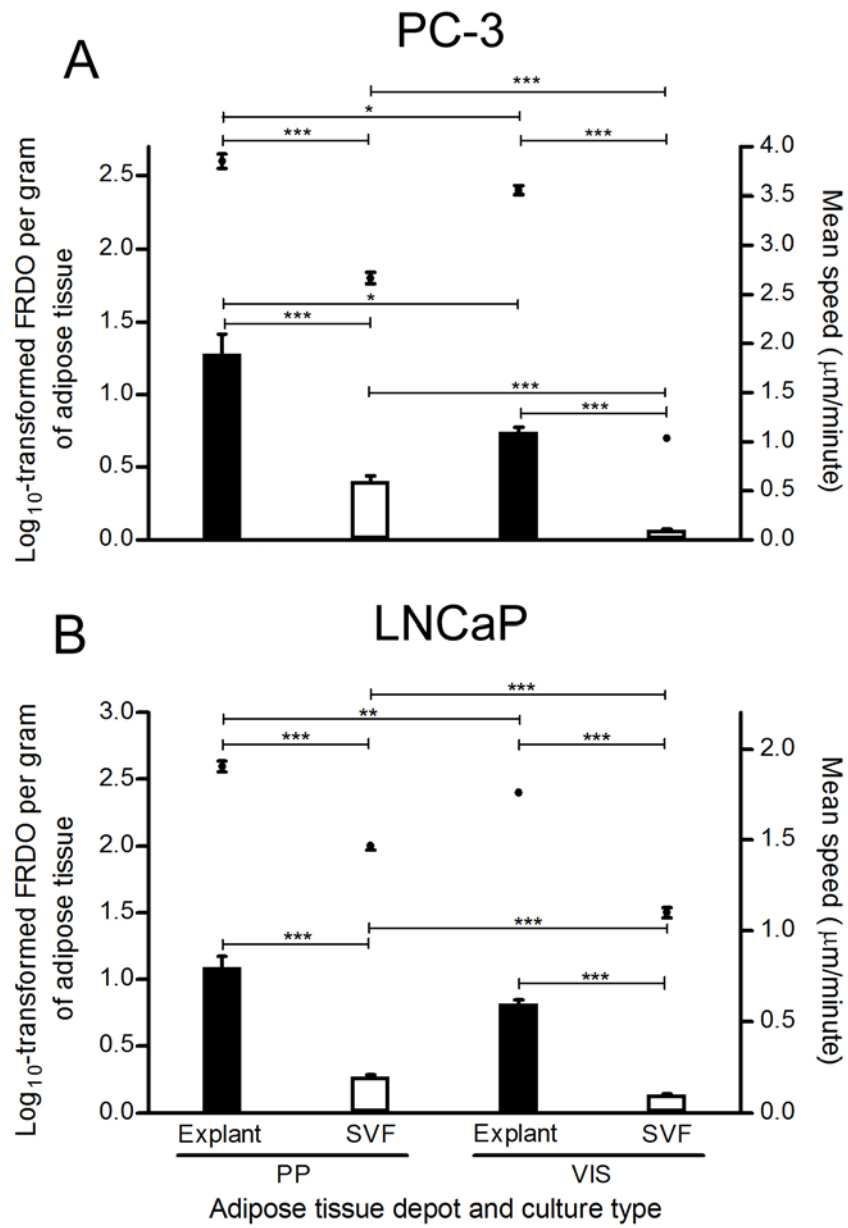
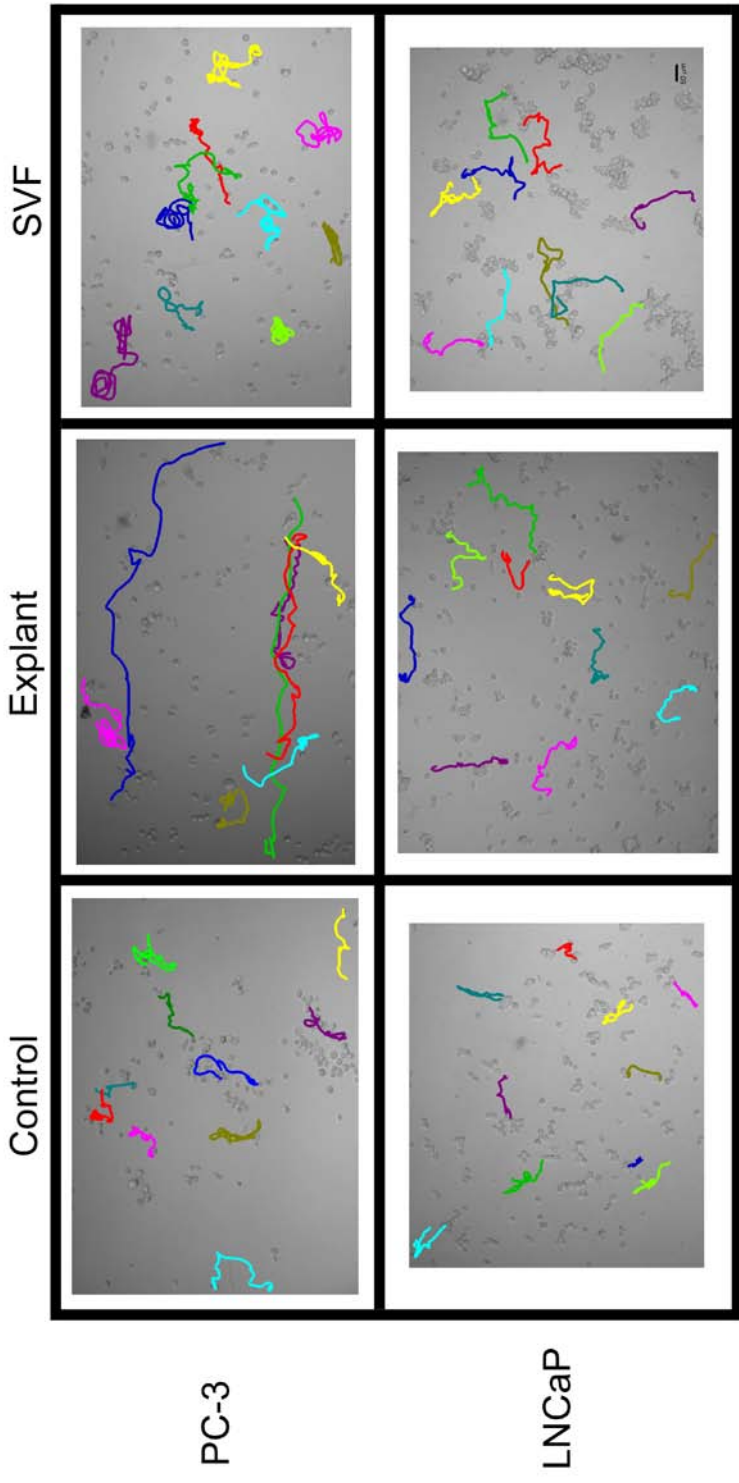


Figure 7



PAPER XI

Obesity and prostate cancer: gene expression signature of human periprostatic adipose tissue

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ABSTRACT

Background: Periprostatic (PP) adipose tissue surrounds the prostate, an organ with high predisposition to become malignant. Frequently growing prostatic tumor cells extend beyond the prostatic organ towards this fat depot. This study aimed to determine the genome-wide expression of genes in PP adipose tissue in obesity/overweight (OB/OW) and prostate cancer.

Methods: Differentially expressed genes in human periprostatic adipose tissue were identified using microarrays. Analyses were conducted according to donors' body mass index characteristics (obese/overweight vs. normoponderal) and prostate disease (extracapsular cancer vs. intra-prostatic cancer vs. benign prostatic hyperplasia). Selected genes with altered expression were validated by real-time PCR. Ingenuity Pathway Analysis (IPA) was used to investigate gene ontology, canonical pathways and functional networks.

Results: In the PP adipose tissue of OB/OW subjects, the majority of the genes with altered expression encode proteins involved in adipogenic/anti-lipolytic, pro-proliferative/anti-apoptotic, and mild immunoinflammatory processes, where *FADS1* (down-regulated), *LEP* and *ANGPT1* (both up-regulated) emerge as interesting target molecules in the regulation of the observed altered biological processes. In the PP adipose tissue of subjects with prostate cancer, the most remarkable findings are related with altered gene expression that likely result in a stimulatory effect to increase adipose tissue cellular activity (proliferation, cell cycle, differentiation and anti-apoptosis), whereas a downward impact in immunity and inflammation was observed, mostly related with the complement (*CFH*). Notably, we found overexpression of *mir-let7-a2* micro RNA in the PP adipose tissue of prostate cancer patients.

Conclusions: Obesity and excess adiposity modifies the expression of PP adipose tissue genes to ultimately foster fat mass growth. In patients with prostate cancer the expression profile of PP adipose tissue may result in a tumor microenvironment with hypercellularity and reduced immunosurveillance. Both findings are liable to promote a favorable environment for prostate cancer progression. In an increasingly obese population, recognizing the mechanisms and role of PP adipose tissue environment may provide an opportunity for preventive and therapeutic strategies to reduce prostate cancer morbidity and mortality.

Keywords: adipose tissue; gene expression; microarray; obesity; periprostatic; prostate cancer

BACKGROUND

Prostate cancer is the most common solid neoplasm and the second cause of cancer death in men in Europe [1]. Age, ethnic background and family history are well-established risk factors. In addition, accumulating evidence over the last years showed that obesity is a relevant risk factor for many types of malignancies, including aggressive prostate cancer [2-4].

Adipose tissue dysfunctional behavior, often seen in obesity, has been widely appreciated as a major cause underlying cancer [5, 6]. The prostate has a capsular-like structure and is surrounded by adipose tissue [7]. Frequently prostate tumor cells infiltrate the periprostatic (PP) fat pad by transposing or infiltrating the capsule [8], resulting in immediate proximity to adipose tissue. Once cancer cells extend beyond the capsule, the PP adipose tissue-secreted factors, extracellular matrix components or direct cell-cell contact may influence the phenotypic behavior of malignant cells. In fact, recent findings in PP adipose tissue showed that tumor-derived factors influence its metabolic activity profile, and that increased local production of interleukin-6 and PP fat thickness were associated with prostate cancer aggressiveness [9-12]. Furthermore, albeit the PP adipose tissue gene expression profile is currently unknown, it is well established that adipose tissue from distinct anatomical origin and obesity status have specific gene expression signatures [13, 14]. Knowledge of the PP adipose tissue genomic profile may uncover molecules and mechanisms linked either with obesity or prostate cancer that can influence prostate cancer progression.

In this study, we aimed to determine in PP adipose tissue the spectrum of genes differently expressed as well as relevant functional clustering, in order to evaluate the influence of obesity/overweight (OB/OW) on prostate cancer and viceversa.

METHODS

Patients

Patients scheduled for retropubic radical prostatectomy or partial open prostatectomy between May and October 2009, without major co-morbidities, were included in this study after informed consent agreement. Inclusion criteria were age (45-75 years) and absence of previous prostatic treatments. Exclusion criteria were: diabetes, family history of prostate cancer, transvesical partial open prostatectomy, other primary malignancies, or pharmacological treatment with drugs that may modify adipose tissue gene expression (e.g. anti-dislipidemics, anti-diabetics).

Anterior-lateral samples of PP adipose tissue were collected during surgery. Adipose tissue samples were immediately sectioned, cleaned and rinsed with pre-warmed phosphate buffer saline and included in RNAlater (Applied Biosystems, Foster City, CA, USA).

Eighteen patients participated in the study and were divided into three groups based on post-surgical diagnosis and pathologic analyses. Six patients with benign prostatic hyperplasia (BPH) and twelve with prostate cancer [six with pT1-T2b, localized prostate cancer (LoPCa) and six with pT2c-T4, locally advanced stage (LAPCa)] met the criteria for inclusion in this study. In each prostatic disease group, three patients were normoponderal [NP, body mass index (BMI) $< 25 \text{ kg/m}^2$] and three obese/overweight (OB/OW, BMI $\geq 25 \text{ kg/m}^2$), resulting in overall nine NP and nine OB/OW. Participants' clinicopathological characteristics and serum PSA concentration at diagnosis were reviewed from clinical charts and are presented in Table 1. The project was approved by the ethics committees of the participating Hospitals. All

reported investigations were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008.

RNA extraction, microarray hybridization and data processing

Total RNA was extracted from PP adipose tissue samples after homogenization with an ULTRA-TURRAX T25 basic (IKA Werke GmbH, Staufen, Germany) in QIAzol reagent (Qiagen, Valencia, CA, USA) and purified through columns (RNeasy Lipid Tissue Mini kit, Qiagen) with DNase I treatment (RNase-free DNase set, Qiagen). Integrity and purity of RNA were assessed by on-chip electrophoresis using Experion (BioRad, USA).

From 1 µg total RNA, cDNA and biotin-labeled antisense cRNA were obtained and hybridized to a high-density oligonucleotide human genome array HG-U133 Plus 2.0 Affymetrix GeneChip Arrays (Affymetrix, Santa Clara, CA, USA). Background correction and normalization were done using robust multi-array average algorithm [15]. Calculation of fold change values was performed using the NP and the non-cancer or localized prostate cancer as reference. We used Linear Models for Microarray Data [16] to identify differentially expressed genes. Briefly, it starts by fitting a linear model for each gene in the data; then an empirical Bayes method is used to moderate the standard errors for estimating the moderated t-statistics for each gene, which shrinks the standard errors towards a common value. This test is similar to a t-test method for each probe except that the residual standard deviations are moderated across genes to ensure more stable inference for each gene. The moderated standard deviations are a compromise between the individual genewise standard deviations and an overall pooled standard deviation. We used False Discovery Rate [17] to evaluate the statistical significance of all genes.

Enrichment analysis using Ingenuity Pathway Analysis software

We evaluated the gene function and network enrichments for selected genes (unadjusted $P < 0.01$) using Ingenuity Pathway Analysis software (Ingenuity Systems, CA, USA). LoPCa group of patients was not included in this analysis. Genes with known HUGO gene symbols and their corresponding fold change were uploaded into the software. Networks of these genes were algorithmically generated based on their connectivity and assigned a score. The score takes into account the number of focus genes in the network and the size of the network to approximate its relevance to the original list of focus genes. The identified networks are presented as a figure indicating molecular relationships between genes/gene products. Canonical pathway analysis identified the pathways which were more significant to the input data set.

Real-Time PCR

To validate the microarray data, a number of representative genes and microRNAs were selected to be studied by Real-Time PCR. For gene expression analysis, cDNA was generated using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA), whereas for microRNAs expression we used the Taqman MicroRNA RT kit (Applied Biosystems), according to the manufacturer's instructions.

Transcript levels of the selected genes and microRNAs were quantified by Real-Time PCR (StepOne, Foster City, CA, Applied Biosystems). The cDNA was amplified using the following conditions, both for genes (Taqman Gene Expression Master Mix, Applied Biosystems) and microRNA (Taqman Universal Master Mix, Applied Biosystems): 95 °C for 10 min, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Results were normalized to the levels of the 18S rRNA for genes and of *mir-103* for

microRNA, according to previous reports with adipose tissue [18, 19]. Assays ID are available upon request to authors. Gene and microRNA expression was calculated using the REST 2009 software, where relative expression was expressed as fold over the reference group. The products of the *PCA3* gene amplification were verified by 1.5% agarose gel electrophoresis, and acquired using the GelDoc XR system (BioRad) and Quantity One software (BioRad).

Plasma leptin measurement

Plasma samples were obtained before surgery after an overnight fast. The concentrations of circulating leptin were quantified using microsphere-based multiplexing technology, as described before [20]. The intra- and inter-assay precisions were 4.2% and 21.4%, respectively. The minimum leptin detectable concentration was 27.4 pg/mL.

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean. Departure from normality was tested using the Kolmogorov-Smirnov test. Accordingly, one-way ANOVA, Kruskal-Wallis or Fisher tests were used for comparisons of clinicopathological variables between prostatic disease groups, whereas differences between OB/OW and NP groups were tested by unpaired t-test, Mann-Whitney and chi-square tests. Data analyses were performed using the software SPSS version 17.0 (SPSS Inc., Chicago, USA) and a $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Clinicopathological characteristics of participants according to prostatic disease are presented in Table 1. Age at diagnosis, body mass index, serum leptin and PSA levels, and combined Gleason grade in subjects with cancer were similar between prostatic disease groups ($P>0.05$ for all comparisons). In this study, as expected, BPH subjects presented heavier prostates ($P<0.05$). The obese and overweight (OB/OW) evidenced higher mean BMI (29.1 ± 1.8 vs. 23.1 ± 1.2 kg/m², $P<0.0001$) and serum leptin levels (8.6 ± 1.3 vs. 1.9 ± 0.7 µg/mL, $P=0.001$) than NP subjects. For each group of prostate disease (BPH, LoPCa and LAPCa), significantly higher BMI was observed in OB/OW subjects compared with NP ($P=0.016$, $P<0.0001$ and $P=0.013$, respectively).

PCA3 gene expression in PP adipose tissue

Frequently, prostate tumor cells infiltrate the PP fat; therefore in order to analyze the PP adipose tissue gene expression signature we needed to ensure the absence of tumor cells. To confirm whether PP adipose tissue samples were free from prostate cancer cells the expression of the *PCA3* gene was examined. Lack of expression of this gene in the PP adipose tissue of cancer patients was demonstrated (Figure 1).

Defining the PP adipose tissue gene expression signature in OB/OW subjects

Comparison of overall transcriptional response revealed 148 of the analyzed gene probe sets as significantly changed ($P<0.001$ and absolute fold change ≥ 1.5). Among these gene probe sets, gene expression features of the overall OB/OW patients

associated with altered 6 genes, whereas OB/OW subjects in restricted BPH, LoPCa and LAPCa groups had respectively 12, 10 and 5 altered genes (Table 2).

The IPA software was used to investigate functions and interactions among altered genes. This analysis revealed a broad spectrum of biological processes for OB/OW vs. NP. Altered functions were predominantly related to nutritional disease, connective tissue development and function, cell death, cellular development and cellular growth and proliferation (Supplementary Table 1). Supplementary figure 1 shows the most significant network in OB/OW men ($P < 0.0001$). In human PP adipose tissue of OB/OW the most relevantly altered canonical pathways was associated with glycerolipid metabolism and leptin signaling (Table 4), and differentially expressed genes encode proteins involved in immunity and inflammation, cell growth and proliferation, fat metabolism and apoptosis. When restricted to the group of subjects with BPH, being OB/OW was associated with changes in the expression of genes involved in cell-to-cell signaling, tissue development and cellular movement functions (Supplementary Table 1).

Defining the PP adipose tissue gene expression signature in prostate cancer patients

Table 3 shows differently expressed genes according to prostatic disease ($P < 0.001$ and absolute fold change ≥ 1.5). We found that in the PP adipose tissue of LAPCa patients 8 genes were up-regulated compared to BPH, while in LoPCa vs. BPH 87% of the genes were down-regulated; 4 genes were up- and 3 down-regulated in LAPCa compared with LoPCa. The PP adipose tissue of cancer patients exhibited increased transcript levels of *MIRLET7A2* and *TC2N* compared with BPH. In the non-OB/OW subjects, features of the LAPCa vs. BPH were matched to only 3 up- and 4

down-regulated genes, while in the OB/OW subjects 86% of the 22 altered genes were overexpressed. The *MIRLET7A2* and *MRPL42* were overexpressed in all analyses (overall, and within NP and OB/OW groups) in the PP adipose tissue of patients with LAPCa. The LoPCa patients presented overall 87% down-regulated genes, 90% down-regulated in NP and 61% down-regulated for OB/OW, when compared with BPH. Conversely, the comparison between LAPCa and LoPCa yielded overall 57% up-regulated genes, and 14 out of 15 and 2 out of 3 genes up-regulated, respectively for NP and OB/OW. In OB/OW subjects 3 genes were consistently overexpressed (*TC2N*, *hsa-miR-let-7a-2* and *CLDN10*) in the PP adipose tissue of men with cancer (LAPCa or LoPCa), compared with BPH.

In the analysis of prostate cancer vs. BPH, OB/OW subjects presented more altered genes in PP adipose tissue. The IPA analysis identified interaction networks between LAPCa vs. BPH and found that functions were more frequently related to cellular growth and proliferation, cell cycle, apoptosis and cell death, cellular movement and to inflammation and immunity (Supplementary Table 2) ($P < 0.0001$). The altered canonical pathways ($P < 0.05$) in the PP adipose tissue of LAPCa are shown in Table 4.

Validation of selected genes by Real-Time PCR

Validation experiments were performed to confirm the accuracy of array gene expression measurements. We selected a set of representative transcripts involved in cell proliferation, immunity and lipid metabolism. Results across the 18 study patients are shown in Figure 2. Overall, PCR results reflected the findings of microarrays.

DISCUSSION

Extra-capsular extension of prostate cancer cells into the PP adipose tissue is a common pathologic finding and a factor related with worst prognosis [21]. Once tumor cells extend beyond the prostatic capsule, the interactions with non-tumor cells in the PP adipose tissue may influence its phenotypic behavior. In fact, accumulating evidence shows that the microenvironment is decisive to determine whether cancer cells progress towards metastasis or whether they remain dormant [22]. To date, the scarce reports on PP adipose tissue support a mechanistic link with prostate cancer aggressiveness [9, 10, 12].

In the present study, forty-six percent of the well-characterized genes included in the array were expressed at detection level in PP adipose tissue, which is comparable to omental adipose tissue [14]. The comparison of PP adipose tissue gene expression of non-diabetic OB/OW to NP men with prostatic disease identified for the first time 34 differently expressed genes.

Two relevant but opponent pathways, lipolysis and adipo/lipogenesis, have a significant role in energy balance. In our study, consistent with other reports on visceral adipose tissue [14, 23], altered expression of genes involved in lipolysis were found in association with obesity and excess adiposity. *NPY1R* and *NPY5R* have anti-lipolytic effects [24], whereas *LEP* expression follows adipo/lipogenesis despite leptin is also a lipolytic factor [25, 26], and were all overexpressed in the PP adipose tissue of OB/OW. Noteworthy, *PDE11*, which has been shown to be involved in adipocyte differentiation and cyclic nucleotides biology [27], was overexpressed in PP adipose tissue of OB/OW subjects. Downstream effects of altered genes reportedly up-regulate adipo/lipogenesis, including *PNPLA3* (also known as adiponutrin), which encodes a triacylglycerol lipase

that mediates triacylglycerol hydrolysis [28], *FADS1*, that regulates unsaturation of fatty acids and SREBP-1 expression [29] or *PCYT2*, that mediates phosphatidylethanolamine synthesis and the availability of di- and tri-acylglycerol [30]. The combined functional dysregulation of these genes suggests that obese PP adipose tissue has anti-lipolytic and adipo/lipogenic gene expression profile.

The number of adipocytes and its volume are determinants of fat mass [31], whilst proliferation/differentiation and apoptosis likely influences adipose tissue growth and regression [32, 33]. In our study, the anti-apoptotic genes *ANGPT1* and *HSPB8* were up-regulated in PP adipose tissue of OB/OW [33, 34]. Furthermore, the expression of *EIF5A*, known to activate the intrinsic mitochondrial pathway [35] was repressed in OB/OW. In agreement, adipose tissue of obese rats presented an anti-apoptotic gene expression profile [36]. Besides altered genes in apoptosis pathways, we found increased expression of genes involved in cell growth and differentiation, such as *LEP* and *ANGPT1*, whose products increase endothelial, mesenchymal and tumor cell growth and differentiation [33, 37-40], or *NPY1R* that mediates proliferative stimulus in progenitor adipose cells [41]. Presented data suggests increased cell growth and anti-apoptosis, extensive to endothelial, progenitor or adult lineages in the PP adipose tissue of OB/OW.

Taken together, these anti-lipolytic, adipo/lipogenic, proliferative and anti-apoptotic effects in the PP adipose tissue of OB/OW likely result in fat mass expansion, conferring increased capacity of enlarged adipocytes to express adipokines and increase fatty acids supplies, which might impact the local energy and growth factors availability, thereby casting the local environment to allow cancer progression [42, 43]. This environment in the PP adipose tissue of OB/OW may at least partially explain the

described association of obesity and excess adiposity with the progression of prostate cancer [3, 44] (Figure 3).

Recent developments in obesity and cancer immunological pathways suggest a previously unappreciated complexity of cancer cell-adipose tissue cell-immunoinflammatory cell cross-talk [45]. We found altered genes in PP adipose tissue of OB/OW that are involved in immunity and inflammation. Overexpressed genes engaged in innate and adaptive components of the immune system, include *LEP*, which up-regulates both innate and adaptive immunoinflammatory response [46], *NPY1R*, that has been shown to inhibit T cell activation [47], and *CYSTLR2*, that increases pro-inflammatory cytokine expression [48]. *FADS1*, known to mediate the formation of inflammatory mediators (e.g. prostaglandin E2, PGE2, thromboxane A2, TXA2, and leukotriene B4, LTB4) [49], and *EIF5A* that is essential for *NOS2* translation [50], are both down-regulated in the PP adipose tissue of OB/OW. Thus, the enhanced local mild immunoinflammatory environment, observed in PP adipose of OB/OW [50] might further influence tissue remodeling and contribute towards tumor progression [44, 45].

The *LEP* and *ANGPT1* encoded proteins may have roles beyond adipose tissue itself. Prostate cancers express the leptin receptor [51] and leptin staining is significantly increased in malignant prostates and poorly differentiated tumors [51]. Also angiopoietin 1 and its receptor Tie-2 were found in both prostate tumor cells and capillaries [52], where they can induce sprouting angiogenesis [39]. These findings along with our own suggest that PP adipose tissue may modulate prostate cancer progression via production of growth factors, that favour proliferative and angiogenic events that in turn are needed for tumor development.

Herein, we showed that the PP adipose tissue gene expression profile of OB/OW subjects may contribute to increased local adiposity, mild immunoinflammatory environment and production of molecules with oncogenic potential (Figure 3).

The PP adipose tissue gene expression signature of men with prostate cancer was determined, in order to find if functional alterations associate with prostate cancer besides the previously reported PP thickness and protein measurements [9-12]. In the present study, when comparing between prostatic diseases the PP adipose tissue of age- and BMI-matched nondiabetic men, 119 altered genes were found. Representative genes and functions are shown in Figure 4. Noteworthy, our findings revealed that altered gene networks pertained to cell cycle and proliferation regulation. Overexpressed genes in the PP adipose tissue of cancer patients that are involved in cell cycle and proliferation include *PLCB1*, that modulates cyclin D3 and CDK4 in response to the IGF-1 mitogenic stimulus [54, 55] or *TPPP3*, that regulates G2-M and G1-S transitions [56]. Furthermore, *HAUS1*, a component of the augmin complex involved in spindle microtubule generation in mitosis [57] and *TSPAN8* (also known as CO-029), that encodes for an integrin-binding glycoprotein that stimulates endothelial cell proliferation [58], are also up-regulated in cancer patients. Noteworthy, the *FGF16* gene that encodes a mitogenic growth factor [59, 60] was overexpressed in the PP adipose tissue of men with prostate cancer. These findings, together with down-regulation of *XAF1*, which influences G2/M arrest through modulation of checkpoint kinase 1 and Cdc2-cyclin B complex [61], support a positive cell cycle regulation and permissive stimulus for growth and proliferation in PP adipose tissue cells. Cumulatively, pro-apoptotic genes, such as *XAF1* and *GADD45B* [62, 63] were down-regulated, whereas *DRAM1* was up-regulated in the adipose tissue of prostate cancer patients [64]. Canonical analysis showed involvement of the p53 pathway in adipose tissue of cancer

subjects, possibly reflecting the relationship of the altered genes *XAF1*, *DRAM1* and *SMG1* with the p53 pathway [65-67]. In adipose tissue biology, cell differentiation also plays an important role in increasing fat mass. Here we show altered expression of genes that associate with cellular differentiation of overall (e.g. *PLCB1*, *GADD45B*), adipocyte (e.g. *PLCB1*, *FFAR2*) and endothelial lineages (e.g. *SCUBE1*) [54, 68-70]. Thus, particularly the adipocyte and vascular biology of PP adipose tissue seem to be committed towards the differentiated state in men with prostate cancer. Consistent with gene expression findings, we observed overexpression of the micro RNA *MIRLETA2* in the PP adipose tissue of prostate cancer patients. The involvement of the *let-7* micro RNA in adipocyte differentiation has been described [71]. Moreover, canonical pathways analysis showed that ERK5 signaling, an evolutionary conserved pathway involved in hypertrophic signaling that regulates adipogenic differentiation [72, 73], was altered in PP adipose tissue of cancer patients. Previously, *in vitro* studies showed that tumor-derived factors induced preadipocyte differentiation [74], supporting a tumor-educated regulation of adipose tissue differentiation program. Taken together, the impact of these gene expression results on cell cycle and proliferation, in apoptosis and differentiation of PP adipose tissue cellular components supports fat mass accrual, which agrees with findings showing increased PP fat pad thickness in prostate cancer patients [9, 11]. Furthermore, bulky adipocytes predispose to increased adipokine secretion and availability of fatty acids, which may influence prostate cancer progression [42, 43].

Immunoinflammatory mechanisms drive both obesity and cancer. Canonical analysis showed that the PP adipose tissue of prostate cancer patients presents altered pathways associated with immunity and inflammation, including antigen presentation, B cell development and T helper cell differentiation. The complement system is important

in immunosurveillance against tumors, albeit malignant cells are usually resistant to complement-mediated lysis [75, 76]. The products of *CFH* and *CFHR1* are soluble complement regulators essential for preventing complement activation, therefore culprits in complement inhibition [76, 77]. Interestingly, we and others report on the expression of *CFH* and *CFHR1* in the adipose tissue [78]. The finding of *CFH* and *CFHR1* up-regulation in PP adipose tissue of subjects with prostate cancer suggests increased inhibitory modulation of the complement activity in prostate tumor cells and evasion to attack. Other altered genes in the PP adipose tissue of prostate cancer patients with repercussion in the local immunoinflammatory environment include *MS4A1* (also known as *CD20*), that plays a functional role in B-cell activation [79] or *FFAR2*, that encodes a protein reported to modulate the differentiation and/or activation of leukocytes [80]. Taken together, these altered genes in PP adipose tissue might contribute to an environment with immunological cellular dormancy and reduced immunosurveillance, which may facilitate prostate cancer progression (Figure 4).

In the present study, we found increased *CLDN10* mRNA transcript abundance in the PP adipose tissue of prostate cancer patients using both microarray and Real-Time PCR analyses. To the best of our knowledge *CLDN10* expression in adipose tissue has not been previously reported. This gene encodes an important tight junction component with an intriguing role in adipose tissue considering its functions in the stroma arrangement and cellular connections [81]. Further studies are required to get insight regarding the cells involved and functional implications of *CLDN10* expression in PP adipose tissue.

In this study we used, for the first time, a global gene expression profiling in PP adipose tissue to unravel genes and regulatory pathways associated with OB/OW and with prostate cancer. Subjects included in this study had histopathological confirmation

of prostatic disease, pathology tumor grade and stage, while contamination with prostate tumor cells in the collected PP adipose tissue samples was excluded by the absence of *PCA3* expression. The high-quality GeneChip data set from RNA specimens of PP adipose tissue, careful patient selection for matching age, race, BMI and clinical variables underscore the strength of major findings of this study.

Our findings likely represent the effects of excess adiposity or cancer and the bi-directional interactions between all cell types that influence adipose tissue function and might affect or be influenced by prostate cancer progression. These hypotheses are grounded on the crosstalk between PP adipose tissue and tumor cells, which ultimately may induce an environment favorable to cancer progression. Better understanding of the mechanisms underlying the association between obesity and aggressive prostate cancer are warranted to gain more insight into the specific contribution of each PP adipose tissue cell type to cancer development and foster the development of new treatment options, at the same time as help to identify malignancies with worst prognosis and encourage the implementation of adjuvant chemoprevention strategies.

CONCLUSIONS

The present study, first in human PP adipose tissue where OB/OW- and prostate cancer-associated gene expression changes were analyzed by microarrays, provides valuable new insight on how local adipose tissue pathophysiology may favor prostate cancer. We identified altered gene expression that might impact components of white adipose tissue overgrowth, including anti-lipolytic, anti-apoptotic, proliferative and mild local immunoinflammatory stimuli in PP fat of OB/OW subjects. The overexpression of *LEP* and *ANGPT1* by PP adipose tissue in OB/OW may contribute towards a favorable environment for prostate cancer progression.

The gene expression signature of PP adipose tissue from prostate cancer patients seems to evidence altered gene expression across distinct cell types, with repercussion in stimulus for cell cycle regulation, cell proliferation and differentiation, and anti-apoptosis. Additionally, we found altered genes involved in immunological cell dormancy and reduced immunosurveillance, namely complement-related *CFH* and *CFHR1* genes. Our findings suggest that PP adipose tissue gene expression profile of both OB/OW and prostate cancer subjects is likely to cause a local environment favorable to prostate cancer progression. Confirmation of PP adipose tissue role in prostate cancer progression and untangling its mechanisms will become increasingly important in the development of adjuvant therapeutic and lifestyle measures.

LIST OF ABBREVIATIONS

BPH, benign prostatic hyperplasia; LAPCa, locally advanced prostate cancer; LoPCa, localized prostate cancer; NP, normoponderal; OB/OW, obese/overweight; PP, periprostatic

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

RR, CM, VC, VC and AR performed most of the experiments. PH performed the microarray analysis and edited the manuscript. AF, PP, CL, FL, AM, VS, JS-M, JO and FP collected adipose tissue and clinicopathologic patient information and edited the manuscript. RR and RM performed the statistical analysis. RR, VC, JG-A, RM and GF designed the experiments and edited the manuscript. RR wrote the manuscript. All authors read and approved the final manuscript.

AUTHORS' INFORMATION

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REFERENCES

1. Boyle P, Ferlay J: **Cancer incidence and mortality in Europe, 2004.** *Ann Oncol* 2005, **16**:481-488.
2. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ: **Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults.** *N Engl J Med* 2003, **348**:1625-1638.
3. Freedland SJ, Banez LL, Sun LL, Fitzsimons NJ, Moul JW: **Obese men have higher-grade and larger tumors: an analysis of the duke prostate center database.** *Prostate Cancer Prostatic Dis* 2009, **12**:259-263.
4. Renehan AG, Soerjomataram I, Tyson M, Egger M, Zwahlen M, Coebergh JW, Buchan I: **Incident cancer burden attributable to excess body mass index in 30 European countries.** *Int J Cancer* 2010, **126**:692-702.
5. van Kruijsdijk RC, van der Wall E, Visseren FL: **Obesity and cancer: the role of dysfunctional adipose tissue.** *Cancer Epidemiol Biomarkers Prev* 2009, **18**:2569-2578.
6. Catalan V, Gomez-Ambrosi J, Rodriguez A, Ramirez B, Silva C, Rotellar F, Hernandez-Lizoain JL, Baixauli J, Valenti V, Pardo F, et al: **Up-regulation of the novel proinflammatory adipokines lipocalin-2, chitinase-3 like-1 and osteopontin as well as angiogenic-related factors in visceral adipose tissue of patients with colon cancer.** *J Nutr Biochem* 2011, **22**:634-641.
7. Hong H, Koch MO, Foster RS, Bihle R, Gardner TA, Fyffe J, Ulbright TM, Eble JN, Cheng L: **Anatomic distribution of periprostatic adipose tissue: a mapping study of 100 radical prostatectomy specimens.** *Cancer* 2003, **97**:1639-1643.
8. Gilliland FD, Hoffman RM, Hamilton A, Albertsen P, Eley JW, Harlan L, Stanford JL, Hunt WC, Potosky A, Stephenson RA: **Predicting extracapsular extension of prostate cancer in men treated with radical prostatectomy: results from the population based prostate cancer outcomes study.** *J Urol* 1999, **162**:1341-1345.
9. van Roermund JG, Hinnen KA, Tolman CJ, Bol GH, Witjes JA, Bosch JL, Kiemeny LA, van Vulpen M: **Periprostatic fat correlates with tumour aggressiveness in prostate cancer patients.** *BJU Int* 2011, **107**:1775-1779.
10. Finley DS, Calvert VS, Inokuchi J, Lau A, Narula N, Petricoin EF, Zaldivar F, Santos R, Tyson DR, Ornstein DK: **Periprostatic adipose tissue as a modulator of prostate cancer aggressiveness.** *J Urol* 2009, **182**:1621-1627.
11. Bhindi B, Trottier G, Elharram M, Fernandes KA, Lockwood G, Toi A, Hersey KM, Finelli A, Evans A, van der Kwast TH, Fleshner NE: **Measurement of peri-prostatic fat thickness using transrectal ultrasonography (TRUS): a new risk factor for prostate cancer.** *BJU Int* 2012.
12. Ribeiro RJ, Monteiro CP, Cunha VF, Azevedo AS, Oliveira MJ, Monteiro R, Fraga AM, Principe P, Lobato C, Lobo F, et al: **Tumor Cell-educated Periprostatic Adipose Tissue Acquires an Aggressive Cancer-promoting Secretory Profile.** *Cell Physiol Biochem* 2012, **29**:233-240.
13. Dolinkova M, Dostalova I, Lacinova Z, Michalsky D, Haluzikova D, Mraz M, Kasalicky M, Haluzik M: **The endocrine profile of subcutaneous and visceral adipose tissue of obese patients.** *Mol Cell Endocrinol* 2008, **291**:63-70.
14. Gomez-Ambrosi J, Catalan V, Diez-Caballero A, Martinez-Cruz LA, Gil MJ, Garcia-Foncillas J, Cienfuegos JA, Salvador J, Mato JM, Fruhbeck G: **Gene**

- expression profile of omental adipose tissue in human obesity.** *FASEB J* 2004, **18**:215-217.
15. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP: **Summaries of Affymetrix GeneChip probe level data.** *Nucleic Acids Res* 2003, **31**:e15.
 16. Smyth GK: **Linear models and empirical bayes methods for assessing differential expression in microarray experiments.** *Stat Appl Genet Mol Biol* 2004, **3**:Article3.
 17. Reiner-Benaim A, Yekutieli D, Letwin NE, Elmer GI, Lee NH, Kafkafi N, Benjamini Y: **Associating quantitative behavioral traits with gene expression in the brain: searching for diamonds in the hay.** *Bioinformatics* 2007, **23**:2239-2246.
 18. Neville MJ, Collins JM, Gloyn AL, McCarthy MI, Karpe F: **Comprehensive human adipose tissue mRNA and microRNA endogenous control selection for quantitative real-time-PCR normalization.** *Obesity (Silver Spring)* 2011, **19**:888-892.
 19. Catalan V, Gomez-Ambrosi J, Rotellar F, Silva C, Rodriguez A, Salvador J, Gil MJ, Cienfuegos JA, Fruhbeck G: **Validation of endogenous control genes in human adipose tissue: relevance to obesity and obesity-associated type 2 diabetes mellitus.** *Horm Metab Res* 2007, **39**:495-500.
 20. Catalan V, Gomez-Ambrosi J, Rodriguez A, Ramirez B, Rotellar F, Valenti V, Silva C, Gil MJ, Fernandez-Real JM, Salvador J, Fruhbeck G: **Increased levels of calprotectin in obesity are related to macrophage content. Impact on inflammation and effect of weight loss.** *Mol Med* 2011.
 21. Cheng L, Darson MF, Bergstralh EJ, Slezak J, Myers RP, Bostwick DG: **Correlation of margin status and extraprostatic extension with progression of prostate carcinoma.** *Cancer* 1999, **86**:1775-1782.
 22. Valastyan S, Weinberg RA: **Tumor metastasis: molecular insights and evolving paradigms.** *Cell* 2011, **147**:275-292.
 23. del Pozo CH, Calvo RM, Vesperinas-Garcia G, Gomez-Ambrosi J, Fruhbeck G, Rubio MA, Obregon MJ: **Expression profile in omental and subcutaneous adipose tissue from lean and obese subjects. Repression of lipolytic and lipogenic genes.** *Obes Surg* 2011, **21**:633-643.
 24. Serradeil-Le Gal C, Lafontan M, Raufaste D, Marchand J, Pouzet B, Casellas P, Pascal M, Maffrand JP, Le Fur G: **Characterization of NPY receptors controlling lipolysis and leptin secretion in human adipocytes.** *FEBS Lett* 2000, **475**:150-156.
 25. Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB, Spiegelman BM: **Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1.** *J Clin Invest* 1998, **101**:1-9.
 26. Fruhbeck G, Gomez-Ambrosi J: **Depot-specific differences in the lipolytic effect of leptin on isolated white adipocytes.** *Med Sci Monit* 2002, **8**:BR47-55.
 27. Aversa A, Caprio M, Antelmi A, Armani A, Brama M, Greco EA, Francomano D, Calanchini M, Spera G, Di Luigi L, et al: **Exposure to phosphodiesterase type 5 inhibitors stimulates aromatase expression in human adipocytes in vitro.** *J Sex Med* 2011, **8**:696-704.
 28. Baulande S, Lasnier F, Lucas M, Pairault J: **Adiponutrin, a transmembrane protein corresponding to a novel dietary- and obesity-linked mRNA specifically expressed in the adipose lineage.** *J Biol Chem* 2001, **276**:33336-33344.

29. Wang Y, Botolin D, Xu J, Christian B, Mitchell E, Jayaprakasam B, Nair MG, Peters JM, Busik JV, Olson LK, Jump DB: **Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity.** *J Lipid Res* 2006, **47**:2028-2041.
30. Fullerton MD, Hakimuddin F, Bakovic M: **Developmental and metabolic effects of disruption of the mouse CTP:phosphoethanolamine cytidylyltransferase gene (Pcyt2).** *Mol Cell Biol* 2007, **27**:3327-3336.
31. Arner P, Spalding KL: **Fat cell turnover in humans.** *Biochem Biophys Res Commun* 2010, **396**:101-104.
32. Ishiko O, Sumi T, Yoshida H, Hyun Y, Ogita S: **Comparison of expression of apoptosis regulatory proteins in the adipose tissue of tumor-bearing and diet-restricted rabbits.** *Int J Mol Med* 2001, **8**:543-547.
33. Dallabrida SM, Zurakowski D, Shih SC, Smith LE, Folkman J, Moulton KS, Rupnick MA: **Adipose tissue growth and regression are regulated by angiopoietin-1.** *Biochem Biophys Res Commun* 2003, **311**:563-571.
34. Hu Z, Chen L, Zhang J, Li T, Tang J, Xu N, Wang X: **Structure, function, property, and role in neurologic diseases and other diseases of the sHsp22.** *J Neurosci Res* 2007, **85**:2071-2079.
35. Sun Z, Cheng Z, Taylor CA, McConkey BJ, Thompson JE: **Apoptosis induction by eIF5A1 involves activation of the intrinsic mitochondrial pathway.** *J Cell Physiol* 2010, **223**:798-809.
36. Qiu J, Cheng R, Zhou XY, Zhu JG, Zhu C, Qin DN, Kou CZ, Guo XR: **Gene expression profiles of adipose tissue of high-fat diet-induced obese rats by cDNA microarrays.** *Mol Biol Rep* 2010, **37**:3691-3695.
37. Sierra-Honigsmann MR, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR: **Biological action of leptin as an angiogenic factor.** *Science* 1998, **281**:1683-1686.
38. Joo HJ, Kim H, Park SW, Cho HJ, Kim HS, Lim DS, Chung HM, Kim I, Han YM, Koh GY: **Angiopoietin-1 promotes endothelial differentiation from embryonic stem cells and induced pluripotent stem cells.** *Blood* 2011, **118**:2094-2104.
39. Satoh N, Yamada Y, Kinugasa Y, Takakura N: **Angiopoietin-1 alters tumor growth by stabilizing blood vessels or by promoting angiogenesis.** *Cancer Sci* 2008, **99**:2373-2379.
40. Park J, Scherer PE: **Leptin and cancer: from cancer stem cells to metastasis.** *Endocr Relat Cancer* 2011, **18**:C25-29.
41. Yang K, Guan H, Arany E, Hill DJ, Cao X: **Neuropeptide Y is produced in visceral adipose tissue and promotes proliferation of adipocyte precursor cells via the Y1 receptor.** *FASEB J* 2008, **22**:2452-2464.
42. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, Romero IL, Carey MS, Mills GB, Hotamisligil GS, et al: **Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth.** *Nat Med* 2011, **17**:1498-1503.
43. Ribeiro R, Lopes C, Medeiros R: **The link between obesity and prostate cancer: the leptin pathway and therapeutic perspectives.** *Prostate Cancer Prostatic Dis* 2006, **9**:19-24.
44. Discacciati A, Orsini N, Wolk A: **Body mass index and incidence of localized and advanced prostate cancer--a dose-response meta-analysis of prospective studies.** *Ann Oncol* 2012.

45. Harvey AE, Lashinger LM, Hursting SD: **The growing challenge of obesity and cancer: an inflammatory issue.** *Ann N Y Acad Sci* 2011, **1229**:45-52.
46. Ribeiro R, Araujo A, Lopes C, Medeiros R: **Immunoinflammatory mechanisms in lung cancer development: is leptin a mediator?** *J Thorac Oncol* 2007, **2**:105-108.
47. Wheway J, Mackay CR, Newton RA, Sainsbury A, Boey D, Herzog H, Mackay F: **A fundamental bimodal role for neuropeptide Y1 receptor in the immune system.** *J Exp Med* 2005, **202**:1527-1538.
48. Thompson C, Cloutier A, Bosse Y, Poisson C, Larivee P, McDonald PP, Stankova J, Rola-Pleszczynski M: **Signaling by the cysteinyl-leukotriene receptor 2. Involvement in chemokine gene transcription.** *J Biol Chem* 2008, **283**:1974-1984.
49. Simopoulos AP: **Genetic variants in the metabolism of omega-6 and omega-3 fatty acids: their role in the determination of nutritional requirements and chronic disease risk.** *Exp Biol Med (Maywood)* 2010, **235**:785-795.
50. Maier B, Ogihara T, Trace AP, Tersey SA, Robbins RD, Chakrabarti SK, Nunemaker CS, Stull ND, Taylor CA, Thompson JE, et al: **The unique hypusine modification of eIF5A promotes islet beta cell inflammation and dysfunction in mice.** *J Clin Invest* 2010, **120**:2156-2170.
51. Hoon Kim J, Lee SY, Myung SC, Kim YS, Kim TH, Kim MK: **Clinical significance of the leptin and leptin receptor expressions in prostate tissues.** *Asian J Androl* 2008, **10**:923-928.
52. Wurmbach JH, Hammerer P, Sevinc S, Huland H, Ergun S: **The expression of angiopoietins and their receptor Tie-2 in human prostate carcinoma.** *Anticancer Res* 2000, **20**:5217-5220.
53. Hsing AW, Sakoda LC, Chua S, Jr.: **Obesity, metabolic syndrome, and prostate cancer.** *Am J Clin Nutr* 2007, **86**:s843-857.
54. O'Carroll SJ, Mitchell MD, Faenza I, Cocco L, Gilmour RS: **Nuclear PLCbeta1 is required for 3T3-L1 adipocyte differentiation and regulates expression of the cyclin D3-cdk4 complex.** *Cell Signal* 2009, **21**:926-935.
55. Xu A, Suh PG, Marmy-Conus N, Pearson RB, Seok OY, Cocco L, Gilmour RS: **Phosphorylation of nuclear phospholipase C beta1 by extracellular signal-regulated kinase mediates the mitogenic action of insulin-like growth factor I.** *Mol Cell Biol* 2001, **21**:2981-2990.
56. Zhou W, Wang X, Li L, Feng X, Yang Z, Zhang W, Hu R: **Depletion of tubulin polymerization promoting protein family member 3 suppresses HeLa cell proliferation.** *Mol Cell Biochem* 2010, **333**:91-98.
57. Uehara R, Nozawa RS, Tomioka A, Petry S, Vale RD, Obuse C, Goshima G: **The augmin complex plays a critical role in spindle microtubule generation for mitotic progression and cytokinesis in human cells.** *Proc Natl Acad Sci U S A* 2009, **106**:6998-7003.
58. Nazarenko I, Rana S, Baumann A, McAlear J, Hellwig A, Trendelenburg M, Lochnit G, Preissner KT, Zoller M: **Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation.** *Cancer Res* 2010, **70**:1668-1678.
59. Katoh M: **Cancer genomics and genetics of FGFR2 (Review).** *Int J Oncol* 2008, **33**:233-237.
60. Konishi M, Mikami T, Yamasaki M, Miyake A, Itoh N: **Fibroblast growth factor-16 is a growth factor for embryonic brown adipocytes.** *J Biol Chem* 2000, **275**:12119-12122.

61. Wang J, Gu Q, Li M, Zhang W, Yang M, Zou B, Chan S, Qiao L, Jiang B, Tu S, et al: **Identification of XAF1 as a novel cell cycle regulator through modulating G(2)/M checkpoint and interaction with checkpoint kinase 1 in gastrointestinal cancer.** *Carcinogenesis* 2009, **30**:1507-1516.
62. Cho HJ, Park SM, Hwang EM, Baek KE, Kim IK, Nam IK, Im MJ, Park SH, Bae S, Park JY, Yoo J: **Gadd45b mediates Fas-induced apoptosis by enhancing the interaction between p38 and retinoblastoma tumor suppressor.** *J Biol Chem* 2010, **285**:25500-25505.
63. Arora V, Cheung HH, Plenchette S, Micali OC, Liston P, Korneluk RG: **Degradation of survivin by the X-linked inhibitor of apoptosis (XIAP)-XAF1 complex.** *J Biol Chem* 2007, **282**:26202-26209.
64. Crichton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM: **DRAM, a p53-induced modulator of autophagy, is critical for apoptosis.** *Cell* 2006, **126**:121-134.
65. Zou B, Chim CS, Pang R, Zeng H, Dai Y, Zhang R, Lam CS, Tan VP, Hung IF, Lan HY, Wong BC: **XIAP-associated factor 1 (XAF1), a novel target of p53, enhances p53-mediated apoptosis via post-translational modification.** *Mol Carcinog* 2011.
66. Lorin S, Pierron G, Ryan KM, Codogno P, Djavaheri-Mergny M: **Evidence for the interplay between JNK and p53-DRAM signalling pathways in the regulation of autophagy.** *Autophagy* 2010, **6**:153-154.
67. Gewandter JS, Bambara RA, O'Reilly MA: **The RNA surveillance protein SMG1 activates p53 in response to DNA double-strand breaks but not exogenously oxidized mRNA.** *Cell Cycle* 2011, **10**:2561-2567.
68. Yang RB, Ng CK, Wasserman SM, Colman SD, Shenoy S, Mehraban F, Komuves LG, Tomlinson JE, Topper JN: **Identification of a novel family of cell-surface proteins expressed in human vascular endothelium.** *J Biol Chem* 2002, **277**:46364-46373.
69. Qiu W, David D, Zhou B, Chu PG, Zhang B, Wu M, Xiao J, Han T, Zhu Z, Wang T, et al: **Down-regulation of growth arrest DNA damage-inducible gene 45beta expression is associated with human hepatocellular carcinoma.** *Am J Pathol* 2003, **162**:1961-1974.
70. Hong YH, Nishimura Y, Hishikawa D, Tsuzuki H, Miyahara H, Gotoh C, Choi KC, Feng DD, Chen C, Lee HG, et al: **Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43.** *Endocrinology* 2005, **146**:5092-5099.
71. Sun T, Fu M, Bookout AL, Kliewer SA, Mangelsdorf DJ: **MicroRNA let-7 regulates 3T3-L1 adipogenesis.** *Mol Endocrinol* 2009, **23**:925-931.
72. Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, Sun Y, Koo S, Perera RJ, Jain R, et al: **MicroRNA-143 regulates adipocyte differentiation.** *J Biol Chem* 2004, **279**:52361-52365.
73. Yang TT, Yu RY, Agadir A, Gao GJ, Campos-Gonzalez R, Tournier C, Chow CW: **Integration of protein kinases mTOR and extracellular signal-regulated kinase 5 in regulating nucleocytoplasmic localization of NFATc4.** *Mol Cell Biol* 2008, **28**:3489-3501.
74. Hirano T, Morii H, Nakazawa K, Murakami D, Yamashita A, Asahi J, Orimo H, Tamae K, Tokura Y: **Enhancement of adipogenesis induction by conditioned media obtained from cancer cells.** *Cancer Lett* 2008, **268**:286-294.

75. Buhe V, Loisel S, Pers JO, Le Ster K, Berthou C, Youinou P: **Updating the physiology, exploration and disease relevance of complement factor H.** *Int J Immunopathol Pharmacol* 2010, **23**:397-404.
76. Wilczek E, Rzepko R, Nowis D, Legat M, Golab J, Glab M, Gorlewicz A, Konopacki F, Mazurkiewicz M, Sladowski D, et al: **The possible role of factor H in colon cancer resistance to complement attack.** *Int J Cancer* 2008, **122**:2030-2037.
77. Ajona D, Hsu YF, Corrales L, Montuenga LM, Pio R: **Down-regulation of human complement factor H sensitizes non-small cell lung cancer cells to complement attack and reduces in vivo tumor growth.** *J Immunol* 2007, **178**:5991-5998.
78. Choy LN, Spiegelman BM: **Regulation of alternative pathway activation and C3a production by adipose cells.** *Obes Res* 1996, **4**:521-532.
79. Tedder TF, Engel P: **CD20: a regulator of cell-cycle progression of B lymphocytes.** *Immunol Today* 1994, **15**:450-454.
80. Nilsson NE, Kotarsky K, Owman C, Olde B: **Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids.** *Biochem Biophys Res Commun* 2003, **303**:1047-1052.
81. Gunzel D, Stuver M, Kausalya PJ, Haisch L, Krug SM, Rosenthal R, Meij IC, Hunziker W, Fromm M, Muller D: **Claudin-10 exists in six alternatively spliced isoforms that exhibit distinct localization and function.** *J Cell Sci* 2009, **122**:1507-1517.

Figure 1. Reverse transcription PCR analysis of *PCA3* in PP adipose tissue samples. GR, gene ruler 100 bp; (+) positive control (prostate tumor sample); (-) negative control (without cDNA); 1-to-6, periprostatic adipose tissue samples of patients with LAPCa.

Figure 2. Validation of selected genes by Real-time PCR. BPH, benign prostatic hyperplasia; LAPCa, locally advanced prostate cancer (pT2c); NP, normoponderal (BMI < 25 kg/m²); OB/OW, obese/overweight (BMI ≥ 25 kg/m²). *ANGPT1*, angiopoietin 1; *CFH*, complement factor H; *CLDN10*, claudin 10; *FAS*, fatty acid desaturase; *LEP*, leptin; *mir-let-7a-2*, microRNA let-7a-2. The gene expression in NP subjects or BHP was assumed to be 1. **P*<0.05 vs NP or BPH groups.

Figure 3. Resume of PP adipose tissue changes in obese/overweight. Genes with altered expression are associated with the regulation of functional biological processes. Altered function likely results in chronic mild immunoinflammatory response and fat mass expansion, which ultimately impact prostate cancer progression. ASCs, adipose-derived stem cells; E-M-T, epithelial-to-mesenchymal transition. Down-regulated genes are in green color and up-regulated are red.

Figure 4. Resume of changes in PP adipose tissue of patients with prostate cancer. Periprostatic adipose tissue of patients bearing prostate cancer likely reveals the interactions between tumor cells and adipose tissue cells. The functional repercussions of altered genes in patients with prostate cancer prompt PP adipose tissue to increased hypercellularity and decreased immunosurveillance. The resulting influence of this favorable microenvironment is to foster prostate cancer progression. ASCs, adipose-

derived stem cells; down-regulated genes are in green color whereas up-regulated are red.

Supplementary Figure 1. Representative network and genes differently expressed in OB/OW vs NP in IPA analysis. Genes are represented as nodes and the biological relationship between two nodes is represented as an edge line. Uncolored genes were not identified as differently expressed in our experiment even though they are relevant to this network. Node shape indicates enzymes ($\diamond$), phosphatases (Δ), kinases (∇), G-protein coupled receptor ($\square$), growth factor ($\square$), transporter ($\triangle$), transcription factor ($\bigcirc$), other ($\circ$).

Table 1. Characteristics of participants included in the study (mean $\pm$ SE)

	BPH (n=6)	Localized PCa (n=6)	Locally advanced PCa (n=6)	P
Age (yrs)	67.4 $\pm$ 3.9	59.4 $\pm$ 2.9	66.9 $\pm$ 2.3	0.140 ^a
BMI (kg/m ²)	26.5 $\pm$ 1.5	25.2 $\pm$ 1.3	26.5 $\pm$ 1.6	0.783 ^a
Serum total PSA (ng/mL)	8.9 $\pm$ 4.7	6.7 $\pm$ 1.6	14.7 $\pm$ 3.0	0.114 ^b
Prostate weight (g)	107.0 $\pm$ 12.9	40.8 $\pm$ 4.2	71.2 $\pm$ 9.5	0.001 ^{a, c}
Serum leptin (μ g/mL)	3.7 $\pm$ 1.0	7.1 $\pm$ 2.5	4.9 $\pm$ 1.8	0.453 ^a
Combined Gleason score				
≤ 7 (3+4), n (%)	---	5 (83%)	4 (67%)	
≥ 7 (4+3), n (%)	---	1 (17%)	2 (33%)	1.000 ^d

^a ANOVA for independent measures; ^b Kruskal-Wallis test; ^c LSD post-hoc analysis: BPH vs. localized PCa, $P < 0.0001$, BPH vs. locally advanced PCa, $P = 0.018$, locally advanced PCa vs. localized PCa, $P = 0.040$; ^d Fisher exact test; BPH, benign prostatic hyperplasia; PCa, prostate cancer; BMI, body mass index; PSA, prostate specific antigen.

Table 2. Altered genes in microarray analysis in OB/OW PP adipose tissue (overall and by prostatic disease)

Probe set	Gene Name	Gene Description	Fold Change	P
<i>All subjects</i>				
8098146	<i>NPY5R</i>	neuropeptide Y receptor Y5	1.80	1.49E-05
7948612	<i>FADS1</i>	fatty acid desaturase 1	-1.73	1.16E-04
8103494	<i>NPY1R</i>	neuropeptide Y receptor Y1	1.97	1.56E-04
7969050	<i>CYSLTR2</i>	cysteinyl leukotriene receptor 2	1.89	2.19E-04
8073633	<i>PNPLA3</i>	patatin-like phospholipase domain containing 3	-1.56	5.74E-04
7930181	<i>AS3MT</i>	arsenic (+3 oxidation state) methyltransferase	1.53	6.54E-04
<i>Benign Prostate Hyperplasia</i>				
8020343	<i>ANKRD20A5</i>	ankyrin repeat domain 20 family; member A5	-1.51	3.95E-05
8163509	<i>ALAD</i>	aminolevulinatase dehydratase	1.63	1.30E-04
8152297	<i>ANGPT1</i>	angiotensinogen 1	1.63	2.08E-04
7937508	<i>CD151</i>	CD151 molecule (Raph blood group)	1.69	2.43E-04
7959102	<i>HSPB8</i>	heat shock 22kDa protein 8	1.51	3.88E-04
8047780	<i>SNORA41</i>	small nucleolar RNA; H/ACA box 41	-1.77	4.94E-04
7906056	<i>MSTO2P</i>	misato homolog 2 pseudogene	1.57	5.78E-04
7912975	<i>ALDH4A1</i>	aldehyde dehydrogenase 4 family; member A1	1.69	7.20E-04
7965206	<i>SLC6A15</i>	solute carrier family 6 (neutral amino acid transporter); member 15	-1.50	7.81E-04
8098146	<i>NPY5R</i>	neuropeptide Y receptor Y5	2.06	7.86E-04
8135909	<i>LEP</i>	leptin	1.95	8.00E-04
7965508	<i>LOC400061</i>	hypothetical gene supported by NM_014886	-1.65	9.88E-04

<i>LoPCa</i>				
7997239	PDXDC2	pyridoxal-dependent decarboxylase domain containing 2	1.99	8.30E-06
7927513	<i>FAM21C</i>	family with sequence similarity 21; member C	1.60	5.02E-05
7941501	<i>BANF1</i>	barrier to autointegration factor 1	-1.50	1.12E-04
7994026	<i>NPIPL3</i>	nuclear pore complex interacting protein-like 3	1.64	1.52E-04
8110417	<i>FAM153C</i>	family with sequence similarity 153; member C	1.63	3.06E-04
8005231	<i>FAM106C</i>	family with sequence similarity 106; member C pseudogene	1.85	3.36E-04
7994371	<i>NPIPL3</i>	nuclear pore complex interacting protein-like 3	1.64	6.53E-04
8019280	<i>PCYT2</i>	phosphate cytidylyltransferase 2; ethanolamine	-1.54	7.55E-04
7961365	<i>MANSC1</i>	MANSC domain containing 1	1.50	7.60E-04
8000537	<i>PDXDC2</i>	pyridoxal-dependent decarboxylase domain containing 2	1.62	9.25E-04
<i>LAPCa</i>				
7902400	<i>SNORD45B</i>	small nucleolar RNA; C/D box 45B	-4.71	2.29E-04
7980861	<i>CATSPERB</i>	cation channel; sperm-associated; beta	1.73	5.81E-04
8057004	<i>PDE11A</i>	phosphodiesterase 11A	2.59	6.68E-04
8004325	<i>EIF5A</i>	eukaryotic translation initiation factor 5A	-1.50	7.72E-04
7969050	<i>CYSLTR2</i>	cysteinyl leukotriene receptor 2	2.50	9.01E-04

Fold change and the correspondent P-value for each significant gene are presented, using the NP as reference. The bold ones with adjusted P<0.2

Table 3. Altered genes in microarray according to prostatic disease status (overall, OB/OW or NP group)

Probe set	Gene Name	Gene Description	Fold Change	P
LOCALLY ADVANCED PROSTATE CANCER vs BENIGN PROSTATIC HYPERPLASIA				
<i>All subjects</i>				
7952313	<i>MIRLET7A2</i>	microRNA let-7a-2	2.00	4.26E-06
7957540	<i>MRPL42</i>	mitochondrial ribosomal protein L42	1.63	5.49E-06
7980891	<i>TC2N</i>	tandem C2 domains; nuclear	2.25	8.26E-06
7915468	<i>CCDC23</i>	coiled-coil domain containing 23	1.70	1.93E-05
8002020	<i>TPPP3</i>	tubulin polymerization-promoting protein family member 3	1.66	8.62E-05
7980861	<i>CATSPERB</i>	cation channel; sperm-associated; beta	1.84	1.48E-04
7964927	<i>TSPAN8</i>	tetraspanin 8	1.91	2.31E-04
8168463	<i>FGF16</i>	fibroblast growth factor 16	1.67	2.93E-04
<i>NP subjects</i>				
126820	<i>GPR110</i>	G protein-coupled receptor 110	-1.56	7.92E-05
7952313	<i>MIRLET7A2</i>	microRNA let-7a-2	2.12	9.04E-05
7995825	<i>MT1F</i>	metallothionein 1F	-1.64	2.59E-04
8148962	<i>OR4F21</i>	olfactory receptor; family 4; subfamily F; member 21	1.57	3.1E-04
7910381	<i>DUSP5P</i>	dual specificity phosphatase 5 pseudogene	-1.74	3.93E-04
8024485	<i>GADD45B</i>	growth arrest and DNA-damage-inducible; beta	-1.56	9.47E-04
7957540	<i>MRPL42</i>	mitochondrial ribosomal protein L42	1.60	9.86E-04
<i>OB/OW subjects</i>				
7980861	<i>CATSPERB</i>	cation channel; sperm-associated; beta	2.50 *	1.91E-06

79080891	<i>TC2N</i>	tandem C2 domains; nuclear	2.98 *	4.31E-06
7908488	<i>CFHR1</i>	complement factor H-related 1	1.88 *	3.16E-05
8078529	<i>STAC</i>	SH3 and cysteine rich domain	1.50 *	5.79E-05
8086615	<i>LRRC2</i>	leucine rich repeat containing 2	1.69	9.02E-05
8126382	<i>C6orf132</i>	chromosome 6 open reading frame 132	1.50	1.1E-04
7901931	<i>EFCAB7</i>	EF-hand calcium binding domain 7	1.54	1.5E-04
7902400	<i>SNORD45B</i>	small nucleolar RNA; C/D box 45B	-5.18	1.9E-04
7957452	<i>ALX1</i>	ALX homeobox 1	1.55	2.0E-04
7903203	<i>SNX7</i>	sorting nexin 7	1.55	2.9E-04
7908459	<i>CFH</i>	complement factor H	1.70	3.0E-04
7952313	<i>MIRLET7A2</i>	microRNA let-7a-2	1.88	4.0E-04
7957540	<i>MRPL42</i>	mitochondrial ribosomal protein L42	1.65	4.0E-04
8045423	<i>SNORA40</i>	small nucleolar RNA; H/ACA box 40	1.56	5.3E-04
7958019	<i>DRAM1</i>	DNA-damage regulated autophagy modulator 1	1.55	5.3E-04
7969640	<i>CLDN10</i>	claudin 10	1.59	6.3E-04
8076586	<i>SCUBE1</i>	signal peptide; CUB domain; EGF-like 1	-1.73	7.2E-04
8146024	<i>ADAM32</i>	ADAM metalloproteinase domain 32	1.67	8.0E-04
8050160	<i>MBOAT2</i>	membrane bound O-acyltransferase domain containing 2	1.55	8.2E-04
7968351	<i>C13orf33</i>	chromosome 13 open reading frame 33	1.73	8.3E-04
7915468	<i>CCDC23</i>	coiled-coil domain containing 23	1.80	8.3E-04
8027862	<i>FFAR2</i>	free fatty acid receptor 2	-1.58	9.2E-04

LOCALIZED PROSTATE CANCER vs BENIGN PROSTATIC HYPERPLASIA

<i>All subjects</i>			
7919349	RNU1-1	RNA; U1 small nuclear 1	-2.18 1.50E-07
7919576	RNU1-1	RNA; U1 small nuclear 1	-1.96 2.47E-07
7952313	<i>MIRLET7A2</i>	microRNA let-7a-2	1.79 6.21E-05
7980891	<i>TC2N</i>	tandem C2 domains; nuclear	1.98 6.26E-05
7978568	<i>RNU1-1</i>	RNA; U1 small nuclear 1	-1.65 9.43E-05
7981964	<i>SNORD116-8</i>	small nucleolar RNA; C/D box 116-8	-1.91 1.07E-04
8019709	<i>RNU2-1</i>	RNA; U2 small nuclear 1	-1.69 1.23E-04
7960052	<i>SNORA49</i>	small nucleolar RNA; H/ACA box 49	-1.73 2.00E-04
7901048	<i>SNORD46</i>	small nucleolar RNA; C/D box 46	-1.90 2.50E-04
7937483	<i>SNORA52</i>	small nucleolar RNA; H/ACA box 52	-1.60 3.97E-04
8165698	<i>MIR1977</i>	microRNA 1977	-2.16 4.19E-04
8044961	<i>RNU4ATAC</i>	RNA; U4atac small nuclear (U12-dependent splicing)	-1.63 4.26E-04
8133106	<i>SNORA22</i>	small nucleolar RNA; H/ACA box 22	-1.82 4.56E-04
7967030	<i>RNU4-1</i>	RNA; U4 small nuclear 1	-2.32 4.64E-04
8086752	<i>SNORD13</i>	small nucleolar RNA; C/D box 13	-1.54 5.22E-04
8026875	<i>SNORA68</i>	small nucleolar RNA; H/ACA box 68	-1.84 6.64E-04
8004184	<i>XAF1</i>	XIAP associated factor 1	-1.64 8.67E-04
<i>NP subjects</i>			
8001067	HERC2P4	hect domain and RLD 2 pseudogene 4	-1.77 1.22E-06
7997239	PDXDC2	pyridoxal-dependent decarboxylase domain containing 2	-2.12 2.69E-06
8000692	BOLA2	bolA homolog 2 (E. coli)	-1.52 3.41E-06

8000651	<i>SMG1</i>	SMG1 homolog; phosphatidylinositol 3-kinase-related kinase (C, elegans)	-1.61	3.76E-06
8019655	<i>TBC1D3B</i>	TBC1 domain family; member 3B	-1.56	1.26E-05
7919349	<i>RNU1-1</i>	RNA; U1 small nuclear 1	-2.30	2.27E-05
7927513	<i>FAM21C</i>	family with sequence similarity 21; member C	-1.66	2.31E-05
7993359	<i>NPIP</i>	nuclear pore complex interacting protein	-1.53	4.30E-05
8014633	<i>TBC1D3</i>	TBC1 domain family; member 3	-1.55	5.53E-05
8014437	<i>TBC1D3G</i>	TBC1 domain family; member 3G	-1.51	6.27E-05
7994026	<i>NPIPL3</i>	nuclear pore complex interacting protein-like 3	-1.69	9.02E-05
8178841	<i>TAP2</i>	transporter 2; ATP-binding cassette; sub-family B (MDR/TAP)	-1.76	1.42E-04
8106025	<i>BDP1</i>	B double prime 1; subunit of RNA polymerase III transcription initiation factor IIIB	-1.53	1.76E-04
7919576	<i>RNU1-1</i>	RNA; U1 small nuclear 1	-1.85	1.96E-04
8124926	<i>BAT1</i>	HLA-B associated transcript 1	-1.55	2.92E-04
8110417	<i>FAM153C</i>	family with sequence similarity 153; member C	-1.61	3.64E-04
8005231	<i>FAM106C</i>	family with sequence similarity 106; member C pseudogene	-1.78	4.64E-04
8000537	<i>PDXDC2</i>	pyridoxal-dependent decarboxylase domain containing 2	-1.67	4.75E-04
7994371	<i>NPIPL3</i>	nuclear pore complex interacting protein-like 3	-1.62	6.59E-04
7972577	<i>RPS26</i>	ribosomal protein S26	1.84	7.61E-04
8068363	<i>C21orf82</i>	chromosome 21 open reading frame 82	1.87	9.14E-04

OB/OW subjects

7969640	<i>CLDN10</i>	claudin 10	2.01	7.04E-06
8115355	<i>GLRA1</i>	glycine receptor; alpha 1	-1.50	3.71E-05
7919576	<i>RNU1-1</i>	RNA; U1 small nuclear 1	-2.08	4.02E-05

7978568	<i>RNU1-1</i>	RNA; U1 small nuclear 1	-2.10	5.36E-05
7967030	<i>RNU4-1</i>	RNA; U4 small nuclear 1	-3.84	6.35E-05
7980891	<i>TC2N</i>	tandem C2 domains; nuclear	2.31	1.10E-04
7919349	<i>RNU1-1</i>	RNA; U1 small nuclear 1	-2.08	1.22E-04
7952313	<i>MIRLET7A2</i>	microRNA let-7a-2	2.06	1.74E-04
8004247	<i>C17orf49</i>	chromosome 17 open reading frame 49	1.50	2.62E-04
8103326	<i>FGG</i>	fibrinogen gamma chain	1.51	2.81E-04
8021101	<i>HAUS1</i>	HAUS augmin-like complex; subunit 1	1.61	3.10E-04
8165698	<i>MIR1977</i>	microRNA 1977	-3.03	3.46E-04
8026875	<i>SNORA68</i>	small nucleolar RNA; H/ACA box 68	-2.26	5.28E-04
8108631	<i>VTRNA1-3</i>	vault RNA 1-3	-1.95	5.83E-04
8060854	<i>PLCB1</i>	phospholipase C; beta 1 (phosphoinositide-specific)	1.53	5.95E-04
8044961	<i>RNU4ATAC</i>	RNA; U4atac small nuclear (U12-dependent splicing)	-2.02	6.39E-04
7925182	<i>SNORA14B</i>	small nucleolar RNA; H/ACA box 14B	-1.76	6.66E-04
7901048	<i>SNORD46</i>	small nucleolar RNA; C/D box 46	-2.32	6.82E-04

LOCALLY ADVANCED PROSTATE CANCER vs LOCALIZED PROSTATE CANCER

All subjects

7919349	<i>RNU1-1</i>	RNA; U1 small nuclear 1	1.71	1.86E-05
8001067	<i>HERC2P4</i>	hect domain and RLD 2 pseudogene 4	1.60	2.36E-05
7919576	<i>RNU1-1</i>	RNA; U1 small nuclear 1	1.58	4.17E-05
7981964	<i>SNORD116-8</i>	small nucleolar RNA; C/D box 116-8	1.88	1.85E-04
7940287	<i>MS4A1</i>	membrane-spanning 4-domains; subfamily A; member 1	-2.15	2.74E-04

7972577	<i>RPS26</i>	ribosomal protein S26	-1.56	6.53E-04
8021081	<i>SLC14A1</i>	solute carrier family 14 (urea transporter); member 1 (Kidd blood group)	-1.51	9.13E-04
<i>NP subjects</i>				
8001067	<i>HERC2P4</i>	hect domain and RLD 2 pseudogene 4	2.06	5.14E-08
8000692	<i>BOLA2</i>	bolA homolog 2 (E, coli)	1.52	3.37E-06
7997239	<i>PDXDC2</i>	pyridoxal-dependent decarboxylase domain containing 2	2.00	6.36E-06
8000651	<i>SMG1</i>	SMG1 homolog; phosphatidylinositol 3-kinase-related kinase (C, elegans)	1.57	6.75E-06
7927513	<i>FAM21C</i>	family with sequence similarity 21; member C	1.74	8.44E-06
7993359	<i>NPIP</i>	nuclear pore complex interacting protein	1.51	5.38E-05
7994026	<i>NPIPL3</i>	nuclear pore complex interacting protein-like 3	1.73	5.61E-05
8119595	<i>RPL7L1</i>	ribosomal protein L7-like 1	1.63	5.71E-05
7990943	<i>GOLGA6L10</i>	golgin A6 family-like 10	1.55	6.08E-05
7919349	<i>RNU1-1</i>	RNA; U1 small nuclear 1	1.93	2.25E-04
7994371	<i>NPIPL3</i>	nuclear pore complex interacting protein-like 3	1.66	4.06E-04
8005231	<i>FAM106C</i>	family with sequence similarity 106; member C pseudogene	1.76	6.19E-04
8031027	<i>MIR519A2</i>	microRNA 519a-2	-1.57	6.16E-04
8110672	<i>OR4F16</i>	olfactory receptor; family 4; subfamily F; member 16	1.78	6.16E-04
8000537	<i>PDXDC2</i>	pyridoxal-dependent decarboxylase domain containing 2	1.62	8.24E-04
<i>OB/OW subjects</i>				
7982088	<i>SNORD115-41</i>	small nucleolar RNA; C/D box 115-41	-1.60	2.04E-04
7980861	<i>CATSPERB</i>	cation channel; sperm-associated; beta	1.78	2.92E-04
7982006	<i>SNORD116-29</i>	small nucleolar RNA; C/D box 116-29	1.77	5.84E-04

The bold ones with adjusted $P < 0.2$

Table 4. Significant canonical pathways ($P < 0.05$) in PP adipose tissue, according to OB/OW and prostate condition

Pathway	$-\log_{10}(P)$
All (OB/OW vs. NP)	
Glycerolipid metabolism	2.20
Lysine degradation	2.08
Leptin signaling in obesity	1.78
BPH (OB/OW vs. NP)	
ERK5 signaling	2.31
β -alanine metabolism	1.83
AMPK signaling	1.81
Parkinson's signaling	1.77
C ₂₁ -steroid hormone metabolism	1.77
LAPCa (OB/OW vs. NP)	
Histidine metabolism	1.91
Eicosanoid signaling	1.77
Linoleic acid metabolism	1.73
All (LAPCa vs. BPH)	
Antigen presentation pathway	3.11
Aminosugars metabolism	2.09
B cell development	1.81
OX40 signaling pathway	1.77
ERK5 signaling	1.59
p53 signaling	1.55
NP (LAPCa vs. BPH)	
T helper cell differentiation	2.24
Galactose metabolism	1.68
Hereditary breast cancer signaling	1.62
Role of osteoblasts, osteoclasts & condrocytes in Rheumatoid Arthritis	1.51
OB/OW (LAPCa vs. BPH)	
Synaptic long term depression	2.89
Urea cycle and metabolism of aminogroups	2.10
p53 signaling	2.10
Amyothropic lateral sclerosis signaling	2.02
Aminosugars metabolism	1.89
Extrinsic prothrombin activation pathway	1.73

p70S6K signaling	1.68
Cardiac β -adrenegic signaling	1.51

OB/OW, obese/overweight; NP, normoponderal; LAPCa, locally advanced prostate cancer;
 BPH, benign prostatic hyperplasia

ADDITIONAL FILES

Additional file 1

File format - .pdf

Title – Supplementary figure 1 - final

Description - Representative network and genes differently expressed in OB/OW vs NP in IPA analysis

Additional file 2

File format - .pdf

Title – Supplementary table 1

Description - Significant functions with altered networks and molecules in PP adipose tissue of OB/OW subjects

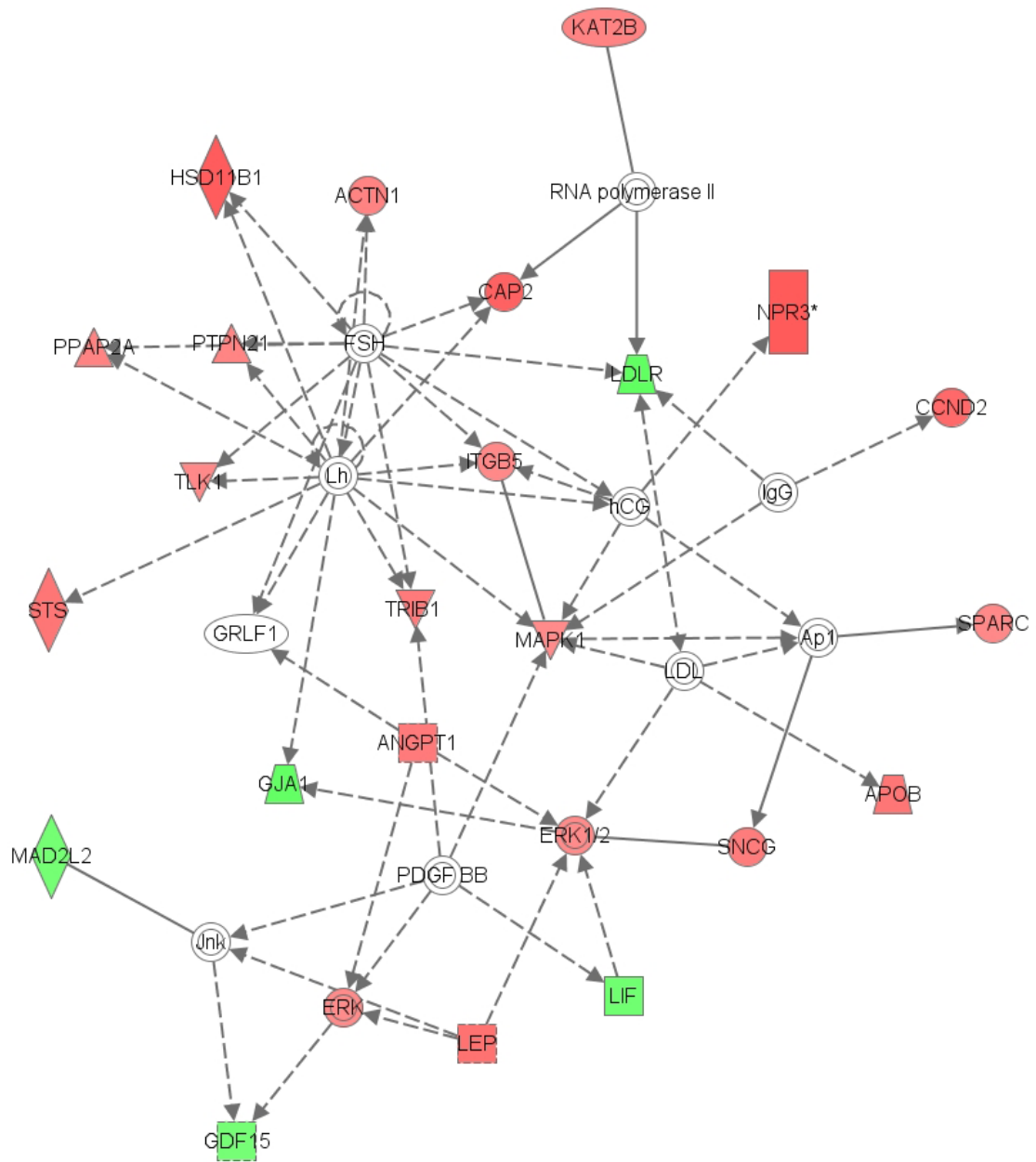
Additional file 3

File format - .pdf

Title – Supplementary table 2

Description - Significant functions with altered networks and molecules in PP adipose tissue of LAPCa vs BPH subjects

Supplementary figure 1



Supplementary Table 1. Significant functions with altered networks and molecules in PP adipose tissue of OB/OW subjects (P<0.0001 for inclusion)

Score	Molecules in Network	Top functions
<i>All subjects</i>		
35	ACTN1 , ANGPT1 , Ap1, APOB , CAP2 , CCND2 , ERK, ERK1/2, FSH, GDF15 , GJA1 , GRLF1, hCG, HSD11B1 , IgG, ITGB5 , Jnk, KAT2B , LDL, LDLR , LEP , Lh, LIF , MAD2L2 , MAPK1 , NPR3 , PDGF BB, PPAP2A , PTPN21 , RNA polymerase II, SNCG , SPARC , STS , TLK1 , TRIB1	Nutritional Disease, Cardiovascular Disease, Connective Tissue Development and Function
24	BUB1B, DAAM1 , EIF5A , EPB41L4B , ESR1, FAM125B, FANCC, FGF2, FHL1 , GLI1, GRB14 , KDM5B, LIMCH1 , MAML2 , NEDD4L , NHP2, NOTCH1, PDLIM4 , PDZRN3 , PPP1R12B , PRDM5, RPL22, RUNX1, SCNN1A, SETD8 , SKAP2 , SNRPE , TCEANC , TERC , TERT, TGFB1, TOM1L1 , TP53, TSG101, UGT2B7	Cell Death, Cellular Development, Cellular Growth and Proliferation
17	AGTR1, BCL2L11, CCNB1, CDK1, CDKN1A, CKAP2 , CTSB , ENDOG , FOXO3, GHRL , Hsp70, ING1, IRF1, LGALS12 , MAP1B , MAPK1 , mir-25 , NFIC, NME5 , NR3C1, PBRM1 , PLEKHF1 , PRKACB , PRL, PRLR, RB1, RNA polymerase II, ROCK1, SMARCA4, SMARCB1, SNCA, SOX4 , TP73, WDR26 , YY1	Cell Death, Cell Cycle, Cellular Development
14	AGPAT9 , AMOT , APOE, BCL6, CCND1, CCND2 , CDK1, CDKN2A, DPT , EGFL6 , EIF4EBP1 , EPB41L2 , FADS1 , FASN , HDAC1, HDAC2, HIC1, HIPK1 , Histone h4, Hsp70, LIF , MBD3 (includes EG:17192), MLH1, NCOR2, NFKB1, PMS2, PP2A, PTTG1, RUNX1T1, SPI1, THRB , TNS3 , TP53, TP63, YY1	Cell Cycle, Cell Death, Tissue Development
14	AICDA , ATAD2 , COL18A1, COL8A1 , COPB1 , ERAP1 , HGF, ID2, IFI16, IRAK1, IRAK2 , MAVS, MAX, Mek, MYC, MYD88, NFKB1, NFkB (complex), NOS2, NTRK2 , PERP , PIM1, RAC1, RASA3 , SETD7 , SH3RF2 , TAF6, TAF7, TAF10, TBP, TCF3, TMEM173 , TNFRSF1A, YY1, ZNF217	Gene Expression, Cell Death, Cellular Development
12	ADCY, ARHGEF6 , ARHGEF7, CCR6, CLINT1 , CXCR4, CYSLTR2 , EIF4A1, EIF4G3 , ELAVL1,	Cell Death, Cell-To-Cell Signaling and

	FOXP3, GIT1, Gm-csf, GNB1, GNG2 , HLA-DQB1 (includes others) , IFNG, IL4, IL23, IL27, IL17A, IL1B, Immunoglobulin, IRS2, LEP , NPY1R , NPY5R , PABPC1, PAK1, PYY, RAF1, RORC , YAP1 , YWHAB, YWHAG	Interaction, Hematological System Development and Function
<i>Nodular Prostatic Hyperplasia</i>		
18	ADRA1D , AKAP13, ANKRD32 , ARHGEF6 , ARHGEF7, CD151 , CKAP2, CLINT1 , CYP2C9 , FADS3 , FOXA3, GIT1, HNF4A, ITGA3, ITGB1, MAP1B , NFIC, NR1I3, NR3C1, PAX3, PERP , PHLDB2 , PI4KA, ROCK1, RORA, RPS6KA6 , SMARCA4, SPI1, TCF7 , TEAD1, TGS1, TP53BP2, YAP1 , YWHAG , ZAK	Cell-To-Cell Signaling and Interaction, Tissue Development, Cellular Movement
18	APOB , CD163, CEBPB, CXCR2, DAB2IP, DEFB4A/DEFB4B, EED, EPC1, EZH2 , FANCC, FOXL2, GRIA1 , HDAC1, HDAC8, HMOX1, HP , Hsp70, HTRA1 , IL6, IL13, IL22, MORF4L1 , MRFAP1, NFkB (complex), NEKBIZ , NR5A2, PCLO , RB1, SERPINB2 , SMG5 , SNX7 , SSX2 (includes others), STUB1, TNS1 , TOM1L1	Inflammatory Disease, Cancer, Connective Tissue Disorders
16	ADM , AMOT , ANXA2 , APOE, ATAD2 , CASP7, CCND1, CD200 , CEACAM1 (includes others), CEBPB, DGKZ , EBI3, HLA-F, HMOX1, HRAS, IFNAR2, IL8, IRF2, LEP , MAPK13 , Mek, MMD , NR1H4 , NR5A2, NRIP1, PLG, PRDM1, PSMB10 , SRC, STAC , STAT5A, TAP1 , TAPBP, TNF, TYK2	Inflammatory Response, Inflammatory Disease, Hematological System Development and Function
16	ADRB1 , ADRB2, AKT1, CAV1, CCNA2, CDKN1B, CDKN2A, CETN2 , COPS5, DLG1, E2f, EPAS1, FHL1 , FYN, HIF1A, HIST1H2AG (includes others) , MBNL2 , MDM2, NUP153, PAK3 , PPM1A , PRL, PTPN11 , RAD23B , RBL2, SLC6A15 , STC2 , TCF4, TERT, TFIIH, TP53, UCHL1 , VCAN , XPC, ZNF217	Cancer, Genetic Disorder, Respiratory Disease
15	ADCY, ANGPT1 , ATP9A , CD55, CTNND1, CXCR4, CYFIP2, DMD , ERK1/2, F7, FSH, GNB1, GNG2 , GRLF1, hCG, HSD11B1 , ITGA3, ITGA4, ITGB5, JMJD6 , LEP , Lh, LIF , Mek, PLAU, PLAUR , PTP4A1 , RASAL2, RGS12, SH3BP4, SNCG , TNFRSF11B, TNFSF11, TRO , VTN	Cell-To-Cell Signaling and Interaction, Tissue Development, Cellular Movement
13	AKR1C1/AKR1C2 , BAX, BCL2L13 , BUB1B, C13orf15 , CASP3, Cbp, CCNA2, CDH2, CDK1,	Cell Death, Renal Necrosis/Cell Death,

	CRYAB , DAAM1 , GLRX , HSPB1, HSPB8 , HTT, Interferon alpha, ITGAV, ITGB3, Jnk, KDM5B, KIF5B , LIMCH1 , MAP3K5, MAPK8, MT1F , NF2, P38 MAPK, PKMYT1, RAC1, TGFB1, TNFSF10, TP73, VEGFA, ZNF124	Embryonic Development
<i>LAPCa</i>		
17	ATF3, BCL3, DAAM1 , DDIT3, DZIP3 , E2F1, EGFR, ERBB2, ERK1/2, FADS1 , HIC1, HIF1A, IL6, IL8, ITGA2, JUN, KDM5B, Lh, MBD3 (includes EG:17192), mir-30 , mir-143 , PRDM5, PSIP1 , RELA, S100A2 , SCD , SMARCA4, SP1, SUZ12, TCEANC , TGFB1, TGFB2, TNS3 , TP53, TP73	Cell Cycle, Cell Death, Cancer
15	ADCY, AGT , ALB, ALOX15, BTC , CCR5, CYSLTR2 , E2F1, EGR1, EIF4EBP1, ERK1/2, IFNG, IL4, IL6, IL8, IL24, IL1B, IL1R1, IRAK1, IRAK2 , ITGB3, MEIS1 , MYD88, NOS2, NPY1R , NPY5R , ORM1/ORM2 , PPARG, PTN , PYY, SERPINE1, STAT1, TRAF6, USF1, VEGFC	Cellular Growth and Proliferation, Inflammatory Response, Organismal Injury and Abnormalities

Down-regulated molecules are in bold and color green, whereas up-regulated are red

Supplementary Table 2. Significant functions with altered networks and molecules in PP adipose tissue of LAPCa vs. BPH subjects (P<0.0001 for inclusion)

Score	Molecules in Network	Top functions
<i>All subjects</i>		
32	ALPL , BECN1 , CCL8 , CCNC , CIITA , DDX58 , EBAG9 , ERK1/2, FSH, GPR56, Histone h3, HLA-DRA , HLA-DRB1, HOXA9 , IDE , IL1F9 , LGALS8 , Lh, MAP2K3, MAPK8IP1 , MT2A , NAE1 , NFkB (complex), NLRP4, PPP2R5C , PRKD1 , RAB4A , RBBP8 , RNA polymerase II, SMAD5 , SMAD7 , TLN1, TNFRSF4, TNFSF4 , TP53BP2	Cell Death, Cellular Growth and Proliferation, Hematological System Development and Function
17	AGTR1, BCL2L1, EFTUD2, ELMOD3 , EPO, FANCC, FN1, FOXO3, GAB1, GULP1 , HNRNP, INSR, MYOC , NR3C1, OAT , p85 (pik3r), PDE3B , PLCB1 , PRKCI, RHOA, SART1, SCHIP1 , SMNDC1 , SNRNP200, SNX7 , SREBF1, SUMO2 , TF , TFRC, TNFRSF21 , TNFSF4 , YWHAG, YWHAH , YWHAQ, ZBTB1	Cell Cycle, Endocrine System Disorders, Gastrointestinal Disease
14	BIRC5, BMI1, CADM1 , CASP8, CASP9, CCL19, CCNG1 , CCRL1 , CD44, CD276 , CDC7, CLCA2 , CLDN7, CLRN1, CREBBP, CXCL10 , DLC1 , E2F3, EPCAM , HOXB6 , HOXB9 , IFN Beta, IFNA4 , IRF1, IRF3, IRF7, ITGB4, MCM3, MCM4 , MCM7, PLK1, ST18 , TCF7L2, TP53, TSPAN8	Cell Death, Cellular Growth and Proliferation, Cancer
14	Actin, ANG, ANTXR1 , ARHGEF3 , CASP1 , CASP3, CCL5, CFHR1 , CREB5 , ENPP2 , Fibrinogen, GAB1, HMGA1, IDI1 , IL6, IL13, IL6R, IL6ST , JAK1, JAK2, KIT, let-7 , NPC2, Ras, RNASE1, RNASE2, S100A8, S100A9, SWAP70 , THNSL2 , TMOD3 , TUBB2C , VASP, VCL, WNT5A	Cellular Movement, Immune Cell Trafficking, Cellular Development
12	Alp, BMP2, CCNA2, CD24 , CD34 , COL18A1, DDIT4 , E2F3, EGF, F2, FGF2, FN1, FOXO1, GADD45B , GDF6 , GLI1, GRIA3 , mir-221 , mir-331 , MIS12, MYB, NDC80, NSL1 , PLA2G4A, PRDM5, S100A10 , SELP, SERPINE1, SMAD4, SNRNP48 , TGFB1, TGFB2, TMEM79 , WISP2, ZWINT	Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function, Cardiovascular System Development and Function

11	ABCA1, BIRC2, CD40, CD40LG, CFLAR, EBI3, ETS1, FABP3 , FADD, HLA-DQA1 , HLA-DQA2 , HLA-DR, IGFBP2, IL27, IRF1, LATS1, LIN7A , MBD4 , PPARG, RASSF1, REL, RIPK2 , ROBO1 , SAV1 , SNTB1 , STAC , STK3, TAB2, TDP2 , TNF, TNFAIP3, TNFRSF1B, TRAF2, TRAF3, TRAF5	Cell Death, Cellular Growth and Proliferation, Hematological System Development and Function
11	ADAMTS5 , AHNAK2 , BCAR1, C14orf45 , CAMK2N1 , CaMKII, CARD16, CASP1 , CCL21, CCND1, DLL4 , EPO, ERK, FYB , HIC1, IL18, LAMA3, LGALS1, MAP2K3, Mek, NR4A2, NTRK3, PI3K (complex), POU2F1, PTK2B, PXN, Ras, RIPK2 , SHC3 , SKAP1, SNAPC1 , SRC, TLE1 , TLR4, TRIP6	Cellular Movement, Cell Death, Inflammatory Response
11	CD9 , CD82, CTNNB1, DEGS1 , EIF4A1, EZH2, FBL, GPA33 , GPR56, HDAC1, HDAC2, IgG, KLF4, KLK3, NME1, NOP58, NUFIP1, ODC1 , PABPC1, PAIP1 , PLEKHA5 , RAD9A, RNU4-1, RPAP3 , RUVBL1, RUVBL2, SVIL , TGFB2, TH, TOPBP1 , TSHZ3 , UGCG , WNT1, WNT5A, YWHAG	Cellular Movement, Cell Cycle, Cellular Development
6	CD3, CD74 , CD247, ERK1/2, FFAR2 , FHL2, FN1, GMNN , HAVCR1, IFN Beta, IFNG, IL4, IL8, ITGA3, ITGA11 , ITGB1, ITK, JUN, LAT, LCK, LGALS1, LGALS3, Lh, NCOR2, P38 MAPK, PI3K (complex), PTK2B, PTPRC, PXN, SH2D2A , SMARCA2, SMARCA4, SPTB , TP53, ZAP70	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking
NP		
22	ABCA1, CLEC4E , CXCR5 , DYNLT3 , Estrogen Receptor, FOXP3, FYB , GRB2, HLA-DRA , IL13, IL1B, ITPR1, LIN7A , Mapk, MEFV , mir-21 , MT2A , NCK1, NFkB (complex), NGFR , PIK3C2B , PLCXD1 , PTK2B, PYCARD, RFX5, SH2D2A , SKAP1, SKAP2, Sos, TGFB2, TIMP3, TNF, TNFRSF11B, TRADD, WAS	Inflammatory Disease, Gastrointestinal Disease, Genetic Disorder
20	BIRC5, CASP3, CDC25A, CDC25C, CDK1 , CTNNB1, Cyclin B, DLGAP5, E2F2, E2F3, E2F4, FEN1, GADD45A, GADD45B , GLRX , ITPR1, KDM5B, let-7 , LZTS1, MSH2, MT1A , MT1B , MT1E , MT1F , MYC, MYCN, SATB1, SFN, SLC25A19 , SLC9A3R1, TNFRSF10C , TNFSF10, TP73, TSPAN8 , UBE2T	Cell Cycle, Cancer, Connective Tissue Development and Function

- 17 **ADM**, APPL1, CASP8, **CCNG1**, **CD9**, **CD151**, **CHRM3**, CLCA2, CTNNB1, EZH2, **GMEB1**, GPR56, **HOXA9**, IgG, Integrin alpha 3 beta 1, **KCNIP3**, KIF23, KLK2, KLK6, **LIN9**, **MST1**, NOP58, **NRCAM**, PSEN1, RNU4-1, **RPAP3**, RUVBL2, **SERPINA3**, SMPD1, TP53, **UGCG**, VCAN, WNT1, WNT5A, ZDHHC2
- 15 **ADM**, **AKT3**, **ATF1**, **BBC3**, CALCA, CASP1, **CASP12**, CD40, **CD44**, CYBB, DIABLO, ELAVL1, **FABP3**, **FCGR2B**, FOXP3, IFNG, IL4, IL22, IL24, **IL13RA1**, MAPK14, MUC5AC/MUC5B, NOD2, PLA2G2A, PPARD, **PPP2R1B**, **RIPK2**, **SCUBE1**, SELE, SELP, SOD2, **STAC**, TNF, TNFRSF1B, TRIP6
- 15 Actin, Alp, **ANTXR1**, BIRC5, CASP3, CCNA2, **CD34**, COL18A1, Collagen(s), **CORO2B**, CREBBP, EP300, F3, FASLG, FGF2, FN1, **GDF6**, **HOXB6**, ITGB2, **KRT81**, **let-7**, **mir-331**, **MLXIPL**, MYB, MYBL2, **NPAS2**, PCNA, **RHOD**, SELE, SELP, **TFPI**, TGFB1, THBS1, **TMOD3**, VCL
- 14 BRCA1, CD40, **CHIC2**, **EBAG9**, ELAVL1, EPO, FANCC, FN1, GAB1, IL1B, MAP3K14, MAPK1, MCL1, **MYOC**, **NAE1**, NR3C1, **OAT**, **PCLO**, **PLEKHA5**, PTGS2, RELA, RHOA, RNA polymerase II, **RPL4**, **SNX7**, SREBF1, **TDP2**, **TF**, TERC, TNFRSF1B, **TP53BP2**, TRAF2, TRAF3, TRAF5, YWHAG
- 14 AGTR1, **AHNAK2**, ATP2A2, CCL5, CD55, CDKN1C, **CSDA**, E2f, FSH, GPR56, hCG, HIC1, ITGA3, Lh, MAPK1, MAPK8, MMP2, NOTCH1, PIK3CD, Pka, **PLA2G10**, **PLCB1**, **PPP2R5C**, **PSIP1**, **SACS**, **SNAPC1**, THBS1, TLN1, **TRAM2**, **TRO**, TRPC3, **TUBB2C**, VCL, VEGFC, **WDR76**
- 14 26s Proteasome, Actin, **ANKRD36B (includes others)**, APP, BAX, BRCA1, **C13orf15**, **CD44**, CDK1, CDT1, CHD4, CTGF, **FADS3**, **FFAR2**, **GLRA1**, **GMNN**, HDAC1, HDAC2, Histone h4, **IVNS1ABP**, Jnk, KDM5B, NCOR2, **PDE3B**, **PSMB5**, PTGS2, REST, SIN3A, SMARCA2,

12 SMARCA4, SMARCB1, **SMC5**, SP1, TFAP4, **TSHZ3**
ABCC4, **BCAP31**, CANX, **CD44**, **CD276**, **CFHR1**, CLDN7, CLRN1, **EPCAM**, ERK1/2, **F10**, Cell-To-Cell Signaling and Interaction, Tissue
F2R, FGF2, Fibrinogen, FN1, Focal adhesion kinase, GRB2, **H19**, HAMP, HFE2, HMMR, ITGAM, Development, Cellular Movement
ITGB2, **LGALS8**, MAPK14, MET, MMP2, MMP7, MMP14, **NEO1**, OSM, Pka, SPP1, **TSPAN8**,
VEGFA

Altered molecules are bold. Down-regulated are in green color, whereas up-regulated are red.

Figure 1

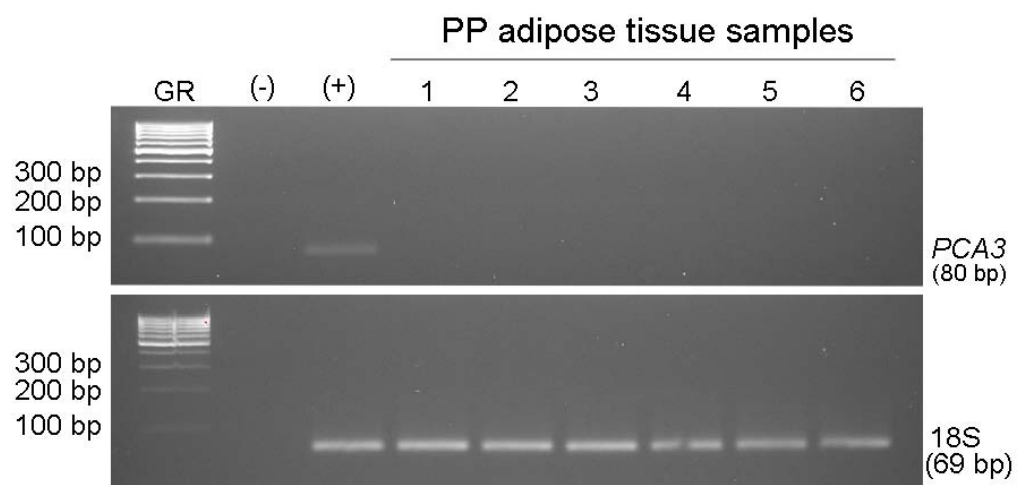


Figure 2

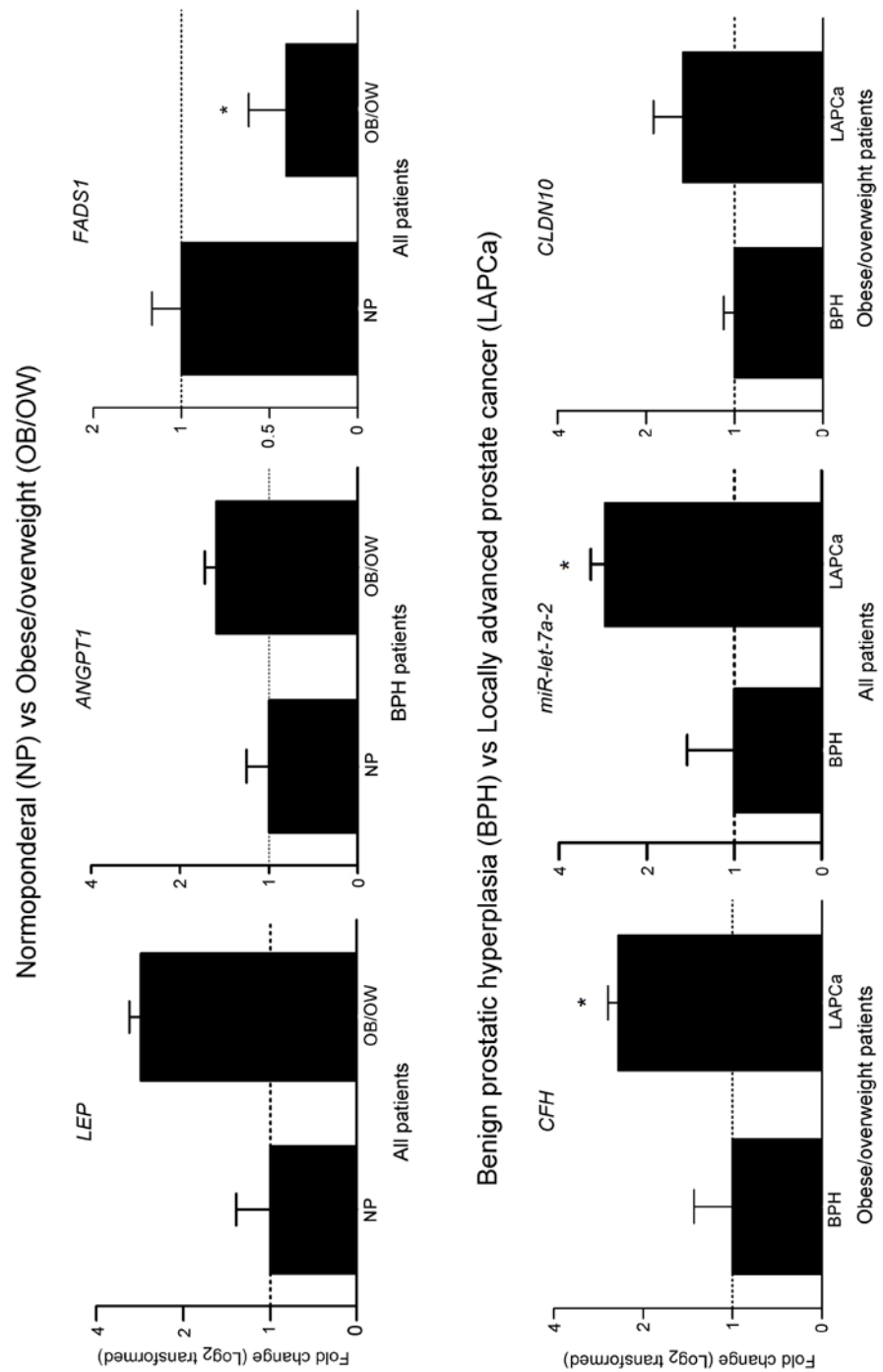


Figure 3

The diagram illustrates the transition from normoponderal to obese/overweight periprostatic adipose tissue and its impact on prostate cancer progression. It is divided into two main sections: the left section shows the transition from normoponderal to obese/overweight periprostatic adipose tissue, and the right section shows the resulting effects on prostate cancer progression.

Normoponderal Periprostatic Adipose Tissue: This section shows a cross-section of adipose tissue with normal-sized adipocytes. The surrounding stroma contains a few immune cells (macrophages, T cells, B cells) and blood vessels. The overall environment is characterized by low levels of inflammation and immune activity.

Obese/Overweight Periprostatic Adipose Tissue: This section shows a cross-section of adipose tissue with significantly enlarged adipocytes. The surrounding stroma is highly inflamed, with a dense infiltration of immune cells (macrophages, T cells, B cells) and increased blood vessel formation (angiogenesis). The overall environment is characterized by high levels of inflammation and immune activity.

Key Molecular Changes in Obese/Overweight Adipose Tissue:

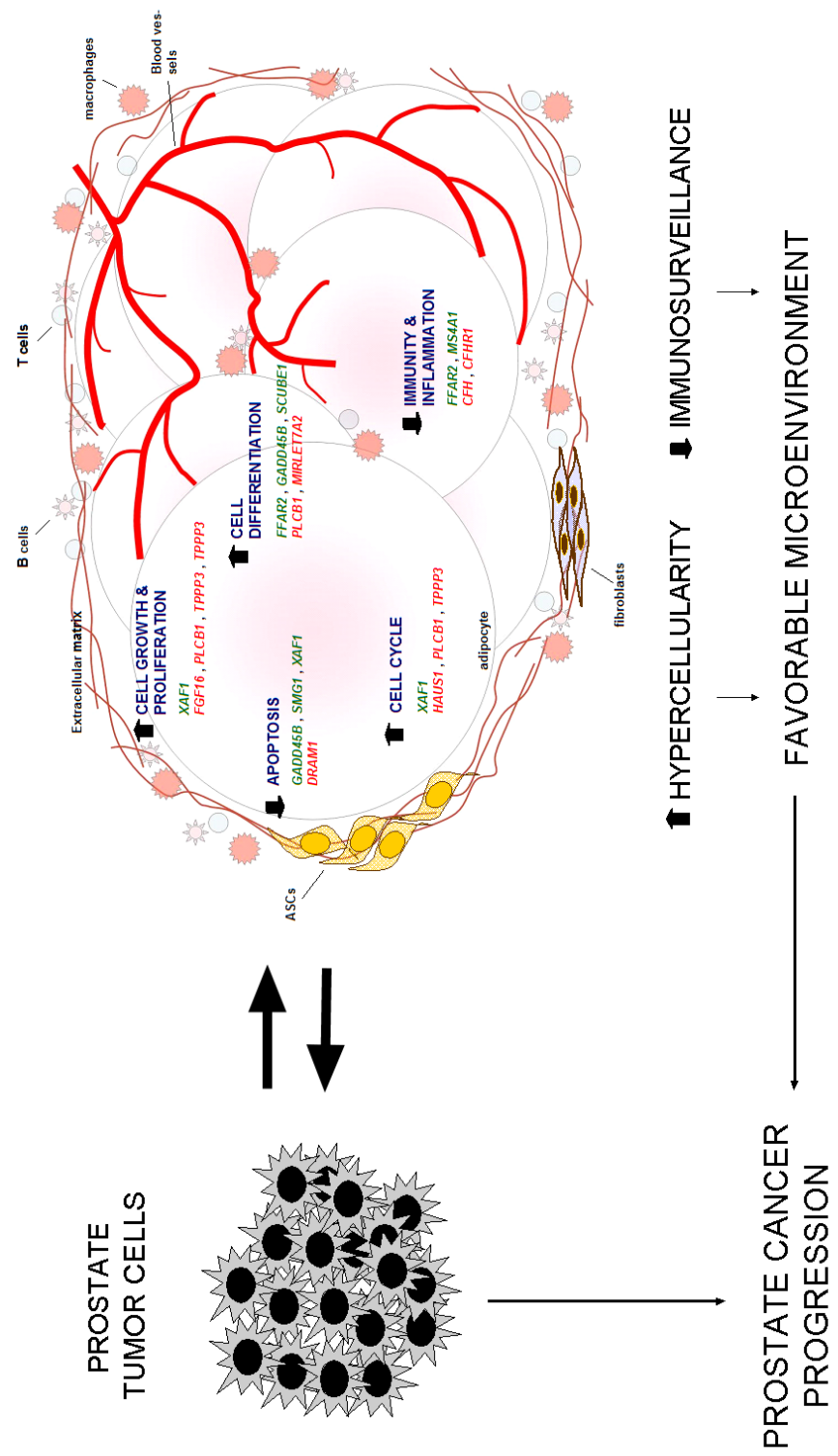
- Immunity & Inflammation:** Increased expression of *EFSA*, *FADS1*, *CYSLTR2*, *LEP*, *NPY1R*, and *NPY5R*.
- Cell Growth & Proliferation:** Increased expression of *ANGPT1*, *NPY1R*, and *LEP*.
- Lipolysis:** Decreased expression of *LEP*, *NPY1R*, *NPY5R*, and *PDE11*.
- Adipogenesis:** Increased expression of *FADS1*, *PCYT2*, *PNPLA3*, and *LEP*.
- Apoptosis:** Decreased expression of *EFSA*, *ANGPT1*, *HSPB8*, *LEP*, *NPY5R*, and *NPY1R*.
- Chemotaxis:** Increased expression of *LEP* and *ANGPT1*.
- Angiogenesis:** Increased expression of *ANGPT1* and *LEP*.
- Stromal Cells:** Increased expression of *CD161* in E-M-T cells and *CD161* in fibroblasts.

Impact on Prostate Cancer Progression:

- Chronic Mild Immunoinflammation:** The increased inflammation in the obese/overweight adipose tissue leads to chronic mild immunoinflammation.
- Fat Mass Expansion:** The increased inflammation leads to fat mass expansion.
- Available energy to provide fatty acids:** Fat mass expansion leads to available energy to provide fatty acids.
- Expression of adipokines:** Available energy to provide fatty acids leads to the expression of adipokines.
- Prostate Cancer Progression:** The expression of adipokines leads to prostate cancer progression.

Figure 4

PERIPROSTATIC ADIPOSE TISSUE



PAPER XII

Human periprostatic white adipose tissue is rich in stromal progenitor cells and a potential source of prostate tumor stroma

Running title: Periprostatic adipose stromal cells and prostate cancer

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ABSTRACT

A body of growing evidence now implicates white adipose tissue as a relevant source of stromal progenitor cells recruited to tumor microenvironment to form supportive tumor stroma. Whilst the role of periprostatic (PP) adipose tissue in prostate cancer progression has been barely appreciated, we sought to determine the ASC population in PP adipose tissue and the association with prostate cancer.

We isolated and characterized $CD34^{+}CD45^{-}CD31^{-}CD146^{-}$ progenitor cells (ASC) in paired samples of PP and pre-peritoneal visceral adipose tissue, from prostate tissue and peripheral blood mononuclear cells of prostate cancer and nodular prostatic hyperplasia patients. ASC were quantified by flow cytometry and confirmed through target gene expression.

Here we show a significantly higher amount of ASC in PP than visceral adipose tissue, independent of body mass index and prostatic disease. In the prostate, ASC are increased in cancer compared with prostatic nodular hyperplasia patients. Concordantly, adipisin gene (*CFD*) expression, which is known to be up-regulated in adipose stem cells, was overexpressed in PP adipose tissue, in the prostate of cancer patients and in prostate $CD34^{+}CD45^{-}CD31^{-}CD146^{-}$ sorted cells. ASC were found at higher levels in the blood of prostate cancer patients simultaneously overweight/obese. Present findings indicate PP adipose tissue is a reservoir of progenitor cells with potential to migrate towards prostate tumors, although its clinical significance merits further evaluation.

KEYWORDS

adipose tissue; obesity; prostate cancer; progenitor cells; tumor microenvironment

INTRODUCTION

Adipose tissue enveloping the prostate has been the focus of recent research in the context of obesity and prostate cancer. Previous work showed that PP adipose tissue has the potential to modulate prostate cancer aggressiveness through effects in tumor cell metastatic behavior, both directly and in response to tumor stimulation (1-3). Moreover, recent clinical reports evidenced an association between PP adipose tissue thickness and prostate cancer severity (4, 5).

Accumulating evidence over the last years demonstrated that interactions between non-tumor cells in the microenvironment and tumor cells influence their phenotypic behavior (6, 7). In demand for tumor microenvironment origins, it was found that most tumor vascular and fibrovascular stroma originates from neighboring adipose tissue (8), once during rapid tumor development local cells may not be capable to meet expanding needs. In fact, white adipose tissue is a rich reservoir of $CD34^{+}/CD45^{-}$ cells containing above two hundred-fold more progenitor cells/mL than bone marrow (9). These adipose tissue-derived stem cells have been described as $CD31^{-}/CD34^{+}/CD45^{-}/CD146^{-}$ (ASC) (10), with high proliferative potential and ability for migration and capillary formation (11). Their activity and amounts are modulated by the degree of adiposity and microenvironment (adipokines, hypoxia, oxidative stress) (12, 13).

Several studies strongly support that ASC home to tumor sites and promote tumor growth, either directly or after differentiation to supportive stroma cells (e.g., endothelial cells, myofibroblasts) (14-18). Furthermore, data specifically from murine prostate cancer models demonstrated that the presence of ASC on prostate tumors was mediated by stromal derived factor – 1/C-X-C chemokine receptor type 4 axis (15, 16, 19, 20).

Taken together, these findings suggest a role for ASC in prostate cancer pathophysiology, albeit no studies in humans have reported the eventual depots of origin or their deposition in prostate. Thus, we sought to investigate human PP and visceral adipose tissue depots as reservoirs of ASC and whether they could be found in the prostate of cancer or nodular hyperplasia (BPH) patients.

MATERIALS AND METHODS

Patients and sample collection

Men eligible for retropubic radical prostatectomy or prostate surgery of BPH, without other major co-morbidities, were included in this study after informed consent agreement. Clinicopathologic characteristics are described in Table 1. This study was approved by the ethics committees of D. Pedro V Military Hospital and Porto Hospital Centre.

Samples of PP (anterolateral fat pad) and visceral adipose tissue (pre-peritoneal visceral fat from the lower abdominal incision), and prostate tissue were collected during surgery. Adipose tissue was washed in phosphate-buffered saline (PBS) (Gibco, Invitrogen, Barcelona, Spain) and divided into two pieces, one immersed in RNAlater (Ambion, Applied Biosystems, Carlsbad, CA, USA); the other maintained in DMEM/F-12 medium (Gibco, Invitrogen, Barcelona, Spain). Immediately after prostate/nodular hyperplasia removal, a fragment of prostate from tumor-bearing region or from nodular hyperplasia, was collected and maintained in pre-warmed RPMI 1640 (Gibco, Invitrogen, Barcelona, Spain) media supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Barcelona, Spain) and 1% penicillin and streptomycin (Gibco, Invitrogen, Barcelona, Spain). Mirror sections of the prostate fragments were stained with hematoxylin-eosin and analyzed by uropathologists to ensure presence or absence of malignancy. Peripheral blood was collected before anesthetic induction.

Adipose tissue samples and prostate fragments were weighted, minced and incubated with collagenase A (2 mg/mL and 3 mg/mL, respectively) (Roche Applied Science, Mannheim, Germany) at 30°C with shaking (120 rpm) (1 and 2 hours, respectively).

Pellets were resuspended in supplemented (with biotin 16 μ M, panthotenate 18 μ M, ascorbate 100 μ M, all from Sigma Aldrich, St. Louis, MO, USA) DMEM/F-12 medium with 10% Newborn Calf Serum (NCS) (Sigma Aldrich, St. Louis, MO, USA) (adipose tissue) or RPMI 1640 with L-Glutamine and Hepes (25mM) (Gibco, Invitrogen, Barcelona, Spain) with 10% FBS (prostate), and filtered through a 100 μ m followed by 40 μ m cell strainers (BD Falcon, BD Biosciences, USA). Cells were cryopreserved in the respective media with 10% DMSO (Sigma Aldrich, St. Louis, MO, USA). Mononuclear cells from peripheral blood were isolated through a density gradient centrifugation (Histopaque 1083, Sigma Aldrich, St. Louis, MO, USA). Cells were cryopreserved in RPMI 1640 supplemented with L-Glutamine and Hepes (25mM) with 10% DMSO.

Flow cytometry analysis and cell sorting

The following directly conjugated mouse anti-human antibodies were used (all from Biolegend, San Diego, CA, USA): FITC- anti-CD31, APC- anti-CD34, PE- anti-CD146, and PerCP- anti-CD45. In short, cryopreserved cell suspension samples of adipose tissues and prostate were rapidly thawed and washed in PBS with 2% FBS (PBS-FBS). Approximately 2×10^6 adipose and cell suspension were stained with a cocktail of previously optimized concentrations of antibodies for 30 minutes at 4°C. For each sample, isotype matched controls were analyzed to acquire the appropriate settings. Data was collected in a BD FACScalibur cytometer (1 million gated events) and analyzed by FlowJo software (Ashland, OR, USA). Figure 1 depicts the identification criteria used for selecting the cellular populations of interest.

Live prostatic cells identified as CD45⁻/CD34⁺/CD31⁻/CD146⁻ were purified by cell sorting in a BD FACS Aria cytometer (n=6). Briefly, 2×10^6 cells were labeled with antibodies and 1 µg/ml of propidium iodide. Cell sorting was performed directly to RNeasy Micro Kit (Qiagen, Valencia, CA, USA) cell lysing solution and kept at -80°C.

RNA extraction and Real-Time polymerase chain reaction

Fragments of adipose tissue were homogenized in QIAzol Reagent (Qiagen, Valencia, CA, USA), and samples purified with the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA). RNA was extracted from an aliquot of prostate cells in tripure reagent (Roche Applied Science, Mannheim, Germany) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Sorted cells were purified with RNeasy Micro Kit. All RNA samples were treated with DNase I (Qiagen, Valencia, CA, USA).

The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA) and quality inspected using Experion RNA StdSens Chips in the Experion™ automated microfluidic electrophoresis system (BioRad Hercules, CA, USA). First strand cDNA was synthesized with 1 µg of total RNA from adipose tissues, 0.25 µg from prostate cell suspensions and 0.10 µg from sorted prostate cells using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). The transcript levels of adipsin (*CFD*) and *CD133* were quantified in duplicate by qPCR using SYBR Green in an iCycler iQ5 Real-Time PCR (BioRad Hercules, CA, USA). The expression of *PCA3* gene was evaluated using Taqman assay (Applied Biosystems, Foster City, CA, USA), in a StepOne Real-Time PCR (Applied Biosystems, Foster City, CA, USA). Results were normalized, for adipose tissues and prostate, to the levels of the ribosomal 18S rRNA and 28S rRNA, respectively; whereas

for *PCA3* we used *18S* and *ACTNB* as reference genes. Relative quantification was calculated using the REST 2009 software (Qiagen, Hilden, Germany), where relative mRNA expression was expressed as fold over the reference group.

Statistical analysis

Data are presented as mean $\pm$ standard error of mean (S.E.M.), except for non-parametric variables where median (min-max) was used. Cell populations evaluated by flow cytometry are expressed as percent of CD45-/CD34+ cells adjusted by gram of tissue or only the percent of cells in blood samples analyses. Departure from normality was tested using Kolomogorov-Smirnov. Values for percent of ASC per gram were log₁₀-transformed to become normally distributed. Unpaired t-test was used for statistical comparison between means. Stratified analyses were performed by comparing one group bearing prostate cancer and simultaneous body mass index (BMI) $\geq$ 25 kg/m² (obese/overweight, OB/OW), against all other subjects (without cancer and BMI < 25 kg/m² (NP), with cancer and NP, and without cancer and OB/OW). Spearman's correlation coefficients were determined to assess the statistical dependence between gene expression data (Cq of target gene normalized to reference gene) and the logarithmically transformed percent of ASC per gram of tissue.

The REST 2009 software (Qiagen, Hilden, Germany) was used to determine gene expression ratios. We used 10000 iterations to evaluate whether significant differences existed between comparison groups. Analyzes were performed using SPSS 17.0 (SPSS Inc., Chicago, USA), and p<0.05 was considered significant.

RESULTS

When comparing the amount of ASC between distinct anatomic origins, we found it was significantly increased in PP versus VIS adipose depot ($P<0.0001$), even after stratified analyses by prostatic disease (BPH, $P=0.001$; prostate cancer, $P=0.012$) (Figure 2A). Interestingly, in PP adipose tissue the quantity of ASC was significantly lower for prostate cancer compared with BPH patients ($P=0.023$), whereas the inverse was observed in VIS adipose tissue ($P=0.012$) (Figure 2B). To strengthen these findings, we evaluated adipon (CFD) mRNA transcripts abundance in adipose tissue, whose expression was shown to be highly up-regulated in ASC. In agreement with figure 2A, CFD was overexpressed by 2-fold in PP compared to VIS adipose tissue ($P=0.008$) (Figure 2C). Figure 2D schematically represents the relative ASC frequencies found in adipose depots, according to prostatic disease.

In the prostate, the amount of ASC cells was increased in cancers compared with BPH ($P=0.043$) (Figure 3A). Furthermore, CFD expression was 10-fold increased in prostate tumors from cancer patients ($P=0.186$), whereas in isolated ASC (sorted cells) from the prostate it was also increased in tumors compared to prostatic tissue of BPH ($P=0.192$) (3B). Moreover, the normalized CFD expression was well correlated with the amount of ASC cells in the prostatic tissue (Spearman coefficient, $r=-0.71$, $P=0.047$). CD133 is a specific stem and progenitor cell marker for a variety of cells, albeit it is not expressed in ASC. Interestingly, CD133 expression was up-regulated in prostatic tumors of cancer patients by 3.6-fold ($P=0.025$) (Figure 3C), but not in isolated ASC (sorted cells) (Figure 3D).

In the peripheral blood, although the ASC cell population was barely detectable, we found a trend for increased ASC-like cells in cancer compared to BPH patients (0.05 ± 0.01 and 0.03 ± 0.005 , respectively, $P=0.068$).

We evaluated *PCA3* mRNA transcript levels, since it informs about the presence of prostate tumor cells, and found that tumor samples used for ASC quantification from prostate cancer patients overexpressed *PCA3* (659-fold, $P=0.011$), thus confirming tumor samples are representative of malignancy areas.

In order to analyze the simultaneous influence of being overweight/obesity and bearing cancer in ASC frequencies, we compared subjects with these characteristics (OB/OW + PCa) to the other patients combined into one group (Others) (Figure 4). Subjects that are OB/OW and have prostate cancer present increased quantities of ASC in peripheral blood ($P=0.032$) and a statistical trend in visceral adipose tissue ($P=0.051$) and prostatic tissue ($P=0.051$). Noteworthy, the amount of ASC in PP adipose tissue was decreased in OB/OW with cancer ($P=0.040$).

DISCUSSION

White adipose tissue is starting to receive further attention, mainly because of accumulating evidence over the years that it is a highly active endocrine organ that secretes numerous factors, results in inflammation and modulates insulin signaling and lipid deregulation (21, 22). Recently, adipose tissue was found to contain also progenitor CD45⁺/CD34⁺/CD31⁺/CD146⁺ cells (ASC), which are highly represented in adipose tissue stromal vascular fraction (SVF), and display multipotency and proliferative capacity (10, 23, 24). One recently published study demonstrated that the neighboring adipose tissue considerably contributes with ASC to tumor vascular and fibrovascular stroma (8), while another work found that different fat depots possess unique capacities to promote tumor growth (25). Hence, we sought to investigate the accumulation of ASC in human PP adipose tissue, and to evaluate if these cells were found in the prostate of cancer or BPH patients.

In prostate cancer, the extension beyond the organ into PP fat has been associated with worst prognosis (26). This anatomic depot of adipose tissue, which lies in close proximity to the prostate, induces tumor cell aggressive behavior and is modulated by prostate tumor cell-derived factors (1-3), therefore resulting in interaction towards cancer progression. Furthermore, clinical studies revealed increased PP thickness in subjects with prostate cancer and advanced disease (4, 5). In present study, we found increased amounts of ASC in PP adipose tissue compared with another fat depot (VIS), regardless of prostatic disease. This finding suggests unique progenitor cell population amounts residing in PP adipose tissue and differences between anatomic adipose depots, which has been previously appreciated for other anatomic origins (25, 27). We hypothesize that the hypoxia, inflammation and oxidative stress conditions arising over

the course of prostatic gland cancerous or hyperplastic development (28, 29), may induce recruitment of ASC to the involving PP adipose tissue from blood and eventually other fat depots or stimulate their local proliferation.

In the prostatic tissue, we found higher amounts of CD45⁺/CD34⁺/CD31⁺/CD146⁺ progenitor cells in tumors, whereas a trend for increased number in circulation was observed for cancer patients compared with BPH subjects. Our findings in the human prostate are concordant with previous results in murine models, which demonstrated that ASC from adipose tissue home to tumors, through the modulation of the stromal derived factor – 1/C-X-C chemokine receptor type 4 axis (15, 16, 30). This axis was shown to be highly activated both in prostate cancer and benign hyperplasia (31, 32), driving the recruitment of ASC.

The most accessible option for tumors that necessitate overcoming growth demands is to exploit resources in close proximity to the site of tumor development (8). In the case of prostate cancer, the enveloping PP adipose tissue may be such a reservoir of ASC. However, local recruitment of these cells towards the tumor may result, as we observed, in reduced amount of ASC in the PP fat depot compared with BPH. Whether ASC migrate from PP adipose tissue into the prostate through the visceral pelvis fascia, which is conceivable in light of their previously described capacity to move and ability to produce matrix proteinases (33) or through the blood, is an interesting issue that remains to be established. Our findings seem to reflect an interactive migratory pattern between PP adipose tissue and prostate tumor cells. In our study, all except one of the prostate cancer men disclosed clinical and pathologically prostate-confined tumors, agreeing with the increased amount of ASC found in prostate tumors while decreased in PP adipose tissue of prostate cancer patients. Larger confirmatory studies are warranted.

As initially suggested (23), a recent study confirmed increased frequency of progenitor CD34⁺ cells in the circulation of obese (BMI $\geq$ 30 kg/m²) patients with colorectal cancer (34). A similar analysis was conducted in our group of patients, although the low number of obese patients prompted us to use a distinct cutoff (overweight and obese subjects with BMI $\geq$ 25 kg/m²), which has been reported before now (35, 36). We found a significantly higher frequency of CD45⁻/CD34⁺/CD31⁻/CD146⁻ progenitors in the circulation of OB/OW patients with prostate cancer, than in lean patients with prostate cancer and OB/OW controls. Since we also observed that ASC frequency is decreased in PP adipose tissue and increased in the prostate of OB/OW men with prostate cancer, their increase in blood may account for one possible route for ASC migration. Some reports indicate that ASC play a role in obesity and are regulated by diet and obesity (27, 37). If confirmed by further studies, the source of tumor stroma from adipose tissue depots that lie in close proximity to cancer foci, besides revealing a mechanistic link between obesity and cancer progression may identify surgical and medical interventions to disrupt tumor-stroma interactions.

To our knowledge, no prior reports evaluated the ASC pool in human prostate and PP adipose tissue. Nevertheless, our initial, hypothesis-generating study should be interpreted in the context of some potential limitations including lack of *in vitro* evaluation of ASC functional characteristics. In addition, the apparent heterogeneity of mesenchymal cells reflected by CD34 expression on stromal and perivascular cells depending on the organ and vasculature type also alerts for cautious interpretations. Nevertheless, CD34 is a frequently used marker to distinguish ASC from other cells in the SVF, and by adding CD31 it is possible to discriminate vascular endothelial cells (38). In blood and prostate, this panel allowed discrimination of ASC (CD45⁻CD34⁺CD31⁻CD146⁻), while excluding hematopoietic mesenchymal cells (CD45⁺).

Moreover, the lack of a specific ASC marker prompted us to investigate adipsin gene (*CFD*) expression, since it is overexpressed in low-passage and fresh ASC, and in comparison with extra-fat mesenchymal stem cells (18, 19). Additionally, *CFD* gene expression was correlated with the amount of ASC cells in the prostate. Conversely, *CD133* mRNA transcripts that are found in both cancer stem cells and stromal progenitor cells of the prostate (39), is an exclusion marker for ASC (40). Noteworthy, *CD133* expression was up-regulated in tumors compared with prostatic tissue of BPH patients, albeit its expression was almost completely abolished in the sorted prostatic CD45⁻CD34⁺CD31⁻CD146⁻ progenitor cells. These findings underpin flow cytometry results, supporting that the identified CD45⁻CD34⁺CD31⁻CD146⁻ progenitors may correspond to ASC in the prostate.

Our findings suggest that the PP fat depot may represent a source of progenitor cells to prostate tumor stroma. The present study further supports ASC migration through the blood in obese men with prostate cancer, although contiguous paths should be considered. If confirmed and extended by following studies, these findings may impact prostate cancer therapeutic interventions aimed at disrupting tumor-stroma crosstalk.

STATEMENT OF AUTHORS CONTRIBUTIONS AND ACKNOWLEDGEMENTS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; RR, CM, RS and AC conducted the experiments, RR wrote the manuscript.

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REFERENCES

1. Ribeiro RJ, Monteiro CP, Cunha VF, Azevedo AS, Oliveira MJ, Monteiro R, et al. Tumor Cell-educated Periprostatic Adipose Tissue Acquires an Aggressive Cancer-promoting Secretory Profile. *Cell Physiol Biochem*. 2012;29(1-2):233-40.
2. Finley DS, Calvert VS, Inokuchi J, Lau A, Narula N, Petricoin EF, et al. Periprostatic adipose tissue as a modulator of prostate cancer aggressiveness. *J Urol*. 2009;182(4):1621-7.
3. Ribeiro R, Monteiro C, Cunha V, Oliveira MJ, Freitas M, Fraga A, et al. Human periprostatic adipose tissue promotes prostate cancer aggressiveness in vitro. *J Exp Clin Cancer Res*. 2012;31(1):32.
4. van Roermund JG, Hinnen KA, Tolman CJ, Bol GH, Witjes JA, Bosch JL, et al. Periprostatic fat correlates with tumour aggressiveness in prostate cancer patients. *BJU Int*. 2011;107(11):1775-9.
5. Bhindi B, Trottier G, Elharram M, Fernandes KA, Lockwood G, Toi A, et al. Measurement of peri-prostatic fat thickness using transrectal ultrasonography (TRUS): a new risk factor for prostate cancer. *BJU Int*. 2012.
6. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell*. 2011;147(2):275-92.
7. Taylor RA, Risbridger GP. Prostatic tumor stroma: a key player in cancer progression. *Curr Cancer Drug Targets*. 2008;8(6):490-7.
8. Kidd S, Spaeth E, Watson K, Burks J, Lu H, Klopp A, et al. Origins of the tumor microenvironment: quantitative assessment of adipose-derived and bone marrow-derived stroma. *PLoS One*. 2012;7(2):e30563.
9. Martin-Padura I, Gregato G, Marighetti P, Mancuso P, Calleri A, Corsini C, et al. The white adipose tissue used in lipotransfer procedures is a rich reservoir of CD34+ progenitors able to promote cancer progression. *Cancer Res*. 2012;72(1):325-34.
10. Tarnok A, Ulrich H, Bocsi J. Phenotypes of stem cells from diverse origin. *Cytometry A*. 2010;77(1):6-10.
11. Suga H, Matsumoto D, Eto H, Inoue K, Aoi N, Kato H, et al. Functional implications of CD34 expression in human adipose-derived stem/progenitor cells. *Stem Cells Dev*. 2009;18(8):1201-10.
12. Maumus M, Sengenès C, Decaunes P, Zakaroff-Girard A, Bourlier V, Lafontan M, et al. Evidence of in situ proliferation of adult adipose tissue-derived progenitor cells: influence of fat mass microenvironment and growth. *J Clin Endocrinol Metab*. 2008;93(10):4098-106.
13. Bellows CF, Zhang Y, Simmons PJ, Khalsa AS, Kolonin MG. Influence of BMI on level of circulating progenitor cells. *Obesity (Silver Spring)*. 2011;19(8):1722-6.
14. Muehlberg FL, Song YH, Krohn A, Pinilla SP, Droll LH, Leng X, et al. Tissue-resident stem cells promote breast cancer growth and metastasis. *Carcinogenesis*. 2009;30(4):589-97.
15. Zhang Y, Daquinag A, Traktuev DO, Amaya-Manzanares F, Simmons PJ, March KL, et al. White adipose tissue cells are recruited by experimental tumors and promote cancer progression in mouse models. *Cancer Res*. 2009;69(12):5259-66.
16. Prantl L, Muehlberg F, Navone NM, Song YH, Vykoukal J, Logothetis CJ, et al. Adipose tissue-derived stem cells promote prostate tumor growth. *Prostate*. 2010;70(15):1709-15.
17. Walter M, Liang S, Ghosh S, Hornsby PJ, Li R. Interleukin 6 secreted from adipose stromal cells promotes migration and invasion of breast cancer cells. *Oncogene*. 2009;28(30):2745-55.
18. Zimmerlin L, Donnenberg AD, Rubin JP, Basse P, Landreneau RJ, Donnenberg VS. Regenerative therapy and cancer: in vitro and in vivo studies of the interaction between adipose-derived stem cells and breast cancer cells from clinical isolates. *Tissue Eng Part A*. 2011;17(1-2):93-106.
19. Donnenberg VS, Zimmerlin L, Rubin JP, Donnenberg AD. Regenerative therapy after cancer: what are the risks? *Tissue Eng Part B Rev*. 2010;16(6):567-75.

20. Zolochovska O, Yu G, Gimble JM, Figueiredo ML. Pigment Epithelial-Derived Factor and Melanoma Differentiation Associated Gene-7 Cytokine Gene Therapies Delivered by Adipose-Derived Stromal/Mesenchymal Stem Cells are Effective in Reducing Prostate Cancer Cell Growth. *Stem Cells Dev.* 2011.
21. Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, Burrell MA. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab.* 2001;280(6):E827-47.
22. Khandekar MJ, Cohen P, Spiegelman BM. Molecular mechanisms of cancer development in obesity. *Nat Rev Cancer.* 2011;11(12):886-95.
23. Zhang Y, Bellows CF, Kolonin MG. Adipose tissue-derived progenitor cells and cancer. *World J Stem Cells.* 2010;2(5):103-13.
24. Rodeheffer MS, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells in vivo. *Cell.* 2008;135(2):240-9.
25. Klopp AH, Zhang Y, Solley T, Amaya-Manzanares F, Marini F, Andreeff M, et al. Omental adipose tissue-derived stromal cells promote vascularization and growth of endometrial tumors. *Clin Cancer Res.* 2012;18(3):771-82.
26. Cheng L, Darson MF, Bergstralh EJ, Slezak J, Myers RP, Bostwick DG. Correlation of margin status and extraprostatic extension with progression of prostate carcinoma. *Cancer.* 1999;86(9):1775-82.
27. Joe AW, Yi L, Even Y, Vogl AW, Rossi FM. Depot-specific differences in adipogenic progenitor abundance and proliferative response to high-fat diet. *Stem Cells.* 2009;27(10):2563-70.
28. Koul HK, Kumar B, Koul S, Deb AA, Hwa JS, Maroni P, et al. The role of inflammation and infection in prostate cancer: Importance in prevention, diagnosis and treatment. *Drugs Today (Barc).* 2010;46(12):929-43.
29. Lee KL, Peehl DM. Molecular and cellular pathogenesis of benign prostatic hyperplasia. *J Urol.* 2004;172(5 Pt 1):1784-91.
30. Lin G, Yang R, Banie L, Wang G, Ning H, Li LC, et al. Effects of transplantation of adipose tissue-derived stem cells on prostate tumor. *Prostate.* 2010.
31. Waugh DJ, Wilson C, Seaton A, Maxwell PJ. Multi-faceted roles for CXC-chemokines in prostate cancer progression. *Front Biosci.* 2008;13:4595-604.
32. Begley L, Monteleon C, Shah RB, Macdonald JW, Macoska JA. CXCL12 overexpression and secretion by aging fibroblasts enhance human prostate epithelial proliferation in vitro. *Aging Cell.* 2005;4(6):291-8.
33. Cho JW, Kang MC, Lee KS. TGF-beta1-treated ADSCs-CM promotes expression of type I collagen and MMP-1, migration of human skin fibroblasts, and wound healing in vitro and in vivo. *Int J Mol Med.* 2010;26(6):901-6.
34. Bellows CF, Zhang Y, Chen J, Frazier ML, Kolonin MG. Circulation of Progenitor Cells in Obese and Lean Colorectal Cancer Patients. *Cancer Epidemiol Biomarkers Prev.* 2011.
35. Capitanio U, Suardi N, Briganti A, Gallina A, Abdollah F, Lughezzani G, et al. Influence of obesity on tumour volume in patients with prostate cancer. *BJU Int.* 2011.
36. Ma J, Li H, Giovannucci E, Mucci L, Qiu W, Nguyen PL, et al. Prediagnostic body-mass index, plasma C-peptide concentration, and prostate cancer-specific mortality in men with prostate cancer: a long-term survival analysis. *Lancet Oncol.* 2008;9(11):1039-47.
37. Mauer MM, Harris RB, Bartness TJ. The regulation of total body fat: lessons learned from lipectomy studies. *Neurosci Biobehav Rev.* 2001;25(1):15-28.
38. Yoshimura K, Shigeura T, Matsumoto D, Sato T, Takaki Y, Aiba-Kojima E, et al. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J Cell Physiol.* 2006;208(1):64-76.
39. Ceder JA, Jansson L, Ehrnstrom RA, Ronnstrand L, Abrahamsson PA. The characterization of epithelial and stromal subsets of candidate stem/progenitor cells in the human adult prostate. *Eur Urol.* 2008;53(3):524-31.

40. Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC. Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. *Stem Cells*. 2005;23(3):412-23.

Figure 1. Flow cytometry quantification of ASC. The gating strategy is represented: debris, dead cells, platelets and granulocytes were excluded in the first gating step (A). Subsequently, hematopoietic cells co-expressing CD45 antigens were excluded (B). Quantification of adipose tissue-derived mesenchymal stem cells (ASC; CD45⁻/CD34⁺/CD31⁻/CD146⁻), while identifying endothelial precursor and mature cells (END; CD45⁻/CD34⁺/CD31⁺/CD146⁺) and pericytes (PER; CD45⁻/CD34⁺/CD31⁻/CD146⁺) was performed based on the surface expression of CD31 and CD146. An isotype control was used in all samples for gating (C). A representative example of visceral adipose tissue from a BPH patient is shown (D).

Figure 2. ASC quantity and *CFD* gene expression in periprostatic and visceral adipose tissue. A. Comparison of log₁₀ percent of ASC per gram of tissue between visceral and periprostatic fat depots, overall and for BPH and prostate cancer groups (* P<0.05); B. Differences in the log₁₀ percent of ASC per gram of adipose tissue between BPH and prostate cancer patients within each fat depot (* P<0.05); C. Comparison of adipsin (*CFD*) gene expression between periprostatic and visceral depots (* P=0.008); D. Scheme depicting the relative quantities of ASC among adipose depots and according to prostatic disease. Bars represent mean±S.E.M. from 12 men (BPH, n=6 and prostate cancer, n=6). PCa, prostate cancer; BPH, nodular prostatic hyperplasia; VIS, visceral adipose tissue; PP, periprostatic adipose tissue.

Figure 3. Quantification of ASC in prostatic tissue and gene expression in prostate tissue and CD45⁻/CD34⁺/CD31⁻/CD146⁻ sorted cells. A. Log₁₀-transformed amount of ASC per gram of prostatic tissue in BPH and prostate cancer patients (* P=0.021); B. Adipsin gene (*CFD*) expression in prostatic tissue and CD45⁻/CD34⁺/CD31⁻/CD146⁻

cells sorted from prostate samples, according to prostatic disease (BPH, $P=0.186$ and prostate cancer, $P=0.192$). C. Comparison of *CD133* gene expression in prostatic tissue between BPH and prostate cancers (* $P<0.025$). Bars represent mean $\pm$ S.E.M. from 12 men (BPH, $n=6$ and prostate cancer, $n=6$). D. Representative gel electrophoresis of target *CD133* and reference *28S* qPCR products from $CD45^-/CD34^+/CD31^-/CD146^-$ cells sorted from prostate cell suspensions (HBP, 1-2 and prostate tumor, 3-4). (+) positive control, using RNA from prostate tumor sample; (-) negative control; GR, gene ruler. PCa, prostate cancer; BPH, nodular prostatic hyperplasia.

Figure 4. $CD45^-/CD34^+/CD31^-/CD146^-$ progenitor cells in adipose tissue, prostate and peripheral blood and prostate cancer-obesity phenotype. OB/OW + PCa, includes obese/overweight (OB/OW) patients with prostate cancer (PCa) ($n=4$); Others, includes OB/OW ($n=5$) and lean controls ($n=1$), and lean patients with cancer ($n=2$). PP, periprostatic adipose tissue; VIS, visceral adipose tissue. * $P<0.05$

Table 1. Clinicopathologic characteristics of participants

	BPH	PCa
Age (years)	68.0±3.1	58.7±2.6
PSA at diagnosis (ng/mL)	5.7±1.4	5.7±0.8
Body mass index (kg/m ²)	28.7±1.3	27.3±1.4
Prostate weight (g) *	127.5 (120-135)	42.3 (34-55)
Stage of disease **		
Localized (T1-T2)	---	83.3
Advanced (T3-T4)	---	16.7
Gleason score **		
≤ 7 (3+4)	---	33.3
≥ 7 (4+3)	---	66.7

Data is presented as mean±S.E.M., except * median (min.-max.) and ** percentage of cases. BPH, prostate nodular hyperplasia patients; PCa, prostate cancer patients.

Figure 1

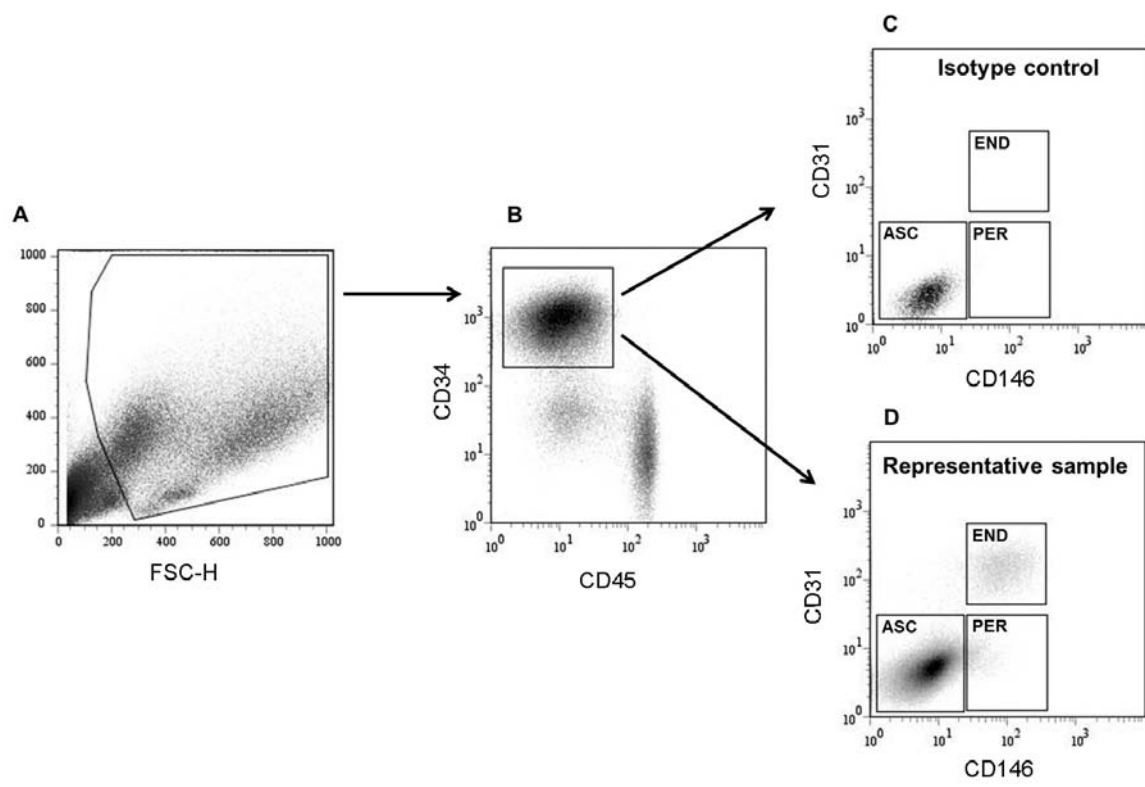


Figure 2

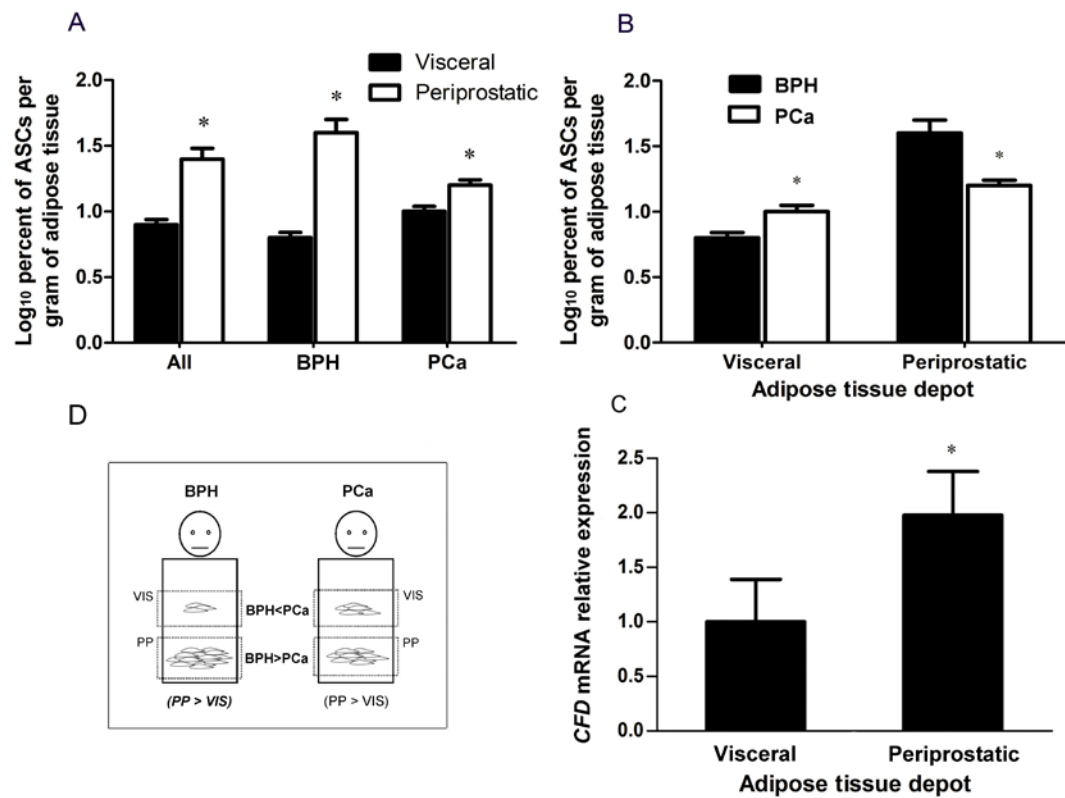


Figure 3

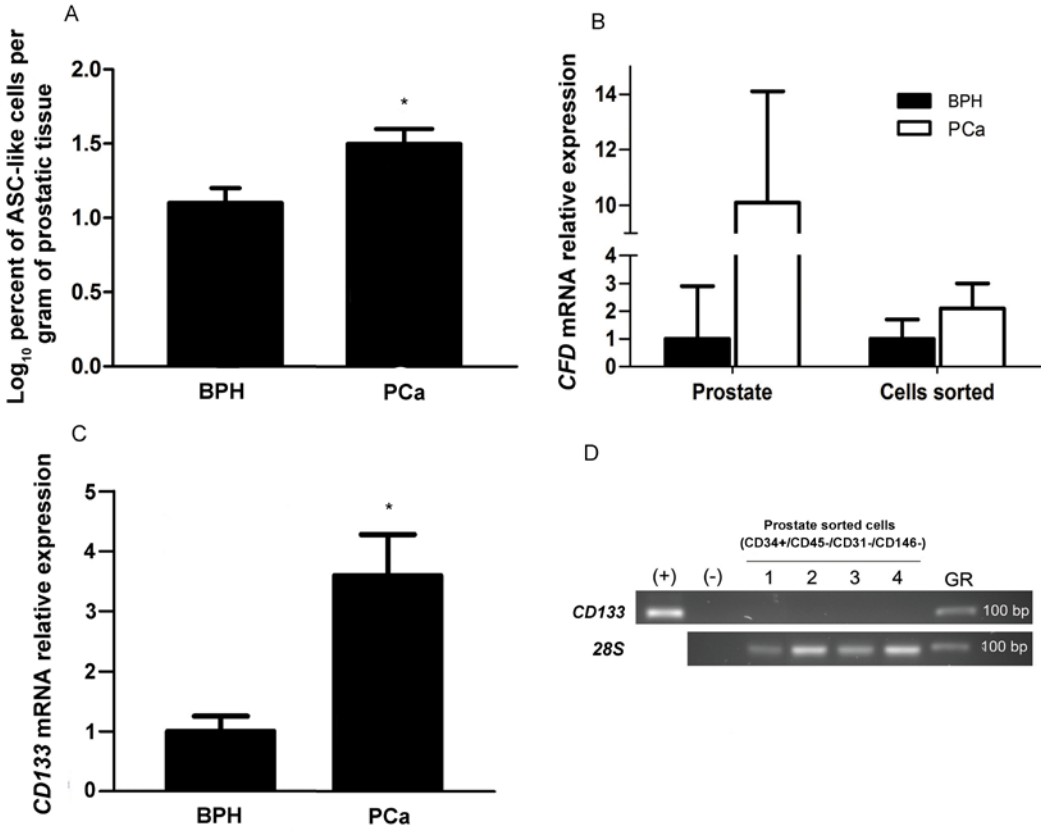


Figure 4

