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HYPOXIA BIOMARKERS IN PROSTATE CANCER: FROM GENETIC POLYMORPHISMS TO INTRATUMORAL PROTEIN EXPRESSION

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Fraga A, Ribeiro R, Príncipe P, Lobato C, Pina F, Maurício J, Monteiro C, Sousa H, Calais da Silva F, Lopes C, Medeiros R. The HIF1A functional genetic polymorphism at locus +1772 associates with progression to metastatic prostate cancer and refractoriness to hormonal castration. *Eur J Cancer 2014; 50: 359-65*.

Ribeiro R, Monteiro C, Azevedo A, Cunha V, Ramanakumar AV, **Fraga A**, Pina F, Lopes C, Medeiros R, Franco EL. Performance of an adipokine pathway-based multilocus genetic risk score for prostate cancer risk prediction. *PLoS ONE 2012; 7 (6): e39236*.

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LIST OF ABBREVIATIONS

ADT, Androgen deprivation therapy
AKT, Protein kinase B
Ang2, Angiopoietin 2
AR, Androgen receptor
ARNT, Aryl hydrocarbon nuclear translocator
BPH, Benign prostate hyperplasia
CaP, Cancro prostata
CAIX, Carbonic anhydrase IX
COX-2, Cyclooxygenase 2
C-TAD, Transcription activation domain of the C-terminal
ECM, extracellular matrix
EGF, Epidermal growth factor
E-M-T, Epithelial-to-mesenchymal transition
EPO, erythropoietin
ERK 1/2, Extracellular signal-regulated kinases 1/2
GLUT-1, Glucose transporter 1
GLUT-3, Glucose transporter 3
GWAS, Genome-wide association study
HIF, hypoxia inducible factor
HIF-1α, hypoxia inducible factor – 1 alpha
HIF-1β, hypoxia inducible factor – 1 beta
HIF-2α, hypoxia inducible factor – 2 alpha
IGF-1, Insulin growth factor 1
IGF-2, Insulin growth factor 2
IGFBP, Insulin growth factor binding protein
IHC, immunohistochemistry
iNOS, inducible nitric oxide synthase
KDR, Kinase insert domain receptor (gene coding for VEGFR2)
LHRH, luteinizing hormone releasing hormone
LOX, Lysyl oxidase
LOXL2, Lysyl oxidase-like 2
LOX-PP, Lysyl oxidase pro-peptide
MRI, Magnetic resonance imaging
NF-KB, Nuclear transcription factor kappa B

NLS, Nuclear localization signals
ODD, Oxygen dependent domain
PCa, Prostate cancer
PH, Prolyl hydroxylase
PHD, Proline hydroxylation domain
PI3K, Phosphoinositide 3-kinase
PIA, Proliferative inflammatory atrophy
PIN, Prostate intraepithelial neoplasia
PSA, Prostate specific antigen
PTEN, Phosphatase and tensin homolog
RP, Radical prostatectomy
ROS, Reactive oxygen species
SNP, Single nucleotide polymorphism
TAD, Transactivation domain
TAM, Tumor-associated macrophage
TGF-α, transforming growth factor alpha
TNM, Tumor, Node and Metastasis, staging system
UICC, Union for International Cancer Control
VEGF, Vascular endothelial growth factor
VEGFR2, Vascular endothelial growth factor receptor 2
VHL, Von Hippel Lindau

ABSTRACT

Current advances in prostate cancer (PCa) steered an increased precision in diagnosis and treatment, even though this disease endures as a significant cause of death for men in Portugal, in Europe and worldwide. Diagnostic tools used today, e.g. PSA test, resulted in overdiagnosis and overtreatment, with significant morbidity and unclear clinical benefit for patients. Conversely, PCa prognosis is established in the clinical setting through tumor and clinical variables, although its precision is far from optimal. Increasing concern has been devoted to uncover novel molecular markers to increase precision for stratifying patients to therapies and the extent of surveillance required either before or after initial treatment.

Here, we studied the relevance of germline genetic variants in genes involved in tumor hypoxia in the determination of PCa aggressiveness profile. Several single nucleotide polymorphisms (SNPs) in *HIF1A* (*HIF1A* +1772 C>T, rs11549465) and in genes of downstream pathways *VEGF/KDR (VEGF* +405 G>C, rs2010963; *VEGF* +936 C>T, rs3025039; *VEGF* -460 C>T, rs833061; *KDR* -604 T>C, rs2071559), *LOX* (*LOX* +473 G>A, rs1800449) and *CA9* (*CA9* +201 A>G, rs2071676) were genotyped, using DNA from approximately 1500 male subjects (754 PCa and 736 cancer-free controls) included in case-control studies. A nested group of over 480 PCa patients eligible for androgen deprivation therapy (ADT) was followed-up using as endpoints: resistance to ADT (primary), all-cause overall survival (secondary) and development of *de novo* bone metastasis while under ADT (tertiary). In addition, representative areas of prostate carcinoma (n=51) and of nodular prostate hyperplasia (BPH) (n=20) were analysed for hypoxia-inducible factor 1 alpha (HIF-1α), carbonic anhydrase IX (CAIX), lysyl oxidase (LOX) and vascular endothelial growth factor receptor 2 (VEGFR2) immunohistochemical protein expression using a tissue microarray, and correlated with putative functional polymorphisms at the corresponding genes (*HIF1A* +1772 C>T; *CA9* +201 A>G; *LOX* +473 G>A; *KDR* -604 T>C).

Findings from molecular epidemiology studies showed that SNPs on both the *HIF1A* (*HIF1A* +1772 C>T) and *VEGF* (*VEGF* +405 G>C, *VEGF* +936 C>T, *VEGF* -460 C>T)/*KDR* (*KDR* -604 T>C) genes were not associated with increased risk for being diagnosed with PCa or high-grade PCa, even on univariate analyses.

Concerning the follow-up study on patients under ADT, results demonstrated an independent effect of *HIF1A* +1772 T-carriers for developing distant metastasis and resistance to ADT (HR, 2.0; 95%CI, 1.1-3.9 and HR, 6.0; 95%CI, 2.2-16.8,

respectively), albeit no association was found with the secondary endpoint (overall survival). Conversely, the VEGF/KDR SNPs were neither individually nor in combination significantly associated with the primary and tertiary endpoints in patients under ADT. Only the VEGF/KDR SNPs combined into the high/intermediate profile of VEGF-VEGFR2 pathway activation were associated with higher risk for all-cause mortality (HR, 1.6; 95%CI, 1.1-2.4) in univariate analysis.
The genotype-phenotype analyses showed higher LOX staining intensity for carriers of the homozygous *LOX +473* G-allele (P=0.011), and that *KDR* -604 T-allele carriers were more prone to have higher VEGFR2 expression in prostate epithelial cells (P<0.006). Immunohistochemistry disclosed predominance of positive CAIX and VEGFR2 expression in epithelial cells of prostate carcinomas compared to BPH (P=0.043 and P=0.035, respectively). In addition, the VEGFR2 expression score in prostate epithelial cells was higher in organ-confined and extra prostatic carcinoma compared to BPH (P=0.031 and P=0.004, respectively). Notably, for LOX protein the immune reactivity score was significantly higher in organ-confined carcinomas compare to BPH (P=0.015).
The expression on prostate epithelial cells of target molecules in hypoxia pathways analysed here (VEGFR2, CAIX and LOX) allowed differentiating malignant from benign prostate disease. Two of the genetic polymorphisms (*LOX* +473 G>A and *KDR* – 604 T>C) accounted for a potential gene-environment effect in the activation of hypoxia-driven pathways in prostate carcinoma. Nevertheless, genetic polymorphism-protein expression relationship in molecules analysed here were not concordant, suggesting that increased complexity might explain the genotype-phenotype association. Upcoming results in LOX and CAIX SNPs from molecular epidemiology studies might add to the comprehension of this association, allowing the development of genetic risk scores combining genetic hypoxia markers and clinicopathological variables. Further research in larger series is warranted to clarify and expand present findings. Ultimately, a different set of genetic variants is likely to influence prognosis, including genetic polymorphisms involved in steroid metabolism, metastasis and drug metabolism.

RESUMO

Os últimos avanços no cancro da próstata (CaP) conduziram a uma maior precisão no diagnóstico e tratamento, embora esta doença permaneça uma importante causa de morte nos homens em Portugal, na Europa e no mundo. Algumas ferramentas de diagnóstico usadas, por exemplo o teste PSA, resultaram em excesso de diagnósticos e tratamento excessivo, com significativa morbilidade e benefício clínico pouco evidente. Por outro lado, o prognóstico do CaP é estabelecido na clínica através da combinação de variáveis clinicopatológicas em nomogramas, embora a sua precisão esteja longe de ser ideal. Uma preocupação crescente tem sido dedicada à descoberta de novos marcadores moleculares com o intuito de aumentar a precisão para estratificar os doentes com CaP para tratamento e em relação à extensão de vigilância necessária, antes e após o tratamento inicial.

Nesta tese estudou-se a relevância das variantes genéticas da linha germinativa em genes envolvidos na hipóxia tumoral na determinação do perfil de agressividade do CaP. Vários polimorfismos genéticos, no *HIF1A* (*HIF1A* 1772 C> T, rs11549465) e em genes de vias relevantes a jusante: *VEGF* / *KDR* (*VEGF* 405 G> C, rs2010963; *VEGF* 936 C> T, rs3025039; *VEGF* -460 C> T, rs833061 ; *KDR* -604 T> C, rs2071559), *LOX* (*LOX* 473 G> A, rs1800449) e *CA9* (*CA9* 201 A> G, rs2071676), foram genotipados, usando o DNA de cerca de 1500 indivíduos do sexo masculino (754 CaP e 736 controlos) incluídos em estudos caso-controlo. De entre os doentes com CaP, um grupo de mais de 480 CaP elegíveis para terapêutica de privação de androgeneos, foi seguido durante vários anos. A resistência à terapêutica de privação de androgeneos, sobrevida global e o desenvolvimento de novo de metástases ósseas, foram respetivamente considerados como *endpoint* primário, secundário e terciário. Para além do estudo genético, num subgrupo de doentes e controlos mais reduzido, áreas representativas de carcinoma da próstata (n = 51) e de hiperplasia nodular da próstata (HBP) (n = 20) foram analisadas por imuno-histoquímica (IHC) utilizando um *microarray* de tecido. A abundância de fator indutível por hipóxia (HIF-1α), anidrase carbónica IX (CAIX), lisil oxidase (LOX) e a expressão da proteína recetor 2 do fator de crescimento vascular endothelial (VEGFR2) foi correlacionada com os polimorfismos estudados dos genes correspondentes (*HIF1A* 1772 C> T; *CA9* 201 A> G; *LOX* 473 G> A; *KDR* - 604 T>C). Achados dos estudos de epidemiologia molecular mostraram que os SNPs nos genes *HIF1A* (*HIF1A* 1772 C> T), *VEGF* (*VEGF* 405 G> C, *VEGF* 936 C> T, *VEGF* -460

C> T) e *KDR* (*KDR* -604 T> C) não estavam associados a maior risco de ser diagnosticado com CaP ou CaP de alto grau em análises uni ou multivariadas.

Em relação ao estudo de follow-up em pacientes em terapêutica de privação androgénica (TPA), os resultados demonstraram um efeito independente do *HIF1A* 1772 portadores alelo T para o desenvolvimento de metástases e resistência à terapêutica privação androgénica (TPA) (HR, 2.0; 95% CI, 1.1-3.9 e HR, 6.0; 95% CI, 2.2-16.8, respetivamente), não tendo sido observada associação com o *endpoint* secundário (sobrevida global). Por outro lado, os polimorfismos nos genes *VEGF e KDR* não estavam individualmente nem em combinação significativamente associados com os endpoints primário e terciário. Apenas a combinação de polimorfismos no *VEGF* e *KDR* como perfil de ativação alta/intermédia da via VEGF-VEGFR2 estava associada a maior risco de mortalidade na análise univariada (HR, 1.6; 95% CI, 1.1-2.4).

As análises genótipo-fenótipo mostraram maior expressão de proteína nas células epiteliais da próstata nos portadores homozigóticos para o alelo G do *LOX* 473 (P = 0.011) e nos portadores do alelo T do *KDR* -604 (P <0.006). Adicionalmente, a análise por imuno-histoquímica evidenciou uma maior frequência de células epiteliais positivas para o CAIX e VEGFR2 em CaP comparativamente com adenomas (P=0.043 e P=0.035, respetivamente). Além disso, o *score* de expressão de VEGFR2 nas células epiteliais da próstata foi mais elevado quer no CaP confinado ao órgão quer no extra-prostárico comparados com os adenomas (P = 0.031 e P = 0.004, respetivamente). Para a proteína LOX o *score* de imunorreatividade foi significativamente maior no CaP confinado ao órgão comparado com os adenomas (P = 0.015).

A expressão de moléculas-alvo por células epiteliais da próstata em vias de hipóxia aqui analisadas (VEGFR-2, CAIX e LOX) permitiu diferenciar a doença prostática maligna da benigna. Dois dos polimorfismos genéticos (*LOX* 473 G>A e *KDR* - 604 T>C) poderão ser responsáveis por um potencial efeito gene-ambiente na ativação de vias induzidas por hipóxia no CaP. No entanto, a relação entre expressão proteica-polimorfismo genético-risco de CaP agressivo nos marcadores aqui analisados não foram concordantes, sugerindo que apenas uma complexidade acrescida poderá explicar a associação genótipo-fenótipo-risco. Os resultados dos estudos de epidemiologia molecular a decorrer com os polimorfismos do *LOX* e *CAIX* poderão acrescentar à compreensão desta associação, permitindo o desenvolvimento de *scores* de risco genético que combinam vários marcadores genéticos e proteicos de hipóxia com variáveis clinicopatológicas. É evidente a

necessidade de estudos em séries maiores para clarificar e expandir os resultados aqui apresentados. De facto, o conhecimento de um conjunto de variantes genéticas maior e mais alargado, é suscetível de influenciar o prognóstico, nomeadamente o conhecimento de polimorfismos genéticos envolvidos no metabolismo de esteróides, metástases e metabolismo de fármacos.

THESIS PLANNING

The present thesis is organized into four different Chapters. In ***Chapter 1***, a short general introduction to review cancer cell singularities and propel Prostate Cancer (PCa) research questions is presented. Then, the literature is reviewed throughout ***Chapter 2***, despite an introductory note revealing our personal clinically-driven approach, focusing on PCa particularities as a research model that took questions from the clinical setting (bedside) towards the bench of basic and translational experimental investigation. These personal thoughts emerged within the frame of classical knowledgeable epidemiology, carcinogenesis and clinicopathology of PCa. Moreover, this chapter included a wide literature review attentive to hypoxia and cancer as a whole, with particular attention to the role of hypoxia-inducible factor – 1 alpha (HIF-1α), which was deepened to focus on PCa and disease aggressiveness through the interplay with key downstream pathways. The *Chapter 2* was upheld on candidate strong clinical background and on two published reviews [*Clin Genitourinary Cancer 2015; 13 (4): 295-301; Actas Urol Esp 2009; 33 (9): 941-951*].

For the sake of clarity, we decided to include experimental works that resulted in scientific publication on a separate ***Chapter 3***. Here, each separate study from a total of four, congruent with literature review and questions identified in clinical practice, was independently described and presented through a short overview, results and discussion, followed by the respective printed paper in appendix. Therefore, in this chapter the most relevant results and specific discussion are concisely depicted, grounded on published papers *[Eur J Cancer 2014; 50: 359-65; PLoS ONE 2012; 7 (6):e39236]* and submitted manuscripts *("Putative functional genetic polymorphisms in key hypoxia-regulated downstream molecules and phenotypic correlation in prostate cancer"*, and *"Inherited variation in adipokine pathway genes may determine prognosis for prostate cancer patients receiving androgen-deprivation therapy"*). An integrative but concise overall conclusion, followed by emerging specific conclusions and prospective investigative remarks were depicted in ***Chapter 4***. We believe this outline will fit the purpose of showing all the major scientific achievements and discoveries made throughout the PhD track, while making it less burdensome to readers.

Hypoxia Biomarkers in Prostate Cancer:
From genetic polymorphisms to intratumoral protein expression

CAP 1 – GENERAL INTRODUCTION AND AIMS

CAP 2 – REVIEW OF LITERATURE	Hypoxia and Cancer Hypoxia and prostate câncer aggressiveness: from pathophysiology to clinical biomarkers	Anexo 1 Anexo 2
CAP 3 – CLINICAL STUDIES.	Clinical studies #1, #2, #3 and #4: Overview and methods. Results. Discussion.	Anexo 3 Anexo 4 Anexo 5 Anexo 6

CAP 4 – CONCLUSIONS AND FUTURE PERSPECTIVES

Anexo 1 - *Papel del factor inductor de la hipoxia. Actas Urol Esp 2009; 33(9): 941-951*

Anexo 2 – *Hypoxia and prostate cancer aggressiveness: different tales with common ending. Clin Genitourinary Cancer 2015; 13(4): 295-301.*

Anexo 3 – *The HIF1A functional genetic polymorphism at locus +1772 associates with progression to metastatic prostate cancer and refractoriness to hormonal castration. Eur J Cancer 2014; 50: 359-65.*

Anexo 4 – *Performance of an Adipokine pathway based multilocus genetic risk score for prostate cancer risk prediction. PLoS ONE 2012; 7(6): e39236.*

Anexo 5 – *Inherited variation in adipokine pathway genes may determine prognosis for prostate cancer patients receiving androgen deprivation therapy. Submitted.*

Anexo 6 – *Putative functional genetic polymorphisms in key hypoxia regulated downstream molecules and phenotypic correlation in prostate cancer. Submitted.*

1. GENERAL INTRODUCTION

Advances in cancer biology research have led to a better understanding of this disease and have shown that it is an extremely complex and dynamic biological phenomenon, characterized by a great capacity for adaptation to evolving environments along its natural history. At the outset, the cancer cell is doomed to failure to die, due to the surrounding environment and because it is against the life cycle, even though if ultimately successful it will result in patient's death. Hence, it is not surprising that throughout cancer development, different biological singularities are happening that will facilitate cancer cell's survival and tumor progression.

Tumor promotion, proliferation, cell instability, deregulation of energy mechanisms, mutations, resistance to apoptosis, invasiveness and metastasis, angiogenesis, the ability to resist and adapt to hypoxia phenomena are all characteristic hallmarks of cancer cells that will lead to its immortality and progression [1]. These singularities are characteristic phenotypic findings that are superimposed on the genetic background and environmental exposure's driving forces, which ultimately determine the diversity among cancers and the different responses of patients even to the same type of cancer and therapies.

Prostate Cancer (PCa) is a very heterogeneous disease with great clinical variability, varying considerably in their genetic profile and biological behavior, making it difficult and complex to decide the best therapeutic approach. Therefore, it is vital to advance our ability to detect the most aggressive cancers. For that reason, it seems imperious to uncover and understand the mechanisms of PCa development, particularly in relation to hypoxia. At our research Group we recognized that raising further the understanding of cancer hypoxia mechanisms and its impact on angiogenesis and tumor microenvironment of PCa will add relevant information to current knowledge and potentially impact clinical reasoning. In fact, although tumor hypoxia is common in urological oncology, particularly in relation to PCa aggressiveness, further research in environmental factors and germline genetic variants as modulators of hypoxia in PCa will foster comprehensive development of predictive and prognostic biomarkers to improve PCa management.

Neoplastic tissues in the prostate gland are highly hypoxic, where the degree of microenvironmental hypoxia largely determines the response to subsequent resistance to treatment and tumor progression, as reflected in the local response evaluated through immunohistochemistry [2].

Several studies have shown a relevant role for hypoxia in androgen dependent PCa, which is supported by the observed clinical impact of anti-androgens involving the downregulation of hypoxia-inducible factor 1 alpha (HIF-1α) transcription and decreased angiogenic potential [3-5]. Studies involving genetic variants, including the *HIF1A* gene, in susceptibility to PCa and hypoxia mechanisms are scarce and often controversial [4-7], thereby fostering further research. Even though some biomarkers are central in hypoxia, we should consider a panel of markers since individually they are unlikely to be assertive enough to be clinically relevant [8,9]. In fact, underlying tumor hypoxia we should notice the existence of a regulatory circuit between molecules (such as vascular endothelial growth factor, VEGF, lysyl oxidase, LOX, carbonic anhydrase, CAIX) and pathways controlled by HIF-1α, which synergistically model the tumor microenvironment and regulate PCa aggressiveness.

During the process of fitting with hypoxia, the microenvironment changes and cells adapt to withstand the hostile environment, where well documented neoangiogenesis regulated by VEGF and its receptors allows new architecture and microcirculation, glycolytic pathways change to avoid cellular acidosis with altered expression of glucose transporters -1 and -3 (GLUT-1 and GLUT-3), changes in pH regulation through carbonic anhydrase IX, and altered lysyl oxidase to modulate extracellular matrix to allow tumor expansion and metastasis. A recent study identified HIF-1α, VEGF and angiogenesis as hypoxia markers that were associated with risk of biochemical failure in patients with localized PCa [2], whereas others showed LOX and CAIX overexpression in PCa compared with BPH and correlation with Gleason grade [10], although additional studies are required to confirm these proteins as useful hypoxia markers in PCa.

Underlying the intricate resulting phenotype of hypoxia microenvironment in prostate tumors, uncovering germline variations in locus of genes coding for molecules in hypoxia pathways likely to play a role in tumor progression and aggressiveness may reveal new potential biomarkers.

The identification of better hypoxia biomarkers can help personalize which patients might benefit more from regulatory hypoxia therapies. Ultimately, being capable of predicting the correlation of local hypoxic phenotypic changes with prognosis, can lead to the application of focal therapy directed to hypoxic areas guided by magnetic resonance imaging or using hypoxia-targeted nanotools [11,12].

This thesis is focused on hypoxia-driven prostate tumor aggressiveness and disease progression. Here we attempt to disclose genetic and phenotypic

characteristics from molecules involved in hypoxia pathways that better define malignancy and discriminate aggressive disease. The ultimate purpose is to congregate enough data from genetic and protein markers (from pathways directly regulated by HIF-1α: CAIX, LOX, VEGFR2) in order to develop a predictive/prognostic model for stratification by risk groups according to the molecular profile. Analysis of genetic variants that may influence the production and action of these molecules, may add new insights into the functional molecular profile of susceptibility to cancer and understanding of its pathophysiology. Accordingly, we expect to study the functional effect of single nucleotide polymorphisms (SNPs) through the verification of protein expression directly in prostatic tissue by immunohistochemistry (IHC). We expect that results presented may help clarify the mechanism of hypoxia in PCa development and will prove to be a step in the need to continue and deepen this line of investigation.

1.1. Aims

- To study the protein expression directly in prostatic tissue corresponding to the functional effect of SNPs in the respective genes, revealing genotype-phenotype relationships.
- To analyze genetic variants in genes coding for molecules that may influence hypoxia downstream effects, therefore adding new insights into the functional molecular profile of susceptibility to prostate cancer and understanding of its pathophysiology.
- To uncover genetic and phenotypic characteristics from molecules involved in hypoxia pathways that better define malignancy and discriminate aggressive disease.
- To identify better hypoxia biomarkers and add to knowledge on stratification of patients who might benefit more from regulatory hypoxia therapies.
- Contribute to the development of new risk scores using molecular markers capable of predicting local hypoxic phenotypic changes, which can lead to the application of focal therapy directed to hypoxic areas.

REVIEW OF THE LITERATURE

2.1 Prostate cancer as a particular research model: from bedside-to-bench

Prostate cancer is one of the most frequent and controversial pathologies of modern medicine. In recent years we have witnessed on the one hand an increased demand for care in this area and on the other hand the development of scientific advances accompanied by great technological progress.

The pronounced progress and new perspectives of cure obtained with the use of prostate specific antigen (PSA) [13] enabled earlier diagnosis of the disease. The technology has progressed immensely allowing easier, faster and more accurate diagnosis, and more comfortable treatments with less sequelae. However, current ultimate treatments have been developed for over fifty years. Indeed, radical prostatectomy (RP) remains the paramount therapeutic opportunity for local disease, and androgen deprivation therapy the everyday treatment for advanced disease.

Prostate cancer is now considered a heterogeneous disease, where efforts should be emphasized towards detecting, identifying and targeting the most aggressive tumors. Existing prognostic algorithms that include clinicopathological variables such as the Gleason grade, PSA, imaging and histological features are limited to crude estimates of disease progression.

Knowledge of the mechanisms of carcinogenesis and tumor biology, including genetic changes associated with tumor initiation and progression, has been an opportunity to add genetic markers to the predictive and prognostic panel, to improve clinical reasoning and aid therapeutic decisions.

For several years it was recognized that a genetic component underlies PCa development and aggressiveness, and approximately 42% higher risk cancer can be ascribed to genetic factors [14]. Only a small proportion of cancers can be attributed to monogenic, high penetrance genes, whereas most have multifactorial etiology, combining environmental and genetic factors. Low penetrance genetic variants, alone or combined as a genetic risk score have been studied, although still inconclusive [15-17].

The identification of susceptibility genes for PCa and its biochemical and metabolic relationship will add to our knowledge contributions in the field of molecular cancer pathophysiology, allowing the closer identification of risk groups and to establish relations with drug response. Notably, several current therapies

(androgen deprivation therapy, chemotherapy, and radiotherapy) may be influenced by genetic variations with functional features in genes coding for regulatory molecules in tissue hypoxia.

2.1.1. Anatomy

Prostate is an exocrine organ measuring approximately 25cc. It is characterized by the presence of tubuloalveolar glands that secrete fluid through ducts that empty into the prostatic urethra. The prostate is located deep in the pelvis below the pubis, above rectum and between bladder and external urinary sphincter. The prostate is involved by a capsule and is divided into a peripheral zone, where most cancers arise, a transition zone where approximately 15% of neoplasias occur, and a central zone with rare malignant transformation [18].

2.1.2. Histopathology

Normal prostatic epithelium contains a heterogeneous group of cells representing several distinct levels of differentiation. Secretory cells are well differentiated epithelial cells that are PSA-producers and androgen receptor (AR) positive. The secretory cells are derived from basal cells through an intermediate proliferating group of cells that are variable in AR and PSA expression. The PSA producing secretory cells are terminally differentiated and incapable of proliferation [19]. Rare neuroendocrine cells are also present in normal prostatic epithelium.

More than 95% of PCa are adenocarcinomas that arise in acinar and proximal ductal epithelium. The typical adenocarcinoma of prostate can be distinguished from others neoplasms using PSA immunohistochemistry. Intraductal proliferation, termed prostatic intraepithelial neoplasia (PIN) is considered a histologic precursor of malignancy; however, an atrophic but highly proliferative condition associated with chronic inflammation, proliferative inflammatory atrophy (PIA), may in fact be the first histologic step in the carcinogenic process [20]. PIN is defined by the presence of cytological atypical epithelial cells within architecturally benign appearing acini and is subdivided into low and high grade; only high grade PIN is considered a precursor of invasive carcinomas and it may precede the development of cancer by 10 years or more [21]. Prostatic adenocarcinomas are often multifocal and heterogeneous, a factor that complicates both prognostication and attempts to develop focal therapies. Patients not only have multifocal tumors but also an average of 2.7 different grades of cancer in each specimen; only 10% of cancers

from RP specimens are comprised of a single histologic grade. The majority of RP specimens contain more than one prostate malignancy focus and type. Genetic studies indicated that multifocality is typically a function of separately arising tumors rather than intraprostatic tumor spread [22]. Adding to the picture, several studies indicate a critical role for stroma cells in supporting the growth of malignant prostate epithelial cells, thereby remaining an area of active investigation [23].

2.1.3. Epidemiology

Considerable changes have occurred in the epidemiology of PCa since the widespread availability of PSA in the early 1990s. In fact, dramatic changes in its incidence have taken place since the PSA became commercially available, as prostate tissue has been increasingly biopsied with better quality in men without symptoms.

Prostate cancer is an important public health issue, being the most frequent non cutaneous male malignancy and the second most common cause of cancer death in men [24]. It was estimated that during the year 2012 in the United States, PCa became the most common cancer in men over 60 years, with about 214 740 new cases and 28 170 deaths [25], accounting for approximately 29% of new malignant cases and 9% of the causes of death by cancer in males. Concurringly in Europe, PCa is the leading cause of death in males, whereas incidence is highest in Northern and Western Europe (> 200 per 100 000) with continuously increasing rates in Eastern and Southern Europe [26]. During the last decade, the five-year relative survival for PCa steadily increased from 73.4% in 1999-2001 to 83,5% in 2005-2007, with an estimated total economic burden of PCa in Europe exceeding 8.43 billion euros [26]. In Portugal, the number of estimated new cases of PCa in 2008 has been 5140 [27,28]. It is the most common malignancy in men after colorectal cancer. The estimated risk for a Portuguese man, below 75 years old, to develop any cancer type is 25.9%, while the risk for PCa is 3.2% and the risk of dying from PCa 3.0 % [29].

There are large differences in incidence of PCa between countries, ethnic backgrounds and populations. Genetic, environmental and social characteristics (access to medical care for example) are likely factors that might influence the development and progression of the disease, despite the persistent clinically-established risk factors, age, race and family history of PCa [30]. Environmental factors have been suggested to influence the risk of progression from so called

latent PCa to clinical PCa, including fat and alcohol consumption, exposure to ultraviolet radiation, chronic inflammation, obesity and metabolic syndrome, but further research is warranted to confirm association.

2.1.4. Molecular mechanisms

Prostate cancer has elevated morbidity, even though its pathogenesis remains unclear. Several putative risk factors and mechanisms have been implicated, but current evidence remains inconclusive.

Normal epithelial cells acquire somatic mutations in critical regions of the genome that result in increased cell proliferation and confer the ability to invade and metastasize; their normal function is modified due to alterations in multiple cellular signaling pathways. Actually, the progression of PCa is known to be caused by deregulation of intracellular signaling pathways such as the AR pathway, PI3K-Akt, NF-kB, Wnt and Notch. It is known that PCa is an endocrine-related cancer driven by androgens, and the mutations at that level are thought to be determinant [31].

A genetic polymorphism has been defined as a commonly occurring (>1%) genetic variation, at the nucleotide level, in the general population. Compared to mutations, SNPs have been perceived as functionally insignificant, albeit current evidence emphasizes that a considerable fraction affects protein intrinsic properties and function to a variable degree. Low penetrance susceptibility alleles are defined as polymorphic genes with specific alleles that associate with altered susceptibility for disease. Usually, variants in these genes are common in the normal population. Therefore, although each variant may be associated with a relatively small attributable fraction risk for prostate cancer, the impact of combining relevant genetic polymorphisms, may add significance to their use as molecular markers and determinants of PCa diagnosis and aggressiveness prediction. Several reports have demonstrated the importance of genetic polymorphisms in the phenotype of several cellular mechanisms. If these genetic variants are combined to induce interactions at gene and protein level, they are likely to influence cancer mechanisms at the cell, with repercussion in the microenvironment and with perception of external environment.

2.1.5. Diagnosis

Screening for Pca is a highly controversial topic. There is no level 1 evidence that PSA screening reduces mortality due to PCa. In the Cochrane review published in 2013, screening was associated with increased diagnosis of PCa (RR, 1.3; 95%CI,

1.02-1.7), with more localized disease (RR, 1.8; 95%CI, 1.2-2.7) and less advanced PCa (T3-4, N1, M1) (RR, 0.8; 95%CI, 0.7-0.9). The early detection of PCa seems to be important because it allows the diagnosis of localized and potentially curable disease, even at the expenses of increased iatrogeny in patients with indolent tumors [32,33]. Nevertheless, controversy exists concerning the cost-effective profile of using PSA widely [34] and the impact on the patient's overall quality of life is still unclear.

A critical issue emerging from the debatable PSA-associated overdiagnosis and overtreatment is the previously unmet need for additional molecular markers that can improve the discrimination of PCa aggressiveness. The patient with advanced disease may receive only palliative treatment.

The diagnosis of PCa is based on PSA values and/or suspicious digital rectal examination, which refer, when adequate, for prostate biopsy. Usually, men with PSA values ≥ 4.0 ng/ml or PSA velocity > 0,75ng/ml/year, are candidates for prostate biopsy. Nevertheless, these PSA cut-offs remain controversial and often lead to false positives and negatives.

Diagnosis of PCa relies on prostate biopsy with subsequent histopathological identification. The accuracy and lower morbidity of prostate biopsy procedure should be taken into account, and are known to influence diagnosis precision.

Currently, the prostate biopsy protocols are increasingly standardized, together with better echography and fusion images obtained from MRI, which have allowed significant advances in diagnosis accuracy and therapeutic guidance (mainly surgical precision and the possibility of focal treatments).

Histopathologically determined Gleason grade and PSA levels are commonly used in nomograms for clinical assessment of the prognosis.

2.1.6. Clinical and pathological staging

Classification of disease staging, either local or regional or systemic, is crucial, since it informs about its progression and extent. Ordinarily, we use the Union for International Cancer Control (UICC) classification of the tumor, node and metastasis (TNM), including the following features: digital rectal examination, multiparametric magnetic resonance imaging (MRI), histopathological findings and PSA values.

The most precise staging is provided when patients are submitted to RP. In this setting the RP specimen, seminal vesicles and locoregional lymph nodes are analyzed together, providing more precise information to better assess risk of

recurrence and necessity of adjuvant treatment. Nevertheless, PCa has a great clinical variability. In cases, PCa patients apparently remain asymptomatic, often without a diagnosis and die of other causes. Frequently, this slow-growing, indolent disease may never elapse as clinically important, and patients are likely to die from another cause [35,36]. This may be due to old age at the time of diagnosis, slow growth seen in many tumors or to therapeutic response. However, sometimes tumors are very aggressive from the start and rapidly progress after a variable period of latency.

Thus, given the elevated prevalence of this disease, its morbidity and mortality, as well as the economic and social repercussions, additional efforts should be undertaken to understand and establish the biological, genetic and environmental mechanisms underlying PCa natural history [37].

2.1.7. Treatments

Therapeutic options in PCa should be reasoned taking into account disease stage (TNM), tumor Gleason grade, PSA level, and patient's age, life expectancy, expected quality of life, in order to modify its natural history, influencing the risk of disease progression and mortality [38]. When such factors are evaluated, diverse treatments are available including active surveillance, surgery, focal therapy, radiotherapy, brachytherapy, hormonal therapy and chemotherapy, according to the moment in the disease natural history.

The development of novel biomarkers for PCa that fit within clinical needs, will certainly improve prognosis and clinical decision-making capacity. In this setting, it is necessary to foster research in this field to upgrade knowledge and accurately provide guidance for different therapeutic options. In recent years, active surveillance and focal treatments in PCa, have emerged as novel therapeutic modalities with strong evidence to take into account. Knowledge of biomarkers and PCa features can help make the right choices for our patients.

2. 2. Hypoxia and cancer

[Fraga A et al. Tumor hypoxia. The role of HIF. Actas Urol Esp 2009; 33 (9): 941-51]

Solid tumors usually occur and progress in a hypoxic environment, suggesting that hypoxia modulates tumor cell resistance to apoptosis and influences neoangiogenesis, making them more aggressive, with invasive capacity and resistant to treatment.

The genetic and biological mechanisms underlying this phenomenon are incompletely clear, even though many studies suggest a role of HIF in this process. Under hypoxic conditions, the alpha subunit is not destroyed, and will activate transcription of a set of genes that ultimately contributes to tumor aggressiveness. Its expression is associated to an increased metastatic potential that has been shown in both animal studies and human tumors.

Hypoxia-inducible factor (HIF) is a transcription factor that regulates cells' response to hypoxia and acts as a regulator of oxygen homeostasis [39-41]. The transcription factor activates genes that codify proteins that increase the availability of oxygen and permit metabolic adaptation in the absence of oxygen; it controls the expression of several genes and proteins involved in angiogenesis, erythropoiesis, glycolysis, invasion, apoptosis, vascular tone, pH regulation, epithelial homeostasis, and drug resistance. More than 60 target genes induced by HIF have been identified [40]; others are suppressed [42]; many functions are HIF-dependent [42].

Tumor hypoxia has emerged as a key factor in tumor progression and is associated to a poor prognosis in urological oncology, particularly kidney and prostate cancer. The purpose of this study was to review the significance of hypoxia in carcinogenesis and tumor progression by reviewing the current knowledge on the subject and the mechanisms of action and activation of hypoxia-inducible factor 1 alpha (HIF-1α).

2.2.1. Molecular structure of HIF-1α

The *HIF1A* gene, which codifies HIF-1α, is located in the 14q21-q24 locus [43], which contains 15 exons [44]. It is a heterodimer composed of alpha chains (regulated by O_2) and beta chains, arranged in a helix-loop-helix (bHLH); it belongs to a family of transcription factors consisting of three alpha subunits (HIF-1α, HIF-2α, HIF-3α) and one beta subunit (HIF-1β), also known as aryl hydrocarbon nuclear translocator (ARNT) [45-47].

There are two nuclear localization signals (NLS), located on the C-terminal (aminoacids 718-721) and on the N-terminal (aminoacids 17-33), but only the C-terminal is responsible for the nuclear accumulation of HIF-1α [48]. It is also known that HIF contains two transactivation domains (TAD) in the C-terminal (aminoacids 531-575 and 786-826), separated by a sequence of aminoacids (575-786) that inhibit transactivation [49] (Figure 1).

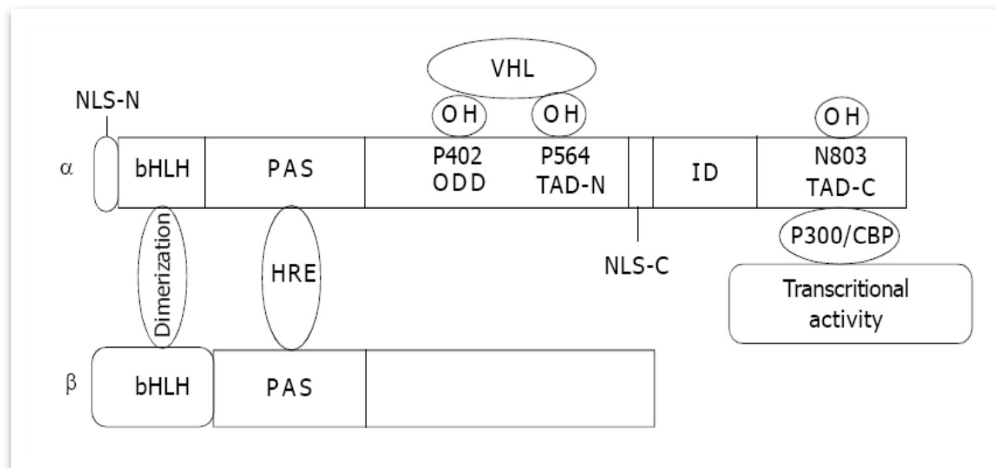


Figure 1. Molecular structure of HIF-1α. Adapted from [45].

The N-terminal of the molecule (aminoacid 1-390) contains the bHLH-PAS domain, necessary for dimerization and binding to DNA [50]. The interaction between the bHLH domains of the two subunits regulates their dimerization [51].
The C-terminal domain's function is to signal the translocation of HIF-1α for the nucleus, protein stabilization, and interaction with coactivator p300 [49]. In the oxygen-dependent domain (ODD) of HIF-1α, proline residues in positions 402 and 564 have an important effect on the stability of the protein in normoxic conditions, as they permit, when hydroxylated, recognition by the von Hippel-Lindau protein (pVHL) and subsequent activation of the ubiquitin degradation pathway [52-57]. The hydroxylation of proline residues in the ODD domain of HIF-1α is the critical point that regulates the protein's stability [58,59] (Figure 2). The transcription activity of *HIF1A* gene is thus regulated by the cellular oxygen tension.

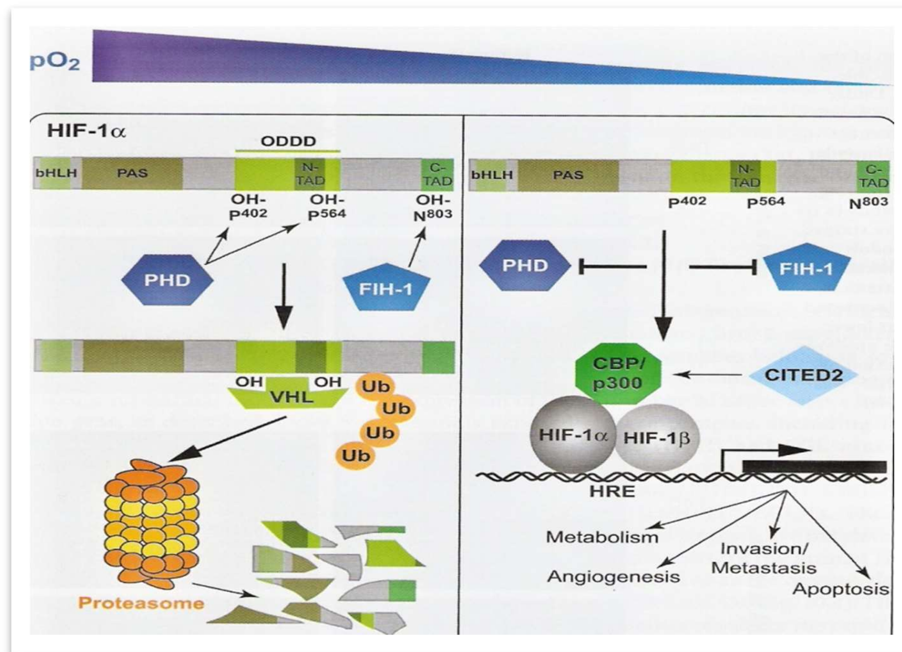


Figure 2. Stability and activity of the HIF. Adapted from [60].

2.2.2. Molecular mechanisms of HIF and of *HIF1A* activation

In the presence of O2, the proline hydroxylation domains (PHD1, 2, 3) provoke specific hydroxylation in two proline residues (P402 and P564) in the HIF-1α ODD, which allows pVHL to recognize HIF-1α; the E3-ubiquitin complex is formed, which will transform HIF-1α into a degradation target [61-64]. Jaakkola et al [63] showed that the interaction between pVHL and the specific HIF-1α domain is regulated by the hydroxylation of the proline residue (HIF-1α P564) by an enzyme called HIF-1α prolyl hydroxylase (HIF-PH), which requires iron and oxygen.

Another O_2 sensor is the factor inhibiting HIF-1 (FIH-1), which hydroxylates HIF-1α in the presence of O_2, at the asparagine residue 803 in the transcription activation domain of the C-terminal (C-TAD), and is inactive in hypoxia, which permits interaction with co-activators CBP/p300 [65,66] (Figure 2).

In hypoxic conditions, molecular O_2 is not available, and thus the enzymes are inactive, which implies elevated levels of HIF-1α [5]. HIF-1α is not hydroxylated, and therefore not degraded; this causes it to accumulate in heterodimerized form with the beta subunit (HIF-β). This heterodimer migrates toward the nucleus, where it binds to the specific DNA sequences, and activates genes involved in the adaptation to hypoxia, cell survival, angiogenesis, and metastasis, such as, for instance, vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGF-

α), glucose transporter 1 (GLUT-1), and carbonic anhydrase IX (CAIX), among many others known to be involved in tumor development and aggressiveness [67,68].
Therefore, the main regulator of HIF is oxygen [54,69]. The second in order of importance are oncogenes, which may contribute to stabilize or degrade protein. For example, protein p53, the product of the tumor suppressor gene *TP53*, inhibits the activity of HIF-1α and becomes a target for proteasomal degradation [70]. However, *TP53* deletions or mutations may facilitate the accumulation of HIF-1α in conditions of hypoxia, increasing the expression of VEGF in tumor cells.
The product of the tumor suppressor gene VHL also regulates the stability of HIF-1α [71], since in the presence of oxygen pVHL can bind to the HIF-1α subunit and become a target for prolyl-hydroxylation [57-59]. Additionally, other oncogenes (v-Src or RasV12) inhibit prolyl-hydroxylation, which implies stabilization of HIF-1α [69-72].
We also know that the expression of the *HIF1A* gene can be regulated through other pathways, mainly those of intracellular signaling, such as protein-kinase B (Akt) and phosphatidylinositol 3'-kinase (PI3K), although their role in these regulation pathways is not yet clear.
Other *HIF1A*-regulating molecules have been described, such as the reactive oxygen species (ROS) involved in carcinogenesis, or cytokines like the tumor necrosis factor alpha (TNF-α) and angiotensin [73-77], which signal pathways such as RAS/RAF1/MEK1/ERK1/2 and/or p53/JNK, activated as a response to oncogenes, growth factors, or hypoxia (Figure 3).

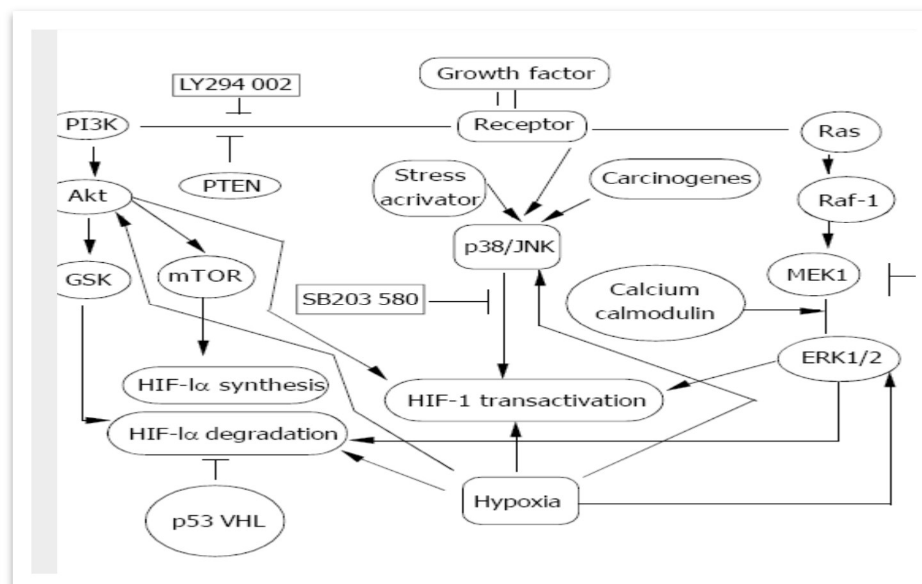


Figure 3. HIF-1α signaling and regulation pathways. Adapted from [78].

2.2.3. General functions of the *HIF1A* gene

Hypoxia is a diminished oxygen tension, defined in clinical terms as a reduction of the availability of oxygen to critical levels (tension under 7%) [77].

HIF-1α is involved in the response to hypoxia, in oxygen homeostasis, and in myocardial, brain and retinal ischemia, pulmonary hypertension, preeclampsia, intrauterine growth retardation, and cancer. It plays a crucial role in physiological homeostatic and etiopathological mechanisms. It acts on target genes because its function is regulated by growth factors and genetic abnormalities involved in tumor progression [79,80].

Aberrant blood vessels can disappear at any time, but they can sometimes be reutilized, causing local reoxygenation, stimulating sudden changes of hypoxia and reoxygenation as a result of local angiogenesis [81-84].

The tumor's environment is well characterized; it is understood as a fluctuation between hypoxia and nutrient deprivation that leads to genetic and epigenetic adaptation of cell clones, which increases its invasion and metastatic capacity.

Additionally, these adaptations to hypoxia make tumors more difficult to treat and more resistant to therapies. An important part of this process is the adaptation of gene products as a response to hypoxia, and the fact that many of these hypoxia-regulated genes are mediated by *HIF1A* [85]; approximately 1% of the genome is estimated to be regulated by hypoxia.

Tumor hypoxia by itself is an important epigenetic factor in the regulation of the HIF-1α protein. In addition to inhibiting PSDs and HIF-1α, hypoxia generates oxygen free radicals capable of stabilizing the HIF-1α protein and of inducing the *HIF* and *VEGF* genes [86,87].

When hypoxia is established, there is a cell response to prevent apoptosis [63], and the HIF-1α transcription factor is activated, which generates a heterodimer with HIF-1β (ARNT) in the hypoxia response element (HRE), which leads to a multiple cell response and the activation of oncogenes [88], increased vascularization with the production of VEGF, increased glucose transport (GLUT1), increased activity of carbonic anhydrase (CAIX), and even the induction of several apoptotic genes [89-91]. HIF is known to act on genes that codify erythropoietin, transferrin, endothelin-1, inducible nitric oxide synthase (iNOS), hemoxygenase 1, insulin growth factor-2 (IGF-2), insulin-like growth factor binding proteins 1, 2 and 3 (IGFBP 1, 2, 3), glucose transporters (GLUT), and glycolytic enzymes [50,92,93] (Figure 4). This promotes metabolic adaptation to hypoxia, and is also regulated by O_2 tension,

depending on the expression of the HIF-1α subunit [94]. Malignant cells' ability to adapt to hypoxia is fundamental for tumor growth (Table 1).

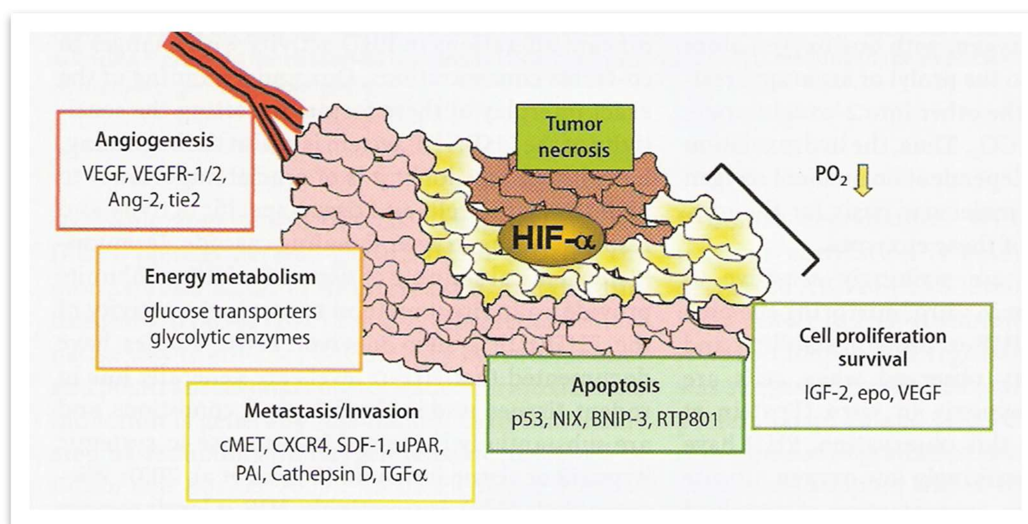


Figure 4. Responses determined by the HIF. Adapted from [95].

Table 1. Examples of molecules regulated by HIF-1α and their pathophysiologic action

Molecule	Function	References
VEGF	Angiogenesis	[5-7] [16] [37,38] [66] [68] [71-78]
Erythropoietin	Erythropoiesis	[5-7][16][66][68][77,78]
GLUT-1	Glycolysis	[5-7][16][37,38][66][68] [77,78]
TGF-α	Invasion and metastasis	[5-7][37,38][78]
Transferrin	Apoptosis	[5-7][16][68][77,78]
Endothelin	Vascular tone	[5-7][16][68][77,78]
CAIX	pH regulator	[5-7][37,38][66][77,78]
iNOS	Drug resistance	[5-7][16][68][77,78]
IGFBP-1, 2, 3	Homeostasis	[5-7][16][68][77,78]

2.2.4. Hypoxia, hypoxia inducible factor, and cancer

Hypoxia is significantly less in tumors in which the average O_2 tension exceeds 1.5% [77,96,97]. In order to survive, tumor cells must adapt to a low pO_2; many genomic products are involved in tumor neoangiogenesis. These adaptations contribute to phenotypic survival and clinical aggressiveness [98]. Tumor hypoxia has been associated with poor prognosis in many kinds of cancer [99].

Tumor cell clones can adapt to hypoxic microenvironments in both primary and metastatic sites. The genetic and epigenetic mechanisms of adaptation to hypoxia (genetic instability, aerobic glycolysis, loss of control of the cell cycle, loss of apoptosis signalling) are characteristic of malignancy [85] (Figure 5).

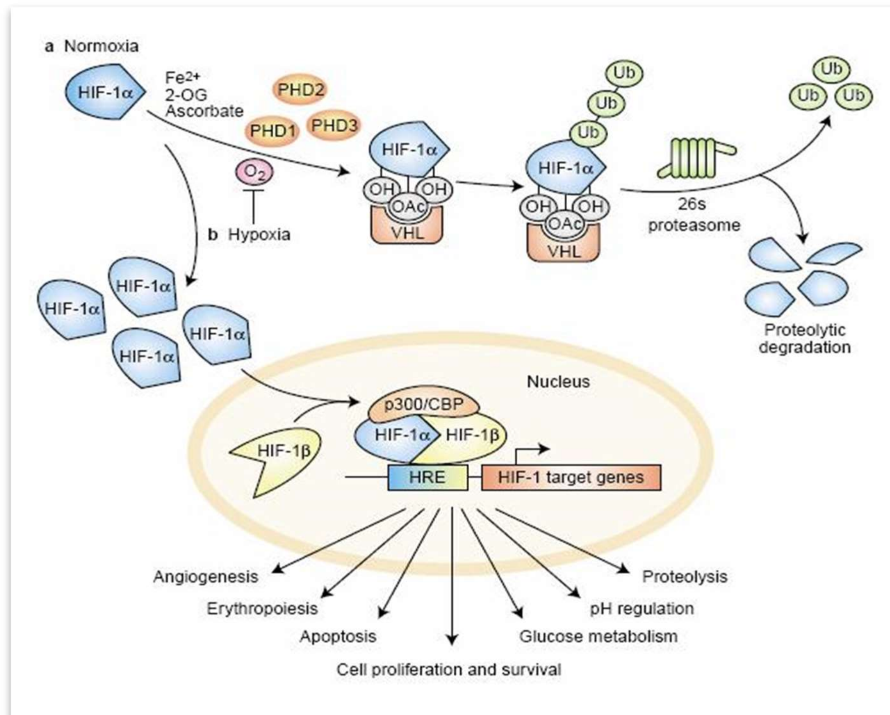


Figure 5. HIF-1α regulation

There is evidence that hypoxia may control and maintain genetic instability. This genetic instability may reduce DNA repair and increase the rate of mutation [90]. Intratumor hypoxia is a factor of poor prognosis observed in prostate, breast, musculoskeletal, head and neck, and cervical cancer [100-102]; it is associated with a higher rate of failure of radiotherapy, chemotherapy, and with increased metastasis [90].
We know that the activation of aerobic glycolysis represents an initial event in the process of neoplastic transformation, probably as a response to increased cell proliferation [103], since rapidly proliferating cells consume more oxygen. Tumors have increased glycolysis, and we know that the concentration of glucose and of components of the glycolytic pathway have an effect on HIF [104,105]. The tumor pH is more acidic due to an increased production of lactate and CO_2. In order to survive, cells must maintain a balance between the intracellular and the extracellular pH; this is achieved thanks to several transporters. Carbonic anhydrase IX plays a fundamental role in this balance; several studies have shown a correlation between hypoxia, angiogenesis, HIF-1α, and CAIX [106].

Therefore, HIF levels are adapted for cells to maintain a high rate of proliferation; on the other hand, the increased cell proliferation may induce an increased expression of HIF [92]. In conditions of hypoxia, where the action of growth factors leads to an increased cell proliferation and thus to an increased oxygen requirement, HIF-1α is more expressed and activated, inducing the expression of genes that codify the pro-angiogenic molecules that permit metabolic adaptation to hypoxia; this is the most powerful activator of genes that codify glycolytic enzymes and pro-angiogenic growth factors [92,107-109,95], since tumors cannot thrive without angiogenesis that allows the diffusion of oxygen, glucose, and other nutrients [110,111].

Angiogenesis is the development of new blood vessels from the pre-existing vessel network, and plays a preponderant role in various pathophysiologic mechanisms, both benign (cicatrization, wounds, ischemia, diabetic retinopathy) and malignant (tumor growth and metastasis); VEGF plays a fundamental role in angiogenesis, and is regulated by HIF [60,112,113].

Currently, there is evidence that tumor blood vessels are disorganized and lack an adequate structure for circulation, which often leads to collapse. Since tumor development requires oxygen, nutrients, and an adequate metabolic function, it is necessary to promote angiogenesis factors in order to inhibit the apoptosis of tumor cells triggered by hypoxia. Therefore, angiogenesis as a response to tumor hypoxia is mediated by HIF-1α [114].

HIF-1α has been considered a key factor in the regulation of VEGF and its receptor (VEGRF), as well as of other angiogenic factors. Several immunohistochemical studies conducted on various tumor models [115] show that the expression of HIF-1α is associated with an increase in VEGF and of vascularization and metastasis, which imply a worse prognosis [116,117]. There seems to be a direct relationship between angiogenesis and metastasis in several kinds of tumors, such as melanoma, glioma, lung, breast, ovary, bladder and prostate cancers [118,119]. It has been proven that HIF-1α target proteins are implicated in the proliferation, survival, adhesion, and mobility of cancer cells.

On the other hand, an increased expression of HIF-1α, in combination with inactivated mutations in suppressor genes such as *VHL*, *p53*, *PTEN* or the amplification of the oncogenes *Akt*, *RAS*, *ERK1/2*, has often been observed in cancer patients; these abnormalities are associated with tumor growth, invasion, and metastasis.

Zhong et al. [120] have demonstrated an increased expression of HIF-1α in approximately 53% of tumors, including cancer of colon, stomach, pancreas, lung, ovary, prostate, kidney, melanoma, and glioblastoma. The increased expression of HIF-1α is associated with a shorter survival in breast and uterine cancer, and with poor response to treatment in nasopharyngeal cancer, highlighting the role of tumor hypoxia in prognosis [116,121-125] (Table 2).

Table 2. Tumors that show overexpression of HIF assessed with immunohistochemistry

	References				
Organ	[99]	[83-85]	[97,98]	[72][100-104]	[48][60][105-108]
Colon	X				
Stomach	X				
Pancreas	X				
Lung	X		X		
Ovary	X		X		
Uterus		X		X	
Prostate	X	X	X		X
Kidney	X				
Glioma	X		X		
Breast		X	X	X	
Head and neck		X			
Melanoma	X		X		

In prostate cancer, it is expressed in the initial stages of carcinogenesis, and this expression is associated with diagnostic and prognostic indicators of early relapse and metastasis; HIF-1α may be a potential poor prognosis biomarker. Its importance in tumor progression becomes a potential target in chemoprevention strategies and in the ability to inhibit angiogenesis [85]. Experimental studies with mice prostate cancer cells show that an overexpression of HIF-1α is associated with more growth and metastatic potential [126]. Similarly, a greater expression of HIF-1α has been found in human prostate tumors [120,127]. The *VEGF* gene, induced mainly by HIF-1α, has been frequently found to be overexpressed in prostate cancer, especially in patients with metastatic or hormone-resistant cancer; this suggests a central action of this molecule in this process [128,129].
The activation of oncogenes and growth factors can induce the HIF system in non-hypoxic cells, or amplify the response to hypoxia. In fact, several growth factors

and cytokines of the stroma and parenchyma also act as regulators and are capable of inducing the expression of HIF-1α, its binding and transactivation capacity, such as the epidermal growth factor (EGF) [130], TGFα [109,131], factors IGF-1 and IGF-2 [132], and interleukin 1beta [83, 133]. Additionally, recent studies show that HIF may play an important role in resistance to treatment [83,134,135].
The HIF system acts as the main regulator of the response to hypoxia, triggering the cascade of mechanisms that permit the tumor to adapt to a hostile environment, and emerges as an important transcription factor in the biology of cancer.

2.3. Hypoxia and prostate cancer aggressiveness: from pathophysiology to clinical biomarkers

[Fraga A et al. Hypoxia and Prostate Cancer Aggressiveness: A Tale With Many Endings. Clin Genitourinary Cancer 2015; 13 (4): 295-301]

Tumors have been reported to possess extensive regions of hypoxia relative to the corresponding normal tissue [96,97]. At least partially, this is due to the rapid proliferation of tumor mass that distances cells from the oxygen carrying vasculature, but is also the consequence of distorted and irregular characteristics of newly formed vessels, ultimately leading to inefficient oxygen transport. It is well established that solid tumors, like prostate cancer, exist under fluctuating oxygen tensions and are exposed to both acute and chronic hypoxia [136-138].

The hypoxic tumor microenvironment correlates with increased tumor invasiveness, metastasis, and resistance to radiotherapy and chemotherapy [97,139-141]. Hypoxia has a detrimental effect on the efficacy of treatment and consequently in the clinical outcomes of patients with prostate cancer, being an independent poor prognostic indicator for patients with prostate and other cancers [97,136].

Over 1% of the genome is transcriptionally responsive to hypoxia, although this varies according to cell type [142]. A large number of endogenous markers of hypoxia which are up-regulated under hypoxic conditions include the vascular endothelial growth factor A (VEGF-A), prolyl hydroxylase 2 (PHD2), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), carbonic anhydrase IX (CAIX), lysyl oxidase (LOX), hypoxia inducible factor 1a (HIF-1a), hypoxia inducible factor 2a (HIF-2a), glucose transporter 1 (GLUT-1), erythropoietin (EPO), E-cadherin, and angiopoietin 2 (Ang2), among others [60,143,144].

Most of these genes have previously been shown to be upregulated by hypoxia in vitro and in vivo tumor models, resulting in a more aggressive, treatment-resistant phenotype [145-148]. Nonetheless, of all these hypoxia biomarkers, none could adequately predict tumor hypoxia [96], even though a biomarker that could reliably and easily identify a man’s prostate cancer oxygen status would be useful for personalized medicine. Current knowledge suggests that rather than considering individual genes, a panel of genes may provide a more accurate reflection of tumor hypoxia [8,9].

Here, we demonstrate linkage with HIF-1α as a tentative explanatory mechanism of prostate cancer aggressiveness. Hypoxia drives a tale where HIF-1α-dependent

effects lead to many influences in distinct key cancer biology features, rendering targeted therapies the endings less efficient. The most appropriate approach would be to inhibit the upstream common driver (HIF-1α) activity. Additional translational and clinical research initiatives in prostate cancer are required to prove its usefulness.

2.3.1. A common tumor hypoxia-driven mechanism (through HIF-1α), with many pathways and therapeutic implications

The hypoxia-inducible factor induces the transcription of numerous genes involved in multiple functions on hypoxia conditions [138,149,150]. HIF-1α is a heterodimeric transcription factor that is the prototypical hypoxia-associated molecule [40]. It is the master key regulator in the hypoxic response of cells by the activity of prolyl hydroxylase domain and orchestrates the hypoxic response (Figure 6). Usually HIF-1α has a cytoplasmic localization, but under hypoxic conditions it is detected and localized in the nucleus, where it binds to HIF-1β and induces transcription causing up-regulation of effector genes by binding to the hypoxia response element within their promoter regions (Figure 6) [151,152].

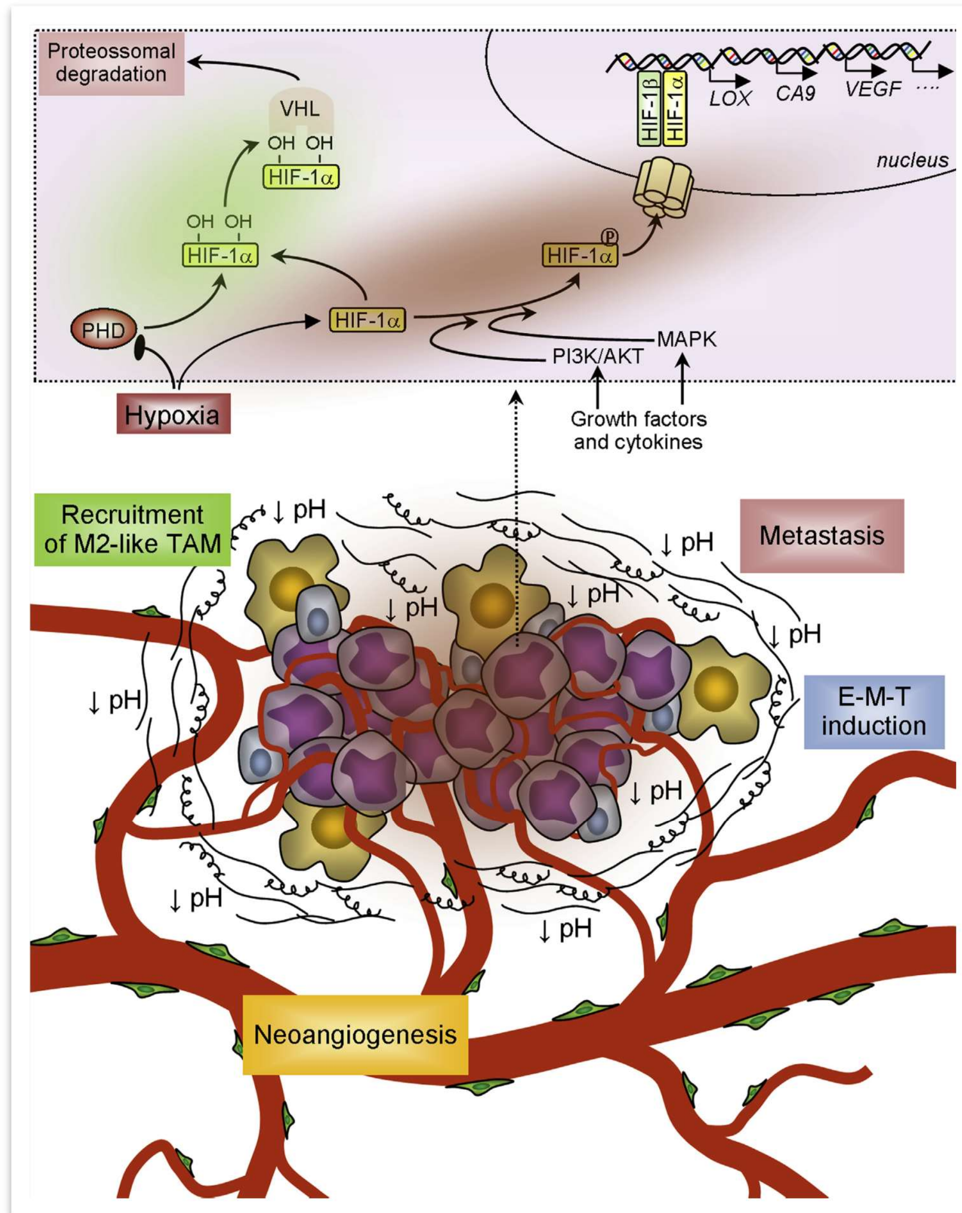


Figure 6. Integration of hypoxia with HIF-1α-associated mechanisms in prostate cancer. specifically downstream-activated LOX, VEGF, and CAIX pathways, and emergence of metastatic traits. The hypoxic environment at the growing prostate tumor primary site conducts HIF-1a toward phosphorylation and translocation to the nucleus instead of the usual proteosomal degradation in normoxia. Here, both the stimulus to increase HIF-1a availability and the suppression of PHD activity concur to hamper HIF-1a degradation. Within the nucleus of the malignant cell, this transcription factor initiates the expression of genes (eg, *VEGF*, *LOX*, *CA9*) notable for their role in driving prostate cancer progression and metastasis. Taken together, these molecules are responsible for modulating the tumor microenvironment through recruitment of tumor-associated macrophages (TAMs), promoting angiogenesis (neoangiogenesis with loss of pericytes, contributing to tortuous and permeable vessels), inducing epithelial-to-mesenchymal transition (E-M-T) and metastasis, thus promoting prostate cancer aggressiveness. Abbreviations: CA9, carbonic anhydrase IX; E-M-T, epithelial-to-mesenchymal transition; HIF-1α, hypoxia inducible factor subunit 1 alpha; HIF-1β, hypoxia inducible factor subunit 1 beta; LOX, lysyl oxidase; MAPK, mitogen activated protein kinase; PHD, prolyl hydrolases; PI3K, phosphoinositol-3-kinase; TAM, tumor-associated macrophages; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

Under hypoxic conditions, HIF-1α induces expression of pro-angiogenic factors and endothelial cell mitogens, eg, vascular endothelial growth factor A (VEGF-A), thus inducing proliferation, sprouting and tube formation of endothelial cells and sustained angiogenesis [153]. Unlike HIF-1α, HIF-2α protein is expressed only in some cell types, can escape degradation, and is transcriptionally active at near-normoxic conditions [154,155]. Still, HIF-2α contributes as HIF-1α to the development of tumor aggressiveness [155,156]. In the prostate, focal HIF-2α expression has been detected in benign neuroendocrinelike and malignant cells [157], being more pronounced in larger prostate tumors [5]. Thus, the role of HIF-2α in hypoxia-associated tumors, particularly prostate cancer, warrants further investigation.

HIF-1α protein has been shown to be increased in prostate cancer tissue sections compared to BPH and to be associated with higher risk for biochemical failure [151,2]. One study reported a trend for higher HIF-1α mRNA expression in prostate cancer versus BPH samples [10]. However, this finding agrees with previous studies showing that HIF-1α is decisively regulated at the posttranslational level [5,158]. Additionally, a direct link between androgen receptors and pro-angiogenic factors may exist, as HIF-1α expression is increased with androgens [5] and decreased in prostatectomy specimen treated with preoperative androgen deprivation therapy [159,2].

Neovascularization is essential for physiologic processes, including in the cancer pathophysiology. In fact, it is well established that tumor growth is associated with increased vascularity [146,160,161]. Mounting evidence from in vitro and in vivo models indicates VEGF is a key regulator of angiogenesis through an effect in endothelial cell growth and proliferation [161]. VEGF binds 2 highly related receptor tyrosine kinases, VEGFR-1 and VEGFR-2. VEGFR-1 expression is upregulated by hypoxia via an HIF-1α dependent mechanism, thereby favouring the activation of VEGF/VEGFR-1 and -2 signalling pathways due to increased availability of both ligand and receptors [162].

It is known that oxygen tension plays a key role in regulating the expression of VEGF [163], whereas VEGF inhibition suppresses pathologic angiogenesis in a wide variety of preclinical models. More specifically, hypoxia may trigger vascular endothelial growth factor (VEGF) expression via the transcription complex of hypoxia-inducible factor HIF-1α (Figure 7). Hypoxia and the consequential

angiogenesis may play a major role in prostate cancer progression [164], as VEGF and HIF-1α is increased in prostate cancer compared to BPH [165,151].

Tumor cells usually have a high rate of glucose uptake accompanied by elevated glucose consumption through the preferential activation of the glycolytic pathway [104]. Several genes involved in glucose uptake and glycolysis (eg, GLUT-1 and most genes coding for enzymes in the glycolytic pathway) have been shown to be targets of HIF-1α [47]. Additionally, HIF-1α activation inhibits mitochondrial metabolism by promoting the expression of pyruvate dehydrogenase kinase 1 to inhibit pyruvate dehydrogenase activity [166], thereby diverting pyruvate to lactate. Noteworthy, despite the decreased flux of glucose-derived pyruvate into the mitochondria, in place of oxidative metabolism, cancers rely on reductive reactions from glutamine carbon [167]. Enhanced lactate production and the production of CO_2 induced by anaerobic conditions contribute to the major acid load in tumor environment. The production of CO_2 induced by anaerobic conditions further contributes to the major acid load in the tumor environment. One of the striking features of cancer cells is their ability to acidify their environment, and the orientation of CAIX suggests that it may serve as one of the mechanisms by which cancer cells regulate extracellular pH and induce cytoplasmic alkalization, playing a role in the adaptation of tumors to hypoxic conditions by regulating the pH of the intracellular and extracellular compartment (Figure 7) [168,169].

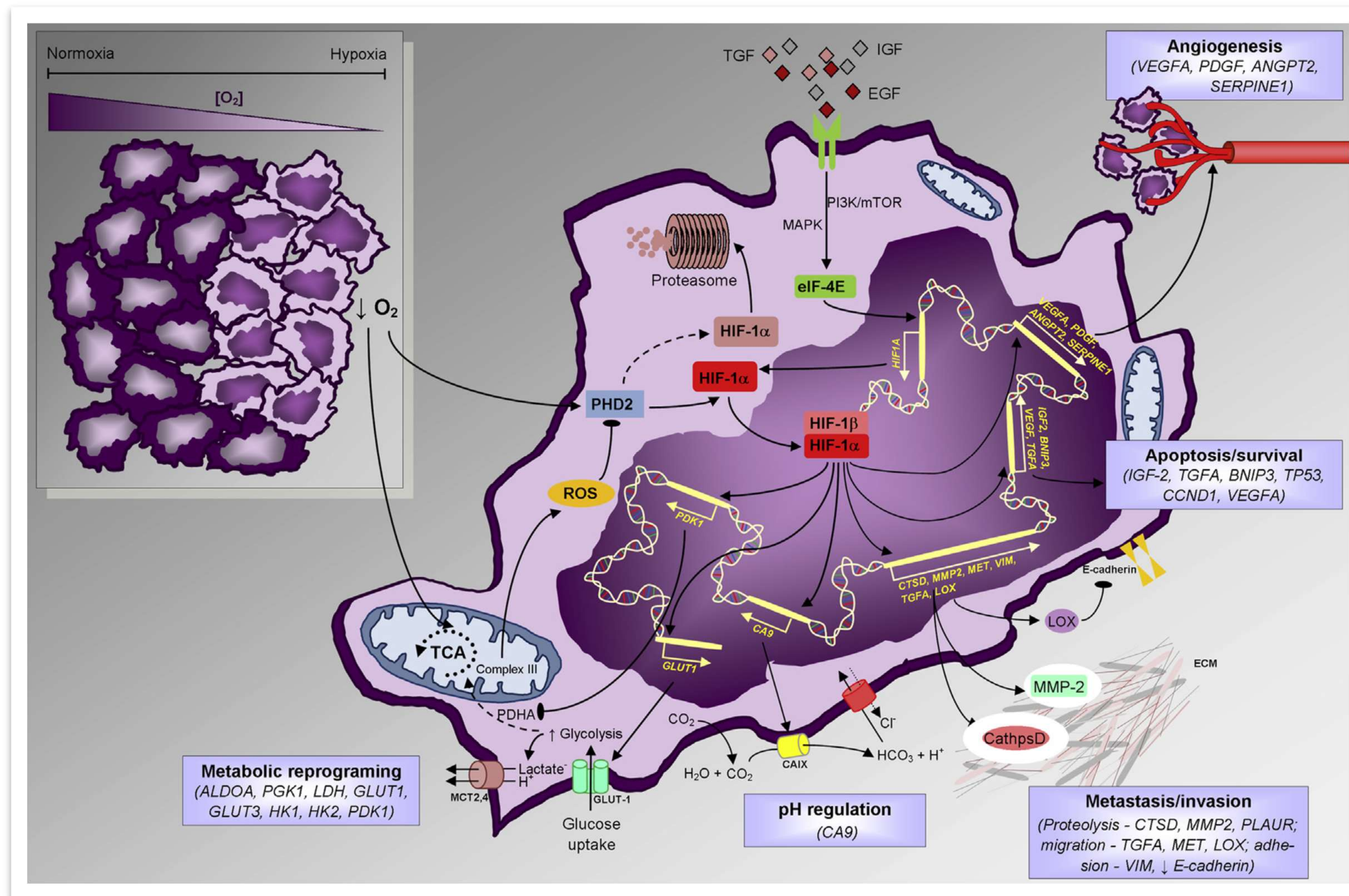


Figure 7. Hypoxia-Induced HIF-1α-Driven Modulation of Key Genes and Resulting Biological Effect. During tumor growth, the unavoidable low availability of oxygen in some areas triggers oxygen-sensing mechanisms, notably prolyl hydrolases (PHDs), which regulate HIF-1α activity (if downregulated, or alternatively proteasomal degradation). In addition, mitochondria-mediated use of oxygen produces reactive oxygen species that suppress PHD2 activity, further stabilizing HIF-1α. Alternative hypoxia-independent or -dependent pathways for HIF-1α up-regulation include binding of growth factors (IGF, EGF, TGF) to tyrosine kinase receptors that signal HIF1A transcription through MAPK and PI3K/Akt/mTOR pathways (by up-regulating the transcription factor eIF-4E). Stabilized and active HIF-1α protein enters the nucleus and binds to HIF-1β to form a complex that regulates the expression of key genes that code for proteins with relevant functions in prostate cancer development and progression. Regulation of genes encoding proteins responsible for metabolic reprograming (eg, GLUT1, ALDOA, PGK1, LDH, PDK1, HK1, and HK2 that switch tumor cell toward glycogenolysis as the main source of energy); genes responsible for pH regulation (eg, MCT1, MCT4, and CA9 that alkalinize the intracellular environment); genes involved in tumor cell apoptosis and survival (eg, IGF2, TGFA, BNIP3, CCND1, TP53, and VEGFA, which down-regulate apoptosis while inducing survival);

genes accounting for neoangiogenesis (eg, VEGF, PDGF, ANGPT2, and SERPINE1 that up-regulate sprouting of new tumor vessel; and genes coding for modulators of invasion and metastasis (eg, the proteolytic CTSD, MMP2, and PLAUR, migration inducers TGFA, MET, and LOX, and adhesion molecules E-cadherin and vimentin). Abbreviations: ALDOA, aldolase A gene; ANGPT2, angiopoietin 2 gene; BNIP3, bcl2/adenovirus e1b 19 kDa protein-interacting protein 3 gene; CA9, carbonic anhydrase 9 gene; CAIX, carbonic anhydrase IX; CathpsD, cathepsin D gene; CCND1, cyclin D1 gene; CTSD, cathepsin D gene; ECM, extracellular matrix; EGF, epidermal growth factor; eIF-4E, eukaryotic translation initiation factor 4E; GLUT1, solute carrier family 2 (facilitated glucose transporter) member 1 or SLC2A1 gene; HIF-1α, hypoxia-inducible factor 1 alpha; HIF-1β, hypoxia-inducible factor 1 beta; HK1, hexokinase 1 gene; HK2, hexokinase 2 gene; IGF, insulin growth factor; IGF2, insulin growth factor 2 gene; LDH, lactate dehydrogenase A gene; LOX, lysyl oxidase; LOX, lysyl oxidase gene; MAPK, mitogen activated protein kinase pathway; MCT1, solute carrier family 16 (monocarboxylic acid transporter) member 1 or SLC16A1 gene; MCT4, solute carrier family 16 (monocarboxylic acid transporter) member 1 or SLC16A3 gene; MET, met protooncogene gene; MMP2, matrix metalloproteinase 2 gene; O_2, molecular oxygen; PDGF, platelet-derived growth factor gene; PDHA, pyruvate dehydrogenase A; PDK1, pyruvate dehydrogenase kinase isoenzyme 1 gene; PGK1, phosphoglycerate kinase 1 gene; PHD2, prolyl hydrolase 2; PI3K/mTOR, phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway; PLAUR, plasminogen activator receptor urokinase-type gene; ROS, reactive oxygen species; SERPINE1, serpin peptidase inhibitor member 1 or plasminogen activator inhibitor type 1 gene; TCA, tricarboxylic acid cycle; TGF, transforming growth factor; TGFA, transforming growth factor alpha gene; TP53, tumor protein p53 gene; VEGFA, vascular endothelial growth factor A gene.

The membrane-bound enzyme CAIX catalyzes the reversible conversion of CO_2 to carbonic acid, contributing to the modulation of pH in tumor cells [170]. The CAIX is HIF-dependent and has been shown to be up-regulated in multiple human cancers [170]. A correlation between hypoxia, angiogenesis, HIF-1α, and CAIX in tumors and metastasis has been reported [106], although the involvement of cancer-associated antigen in prostate tumor progression and metastasis through the modulation of pH remains elusive.

Despite being normally expressed in normal tissues, CAIX becomes highly expressed when tumor cell hypoxia occurs in malignancies [171]. CAIX is up-regulated by hypoxia [172], and its gene is a target of HIF-1α (Figure 7) [173]. Interestingly, the degree of CAIX expression was found to be a prognostic factor of poor survival in many cancer types [174-179]. Prostate cancer cell lines can express CAIX during severe hypoxia [180], which is a good marker of hypoxia particularly for androgen-independent cell lines, with reliable increases in *CA9* mRNA expression after hypoxia exposure [10]. Even though initial findings showed an absence of CAIX expression in primary prostate cancers [180,181], others have observed moderate expression in both BPH and malignant prostatic tissue [10]. Thus, the clinical usefulness of CAIX as a diagnostic tool with implications for therapy and patient outcome remains to be elucidated.

The clinical and pathologic heterogeneity found in cancers highly depends on reciprocal interactions between malignant cells and their dynamic microenvironment [1]. The cross-talk between cells and with extracellular matrix (ECM) in tumor microenvironment seems to be critical in many aspects of cancer

development, including maintenance of cancer cell dormancy, cancer progression and metastasis, and drug resistance [1]. The ECM of solid tumors is composed of a complex meshwork of fibrillar collagens, glycoproteins, and proteoglycans [182,183], which affect metastasis, proliferation, angiogenesis, adhesion, migration, invasion, and drug delivery [184,185].

Hypoxia is an important microenvironment factor in the development of cancer, and while HIF-1α has been shown to be the key regulator of the cellular response to hypoxia [1,186], the relationship between tumor hypoxia and components of ECM is far less known. The role of ECM components and remodeling in cancer has only been a focus of research during the last years. Recent findings suggest that hypoxia mediates collagen 1 fiber remodeling in the ECM of tumors, which may impact delivery of macromolecular agents and the dissemination of cells [184,187-189]. Collagen type I is the major structural ECM component in prostate tumors, with cancer cell invasion occurring radially along its fibers [187]. Moreover, cells of myofibroblast phenotype in the reactive stroma of Gleason 3 scored prostate cancers exhibited elevated collagen type 1 synthesis, which was first observed in activated periacinar fibroblasts adjacent to prostatic intraepithelial neoplasia [184]. In a previously described hypoxia gene signature [190], LOX was shown to be directly regulated by HIF-1α and essential for hypoxia-induced metastasis in several cancer models [191,192]. In agreement with this finding, hypoxia-induced cancer cell invasion was severely impaired through inhibition of LOX expression [193,194]. Cancer cell proliferation was stimulated by LOX in a HIF-1α-dependent manner both in vitro and in vivo [194]. Thus, the regulatory circuit between LOX and HIF-1α act in synergy to foster tumor formation in the adaptation of tumor cells to hypoxia (Figure 7).

The LOX family of oxidases oxidizes lysine residues in collagens and elastin, resulting in the covalent cross-linking and stabilization of these ECM structural components, thus providing collagen and elastic fibers with most of their tensile strength and structural integrity [195]. The accurately regulated expression and activity of the LOX family of oxidases are a prerequisite for them to exert critical functions in connective tissue homeostasis. *LOX* mRNA level is highly up-regulated under hypoxic conditions mediated by HIF-1α at the transcriptional level [183]. In addition to the well-documented roles in connective tissue homeostasis, the LOX family of oxidases participates in other critical biological functions, including cell migration, cell polarity, epithelial-to-mesenchymal transition (EMT), and angiogenesis [196-200].

LOX is synthesized as a pro-enzyme (Pro-LOX) from stromal cells, from normal epithelial cells, or from tumor cells under hypoxic conditions, and is secreted where it undergoes extracellular proteolytic processing by pro-collagen C-proteinases to a functional enzyme and a pro-peptide (LOX-PP) [201,202]. Levels of Pro-LOX production in prostate cancer epithelium are decreased as a function of prostate cancer progression [203]. A recent study proposed that Pro-LOX, but not LOX-PP, is a tumor suppressor [204]. Further studies showed that LOX-PP is an active inhibitor of prostate cancer and other tumor cells growth and of RAS-dependent signalling [194,205,206].

Although LOX was initially implicated as a tumor suppressor, now it is accepted as a poor prognosis marker, particularly in promoting metastasis in breast, lung, prostatic, head and neck, and bronchogenic carcinomas [207,208,203,186,191,149]. Cancer invasion is facilitated by stromal collagen reorganization, and this behavior is significantly increased in collagen-dense tissues (Figure 7) [209]. Many ECM modifying enzymes, including matrix metalloproteinases and LOX family oxidases, are aberrantly expressed during malignant transformation, progression, and metastasis of cancers [186].

Lysyl oxidase-like 2 (LOXL2), a LOX oxidase family member, accumulates in the endothelial ECM and regulates sprouting angiogenesis through assembling type IV collagen in the endothelial basement membrane [210]. Therefore, oxidases of the LOX family play roles in cancer progression and metastasis, promoting not only cancer cell migration and invasion but also angiogenesis in concert with pro-angiogenic factors under hypoxia. Furthermore, inhibition of LOXL2 resulted in a marked reduction in activated fibroblasts and endothelial cells, as well as decreased production of growth factors and cytokines [211]. In agreement, a recent report in advanced renal cell carcinoma patients receiving therapy with angiogenesis inhibitors (pazopanib and sunitinib) disclosed an association of a LOXL2 intronic single nucleotide polymorphism (rs4872122) with overall survival, suggesting its potential role as a predictive biomarker for antiangiogenic drugs and as a therapeutic target in cancer [212].

LOX is a potent chemokine inducing directional migration of varied cell types; when it is present, it strongly induces directional migration of cells [186], and it regulates cell polarity and the E-M-T process (Figure 7) [186,194]. Hypoxia represses E-cadherin expression and promotes E-M-T [198,200]. HIF-1α enhanced E-M-T in vitro and induced epithelial cell migration through up-regulation of LOX [198-200,213]. The up-regulated expression of LOX and LOXL2 under hypoxia is required and

sufficient for hypoxic repression of E-cadherin, possibly through stabilization of the SNAIL transcription factor [198,199]. Further studies are warranted to investigate the contribution of individual LOX family members to the induction of E-M-T in the context of dynamic microenvironment during cancer cell invasion and metastasis.

3.1. Clinical study 1

[Fraga A et al. The HIF1A functional genetic polymorphism at locus +1772 associates with progression to metastatic prostate cancer and refractoriness to hormonal castration. Eur J Cancer 2014; 50: 359-65]

3.1.1. Summary

The hypoxia inducible factor 1 alpha (HIF1α) is a key regulator of tumour cell response to hypoxia, orchestrating mechanisms known to be involved in cancer aggressiveness and metastatic behaviour.

In this study we sought to evaluate the association of a functional genetic polymorphism in *HIF1A* with overall and metastatic prostate cancer (PCa) risk and with response to androgen deprivation therapy (ADT). The *HIF1A* +1772 C>T (rs11549465) polymorphism was genotyped, using DNA isolated from peripheral blood, in 1490 male subjects (754 with prostate cancer and 736 controls cancer-free) through Real-Time PCR. A nested group of cancer patients who were eligible for androgen deprivation therapy was followed up. Univariate and multivariate models were used to analyse the response to hormonal treatment and the risk for developing distant metastasis. Age-adjusted odds ratios were calculated to evaluate prostate cancer risk.

Results showed that patients under ADT carrying the *HIF1A* +1772 T-allele have increased risk for developing distant metastasis (OR, 2.0; 95%CI, 1.1–3.9) and an independent 6-fold increased risk for resistance to ADT after multivariate analysis (OR, 6.0; 95%CI, 2.2–16.8). This polymorphism was not associated with increased risk for being diagnosed with prostate cancer (OR, 0.9; 95%CI, 0.7–1.2).

The *HIF1A* +1772 genetic polymorphism predicts more aggressive prostate cancer behaviour, supporting the involvement of HIF1a in prostate cancer biological progression and ADT resistance. Molecular profiles using hypoxia markers may help predict clinically relevant prostate cancer and response to ADT.

3.1.2. Overview and methods

Currently, only incipient but scarce markers help to predict whether PCa will be an aggressive, fast growing disease or an indolent slow growing type of cancer [214]. The hypoxia inducible factor 1 alpha (HIF-1α) is a transcription factor coded by the *HIF1A* gene that regulates cellular response to hypoxia [215,216], inducing cancer progression through activation of many genes involved in regulatory cancer

biology (angiogenesis, cell metabolism, cell survival, and epithelial-to-mesenchymal transition) [217]. The *HIF1A* gene harbours several SNPs, including a C-to-T substitution at locus +1772 that result in aminoacid modification (proline by serine). Previous in vitro studies showed higher transcriptional activity of the variant allele under both normoxic and hypoxic conditions [217,215], whereas additional research associated this SNP with increased tumour microvessel density [215,217,120].

In prostate cancer, the few molecular epidemiology studies in this SNP were conducted in distinct ethnic populations and clinicopathological characteristics, leading to conflicting results [218-220]. Furthermore, the association of *HIF1A* +1772 C>T with prostate cancer progression, metastasis and refractoriness to androgen deprivation therapy (ADT) merits further evaluation in larger series of patients. In the present study we sought to analyse the association of the functional SNP +1772 C>T in *HIF1A* with PCa using prostatic biopsy-proven controls, and to predict the response to treatment in men receiving ADT.

Histologically confirmed prostate cancers (n = 754) or non-cancers (n = 736) were included in a case-control study. Patients were recruited from five Hospitals in Portugal between 1990 and 2009: Portuguese Institute of Oncology – Porto Centre, S. João Hospital, Porto Military Hospital, Porto Hospital Centre, and Central Lisbon Hospital Centre. The study was approved by hospital's research ethics committees and consent obtained from participants. The non-PCa control group comprises men referred for prostate biopsy, but with normal or benign prostatic histology. Patients with highgrade prostatic intraepithelial neoplasia or a biopsy suspicious of cancer were excluded. A nested sample of subjects from the group of PCa patients (those eligible for androgen deprivation therapy, ADT, (n = 429) was followed up for several years. These patients were submitted to orchiectomy or luteinising hormone releasing hormone agonist (LHRHa) (with or without anti-androgen) immediately after diagnosis or after relapsing from surgery/radiotherapy. Resistance to ADT was defined as the time from ADT initiation to two consecutive rises of PSA greater than the PSA nadir or progression of bone lesions [221,222].

The white cell fraction of blood samples was used to extract DNA (QIAmp DNA Blood Mini Kit, Qiagen). The *HIF1A* +1772 C>T (rs11549465) genetic polymorphism was genotyped by Real-Time PCR using a pre-designed validated Taqman assay (Applied Biosystems). Procedures implemented for quality control included double sampling in about 5% of samples and the use of negative controls in every run.

3.1.3. Results

One-thousand four hundred ninety individuals were included in this study, 736 cancer-free controls and 754 with a positive biopsy for prostate cancer (median age, 66.8 and 68.0 years old, respectively, p = 0.001). Biopsy findings in the control cancer-free group revealed normal histology (10.9%), benign prostatic hyperplasia (33.4%), chronic prostatitis (55.2%) and atrophy (0.5%). As expected, PCa patients presented significantly higher serum PSA levels at diagnosis (p < 0.0001). *HIF1A* +1772 (rs11549465) genotype distributions by group and risk analysis is shown in Table 3.

Table 3. *HIF1A* +1772 genotype distribution and risk for prostate cancer

		Prostate cancer			
	Control	All		High-grade (Gleason<7)	
HIF1A genotypes	N	N	aOR (95%CI)	N	aOR (95%CI)
Additive model					
CC	566	579	Referent	333	Referent
CT	156	164	1.0 (0.8-1.3)	83	0.9 (0.7-1.2)
TT	14	11	0.9 (0.4-2.1)	7	1.0 (0.4-2.5
Dominant model					
CC	566	579	Referent	333	Referent
T carriers	170	175	1.0 (0.8-1.3)	90	0.9 (0.7-1.2)

aOR (95%CI), age-adjusted odds ratios and the respective 95% confidence intervals

Both additive and dominant genetic models were not associated with prostate cancer risk or high grade disease. The distribution of *HIF1A* +1772 C>T genotypes among the non-cancer control subjects were in agreement with Wardy–Weinberg equilibrium (p = 0.988). Furthermore, we found that this SNP was not associated to earlier onset of disease, using Kaplan–Meier plots and functions (data not shown). In the group of prostate cancer patients, analyses of the association between *HIF1A* +1772 genetic variants and patient's clinicopathological characteristics showed over-representation of T-allele in the group of patients not treated with definitive therapy (p = 0.05) and who developed metastasis at any time during the course of malignant disease (Table 4).

Table 4. Genotype distribution in PCa subjects (n=754) according to clinicopathological characteristics

	HIF1A +1772 C>T genotypes			
	CC (n=579)	CT (n=164)	TT (n=11)	p
Definitive therapy				
No	228 (75.0)	69 (22.7)	7 (2.3)	
Yes	281 (78.5)	76 (21.2)	1 (0.3)	0.05*
Clinical stage				
Localized	262 (78.9)	67 (20.2)	3 (0.9)	
Advanced	222 (76.0)	66 (22.6)	4 (1.4)	0.639*
Gleason score				
< 7	177 (75.0)	56 (23.7)	3 (1.3)	
≥ 7	333 (78.7)	83 (19.6)	7 (1.7)	0.443*
Tumor percent[a]	17.0 (6.0-40.0)	20.0 (5.0-38.5)	65.0 (50.0-80.0)	0.185**

Data are presented as number of cases and respective percentage.
[a] Median (interquartile range). * Chi-square test; ** Kruskal-Wallis test.
Columns do not sum up because of missing data.

From the group of 754 patients with prostate cancer, 429 were eligible for androgen deprivation therapy, either due to advanced disease at diagnosis or due to disease progression. The clinicopathological characteristics of this nested group are shown in Table 5.

Table 5. Clinicopathological characteristics features of the group of patients under ADT (n=429)

	n (%)
Age at diagnosis, yrs	
Median (IQR)	70.0 (64.9-75.4)
PSA at diagnosis, ng/ml	
Median (IQR)	19.0 (8.9-51.6)
Gleason score	
<7	128 (32.2)
≥ 7	269 (67.8)
Clinical stage	
Localized	156 (38.7)
Advanced	247 (61.3)
Metastasis at ADT initiation	
No	286 (75.9)

Yes	91 (24.1)
Definitive therapy	
No	299 (69.7)
RP/RT	130 (30.3)
ADT pharmacological group	
aLHRH alone	91 (21.2)
aLHRH + antiandrogen	338 (78.8)

ADT, androgen deprivation therapy; aLHRH, luteinising hormone releasing hormone agonist; RP/RT, radical prostatectomy/radiotherapy; IQR, interquartile range

From the group of patients on ADT, 194 (45.2%) developed resistance to hormonal therapy. The median (95%CI) follow up time was 91.8 (79.8–103.7) months. Univariate age-adjusted empirical time-to-ADT resistance analysis on clinical covariates showed that Gleason grade > 7 (HR, 2.8; 95%CI, 2.0–4.1), advanced clinical stage (HR, 3.7; 95%CI, 2.5–5.3), definitive treatment (HR, 0.6; 95%CI, 0.4–0.8), PSA > 20 ng/ml (HR, 1.9; 95%CI, 1.5–2.6) and presence of metastasis at ADT initiation (HR, 2.9; 95%CI, 2.1–3.9) were all significantly associated with resistance to ADT. The associations between HIF1A +1772 C>T genotypes and the time-to-event age-adjusted univariate and multivariate analyses are shown in Table 6.

Table 6. Association of *HIF1A* +1722 C>T polymorphism with resistance to ADT

	Resistance to ADT				
		Univariate		Multivariate*	
HIF1A +1722 C>T	LR	HR (95%CI)	p	HR (95%CI)	p
Additive model	2.24				
CC		Referent		Referent	
CT		0.8 (0.6-1.2)	0.288	1.0 (0.7-1.5)	0.918
TT		1.8 (0.7-4.6)	0.183	6.1 (2.2-17.0)	0.001
Dominant model	2.70				
CC		Referent		Referent	
T carriers		0.9 (0.6-1.2)	0.460	1.1 (0.8-1.7)	0.536
Recessive model	3.86				
C carriers		Referent		Referent	
TT		1.9 (0.8-4.8)	0.149	6.0 (2.2-16.8)	0.001

LR, likelihood ratio; ADT, androgen deprivation therapy; HR, hazard ratio; 95%CI, 95% confidence interval. * Cox regression using as covariates: Gleason grade, clinical stage, PSA ≥ 20 ng/ml, definitive therapy and existence of metastasis at the time of hormonal castration initiation.

Although we have not found association of HIF1A +1772 C>T polymorphism with resistance to ADT on univariate analysis, in the recessive model the T homozygous genotype was associated with a 6-fold higher risk for developing resistance to ADT, after adjustment for relevant clinicopathological variables (Gleason grade, clinical stage, PSA > 20 ng/ml, definitive therapy and existence of metastases at the time of hormonal castration initiation) (Table 6). The risk of developing metastasis at any time during the course of disease in patients under ADT was significantly higher for T-allele carriers, still after adjustment for other clinical covariates (Gleason grade, clinical stage and PSA > 20 ng/ml) (Table 7).

Table 7. Risk for metastasis in patients receiving androgen deprivation therapy

	Univariate analysis*			Multivariate analysis**		
HIF1A +1722	n	OR (95%CI)	P	n	OR (95%CI)	p
Additive model	380			323		
CC		Referent			Referent	
CT		1.7 (1.0-2.7)			1.9 (1.0-3.6)	
TT		3.5 (0.6-19.4)	0.055		14.9 (1.0-223.1)	0.031[a]
Dominant model	380			323		
CC		Referent			Referent	
T carriers		1.7 (1.1-2.8)	0.023		2.0 (1.1-3.9)	0.027
Recessive model	380			323		
C carriers		Referent			Referent	
TT		3.1 (0.6-17.1)	0.199		12.9 (0.9-190.1)	0.063

[a] *p* for trend. OR (95%CI), odds ratio with 95% confidence interval.
* Age-adjusted ORs.
** Multivariate logistic regression analysis using Gleason grade, clinical stage and PSA ≥ 20 ng/ml as covariates.

3.1.4. Discussion

Hypoxia is a frequent event during prostate cancer progression, while the hypoxia-responsive gene *HIF1A* codes for a key transcription factor that has been proposed as a modulator of PCa initiation and progression [85,151,223]. We analysed a functional SNP (+1772 C>T) in the *HIF1A* gene in prostate cancer patients and controls and found lack of association, although a relatively large population with approximately 1500 men was analysed. Concordantly, two large case-control studies from the United States of America and China also observed no risk for having PCa in carriers of this polymorphism [224,220], even though

opposite results have been also reported [218,225]. The C-by-T substitution in the +1772 locus at the oxygen-dependent domain of the *HIF1A* gene results in a proline-to-serine substitution and was shown to stabilise *HIF1A* and enhance its activity as a transcription factor in both normoxia and hypoxia [215,226]. In agreement, albeit we hypothesised those carriers of T allele were more susceptible to have cancer, our data, together with other, suggest no influence in earlier stages of prostate cancer development. As PCa natural history usually reveals slow growing indolent tumours, the initial steps of carcinogenesis are not likely to be relevant sources of hypoxia, thereby inducing the activation of other than the HIF-1α pathway. Actually, a previous report found that *HIF1A* +1772 C>T genotypes were not correlated with HIF-1α and VEGF expression in localised prostatic tumours [218]. However, HIF-1α overexpression has been reported in cancer precursor lesions, high grade prostate intraepithelial neoplasia, and early stage PCa, compared with normal prostate epithelium [151].

Previous studies have shown overexpression of HIF-1α in many tumours with advanced grade, implying HIF-1α as an independent prognostic factor in cancer [120]. In addition, increasing evidence suggests that genetic markers may be independent predictors of outcome in PCa with various SNPs predicting decreased progression-free and overall survival [227-229]. Data presented here show that the homozygous T genotype and T-allele of *HIF1A* +1772 C>T is associated with increased relapsing after ADT, whereas the T allele is prone to higher risk for having distant metastasis, still after adjustment for empirical covariates (adjusted by Gleason grade, clinical stage and PSA > 20 ng/ml for the risk of metastasis; and by Gleason grade, clinical stage, PSA > 20 ng/ml, definitive therapy and existence of metastases at the time of hormonal castration initiation for the risk of disease recurrence after ADT). While the recessive model (TT versus CT/CC) was significantly associated with resistance to ADT, the dominant (TT/CT versus CC) and additive models were significant for metastasis development under ADT. A recently published meta-analysis suggests that both the T allele and TT genotype were significantly associated with increased cancer risk [230]. Experimental data also support a functional role for the C-by-T substitution at the allele and homozygous genotype level [215,226,231]. We found that additivity was better fitted for metastasis but not to ADT resistance, even though the low number of patients carrying the TT genotype in metastasis analyses yielded a very wide CI, hence deserving careful interpretation.

Our findings in a large cohort of patients that received ADT, support a role for HIF-1α in the pathophysiology of castration resistance and the *HIF1A* +1772 C>T polymorphism as a potential pharmacogenomics predictor of the response to ADT. Concordantly, a recent study demonstrated that HIF1a expression contributed both to metastasis and chemo-resistance of castration resistant prostate cancer [232]. A study comparing *HIF1A* +1772 C>T genotypes between castration-resistant PCa and non-cancer men showed that the T-allele was overrepresented in the cancer group, although it was not associated with survival [219]. Noteworthy, this report presents data from 196 castration-resistant patients using univariate analysis. Another study observed a somatic rare mutation at the same locus in 1/15 androgen-independent prostate tumours, whereas functional studies demonstrated in androgen-independent prostate cancer cells that the T-allele is associated with increased transcriptional activity and protein expression [226]. Therefore, we hypothesise that carrying the T-allele, which stabilises HIF-1α protein and upregulates the *HIF1A* gene expression, may offer a selective advantage to androgen-independent tumour cells through the upregulation of several genes involved in metastasis, angiogenesis, epithelial-to-mesenchymal transition or in other cancer-associated mechanisms [138,85,233-235]. The SNP in *HIF1A* at locus +1772 represents a germline variant, suggesting a cumulative impact of higher HIF-1α expression since birth. However, we hypothesise that *HIF1A* +1772 functional SNP repercussion when combined with hypoxic environmental events or with other genetic risk factors is triggered to higher extent in response to hypoxia-inductive treatments such as ADT. When confirmed in larger and independent samples, additional therapeutic schemes (such as CYP17A1 inhibitors or chemotherapy) could be offered to carriers of the poor responder TT genotype as alternative to ADT. These patients could also be enrolled in clinical trials with drugs that target HIF-1α function (e.g. tasquinimod and other agents that target HIF-1α or its downstream products) [236-239].
Present findings should be further extended and replicated by future studies focusing on genetic polymorphisms as predictors of treatment response to allow tailored therapy in PCa patients. Using this focused candidate gene approach to evaluate the *HIF1A* +1772 C>T SNP gives us an incomplete analysis of hypoxia mechanism. Other hypoxia-related SNPs were not included in this study. However, our study has several strengths such as the selection of the candidate gene based on biological evidence of functional importance; statistical analyses accounted for relevant clinical and pathological factors. In this study all men (including the

controls) 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 or surgical sample, thus making outcome misclassification unlikely.

Our findings suggest that the *HIF1A* +1772 C>T might be a useful marker of aggressive PCa, particularly a predictor of the response to ADT, thus a plausible candidate to include in a panel of risk prediction SNPs in combination with clinical and pathologic features.

3.2. Clinical study 2

[Ribeiro R, Monteiro CP, Azevedo A, Cunha V, Ramanakumar AV, Fraga A, Pina F, Lopes C, Medeiros R, Franco EL. Performance of an Adipokine Pathway-Based Multilocus Genetic Risk Score for Prostate Cancer Risk Prediction. PLoS ONE 2012; 7 (6): e39236]

3.2.1. Summary

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. 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, which included data from putative functional SNPs from the *VEGF/KDR* pathway, since VEGF is produced in adipose tissue and VEGFR2 expressed in tumors and surrounding vessels. 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 (Gleason≥7), whereas three variants were associated with high metastatic-risk prostate cancer (PSA≥20 ng/mL and/ or Gleason≥8). All the examined SNPs in *VEGF* (3 SNPs) and *KDR* (1 SNP) genes did not reach significance in association analysis, therefore they were not further included in multilocus genetic risk analyses. Nevertheless, 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.

3.2.2. Overview and methods

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 [240].

Many prostatic biopsies are unnecessary [241], which underscores the need for better prediction models with increased specificity to aid clinicians decide whether

or not to recommend biopsy. After diagnosis, some cancers are indolent and cause no clinical problems, whereas others progress and may be fatal [38]. 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 [242,243].

Common polymorphisms in adipokine pathways including SNPs in genes coding for VEGF/VEGFR2 pathway are plausible candidates that may help predict prostate cancer susceptibility. 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.

Participants were enrolled 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. We selected a control group of patients with non-prostate cancer (benign prostate hyperplasia [BPH] or chronic prostatitis) from the prospectively enrolled men undergoing prostate biopsy. Prostate pathology and Gleason scores were determined via biopsy. None of the participants had undergone prostate cancer treatment (hormonal castration, surgery, chemotherapy, or radiotherapy). All remaining 1006 eligible Caucasian patients were included for molecular analysis.

Candidate SNPs were selected from the best evidence from published studies and through public databases that provide information on the phenotypic risks. From a total of 29 literature-defined putative functional SNPs in 19 different genes and corresponding to 9 adipokine pathways, 4 SNPs were related with VEGF/VEFR2 pathway (Table 8).

Table 8. Characteristics of SNPs from the VEGF/KDR pathway included in this study

Gene	SNP ID	Substitution	Locus	Region
KDR	rs2071559	T>C	- 604	promoter
VEGF	rs2010963	G>C	+ 405	5'-UTR
VEGF	rs833061	C>T	- 460	promoter
VEGF	rs3025039	C>T	+ 936	3'-UTR

SNP, single nucleotide polymorphism; VEGF, vascular endothelial growth factor gene; KDR, VEGFR2 gene.

SNPs were genotyped using TaqMan allelic discrimination (Applied Biosystems) or polymerase chain reaction - restriction fragment length polymorphism analysis. SNPs in *VEGF* and *KDR* were studied by Taqman.

3.2.3. Results

A total of 449 histologically confirmed prostate cancer and 557 non-prostate cancer patients were included in the analyses. We evaluated the associations between each individual SNP on prostate cancer susceptibility. No association was found for *VEGF* and *KDR* SNPs with overall, high-grade and high-risk for metastasis PCa (Table 9).

Table 9. Age-adjusted Odds Ratios and 95% CI of prostate cancer according to VEGF/KDR pathway polymorphisms

		Age-adjusted ORs					
Genetic	NPC	All PCa		HGPCa[a]		HRPCaM[b]	
Polymorphism	n	n	OR (95%CI)	n	OR (95%CI)	n	OR (95%CI)
KDR -604 T>C							
Additive model							
TT	154	127	Referent	105	Referent	48	Referent
CT	281	215	0.9(0.7-1.2)	177	0.9(0.7-1.3)	72	0.8(0.5-1.3)
CC	122	107	1.1(0.7-1.5)	92	1.1(0.8-1.6)	35	1.0(0.6-1.6)
Dominant model							
TT	154	127	Referent	105	Referent	48	Referent
C carriers	403	322	1.0(0.7-1.3)	269	1.0(0.7-1.3)	107	0.9(0.6-1.3)
Recessive model							
T carriers	435	342	Referent	282	Referent	120	Referent
CC	122	107	1.1(0.8-1.5)	92	1.3(0.9-1.6)	35	1.1(0.7-1.7)
VEGF -460 C>T							
Additive model							
CC	131	114	Referent	99	Referent	46	Referent
CT	274	201	0.9(0.6-1.2)	166	0.8(0.6-1.1)	74	0.8(0.5-1.2)
TT	151	13	1.1(0.8-1.5)	108	1.0(0.7-1.5)	34	0.7(0.4-1.1)
Dominant model							
TT	151	133	Referent	108	Referent	34	Referent
C carriers	405	315	0.9(0.6-1.1)	265	0.9(0.6-1.2)	120	1.2(0.8-1.9)

Recessive model							
T carriers	425	334	Referent	274	Referent	108	Referent
CC	131	114	1.1(0.8-1.4)	99	1.1(0.8-1.5)	46	1.3(0.9-2.0)
VEGF +405 G>C							
Additive model							
GG	251	200	Referent	169	Referent	77	Referent
GC	252	197	1.0(0.8-1.3)	162	1.0(0.7-1.3)	66	0.8(0.6-1.2)
CC	54	50	1.2(0.8-1.9)	41	1.2(0.8-1.9)	10	0.7(0.3-1.4)
Dominant model							
GG	251	200	Referent	169	Referent	77	Referent
C carriers	306	247	1.0(0.8-1.3)	203	1.0(0.8-1.3)	76	0.8(0.6-1.2)
Recessive model							
G carriers	503	397	Referent	331	Referent	143	Referent
CC	54	50	1.2(0.8-1.9)	41	1.2(0.8-1.9)	10	0.7(0.4-1.5)
VEGF +936 G>C							
Additive model							
CC	421	341	Referent	282	Referent	114	Referent
CT	123	100	1.0(0.7-1.3)	87	1.0(0.7-1.4)	39	1.1(0.7-1.6)
TT	11	8	0.9(0.3-2.2)	5	0.7(0.2-2.0)	2	0.7(0.1-3.2)
Dominant model							
CC	421	341	Referent	282	Referent	114	Referent
T carriers	134	108	1.0(0.7-1.3)	92	1.0(0.7-1.3)	41	1.0(0.7-1.6)
Recessive model							
C carriers	544	441	Referent	369	Referent	153	Referent
TT	11	8	0.9(0.4-2.2)	5	0.7(0.2-2.0)	2	0.7(0.1-3.1)

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 $ng.mL^{-1}$)

When we estimated the overall mutually-adjusted effects by stepwise multivariate logistic regression, only the SNPs in *LEPR* Gln223Arg, *SPP1*-66 T>G, *IGF1R*+3174 G>A, *IGFBP3*-202A>C, *FGF2*+223C>T and *IL6*-597G>A, plus age and PSA remained independently associated with risk for overall, and for high-grade prostate cancer. The SNPs in VEGF/KDR analysed didn't reach significance for inclusion in the risk score.

The inclusive (age and PSA added to the multi-locus genetic set) linear risk scores computed on the basis of the above logistic regression models were tested using goodness of fit, were significantly greater than for the models based on the restricted age plus PSA score, for all prostate cancers (P=0.0002) and high-grade prostate cancers (P=0.0001), after likelihood ratio test and confirmed via the net reclassification improvement (NRI) and integrated discrimination improvement (IDI) comparisons.

3.2.4. Discussion

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. Nevertheless, 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, namely in VEGF-460, VEGF+405, VEGF+936, were in agreement with other studies [244-247]. To our knowledge, there have been no prior reports of null associations of KDR-604 and other functional SNPs in other genes with prostate cancer. Although a wealth of evidence demonstrates the effects of individual VEGF on prostate carcinogenesis, it is unlikely that the overall pathophysiological impact is due to the influence of a simple genotypic variation *in vivo*. Here, we showed that consideration of the cumulative susceptibility contributed by SNPs from adipokine pathways helps in risk stratification. Our analyses indicated 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. The effect of the studied SNPs in *VEGF* and *KDR* were not strong enough to be included as risk genotypes in the inclusive model, therefore other more robust genetic markers may cooperate to influence the endocrine and paracrine activity of adipokine pathways that leads to tumor development and progression. However, we cannot exclude that other SNPs in VEGF/KDR pathway may prove to exert a more solid effect in PCa.

3.3. Clinical study 3

[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]

3.3.1. Summary

Androgen deprivation therapy (ADT) is commonly used to treat advanced and recurrent prostate cancer, although prognosis varies widely among individuals. We evaluated whether polymorphisms in adipokine pathway genes may predict clinical outcomes among prostate cancer patients. We enrolled 483 patients who underwent ADT and genotyped them for 27 functional single nucleotide polymorphisms (SNPs) in 17 genes from 9 adipokine pathways, including SNPs from the VEGF/KDR pathway. SNPs were also combined by pathway according to functional characteristics.

The *ADIPOQ* +45 T>G G homozygous carriers were more likely to present biochemical progression and to die than T-allele carriers. Having the *ADIPOQ* +276 G>T G homozygous genotype and the tumor necrosis factor high activation genetic profile were associated with reduced likelihood of resistance to ADT. Presence of the *IL6* -572 G>C C-allele was independently associated with all-cause mortality. The *LEPR* Gln223Arg G-allele variant was associated with a more than twofold increased risk of developing metastasis. The SNPs in VEGF and KDR genes were not associated with any of the clinical outcomes studied after adjustment for other relevant variables. Genetic polymorphisms in specific adipokine pathways might have a clinical role in evaluating prognosis among men treated with ADT, as opposite to the effect of SNPs in VEGF/KDR pathway, either alone or in combination.

3.3.2. Overview and methods

In the last decades, depletion or blockage of androgen action has been the standard of care for men with advanced prostate cancer [248]. Response to treatment is not durable since patients become resistant to ADT, leading to castration-resistance status, an invariably fatal condition [249]. Although mechanisms responsible for prostate cancer cell survival after ADT are not entirely understood, there is evidence that AR-dependent and AR-independent pathways may be implicated [250,251].

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. Some functional SNPs in genes encoding molecules of these pathways (e.g. VEGF/KDR among others) have been shown to be associated with prostate cancer risk [244,252-254] and a recent study found that obese men were at increased risk of developing castration-resistant prostate cancer and metastasis [255]. We studied a cohort of prostate cancer patients treated with ADT to examine the prognostic significance of 27 functional adipokine pathway SNPs with risk of metastasis, response to chemical/surgical castration, and all-cause mortality (ACM).

Patients with histopathologically confirmed prostate cancer and treated with ADT between 1990 and 2009 were included in this study (n=483). Patients were recruited from 4 Hospitals in Portugal. ADT consisted of orchiectomy or treatment with luteinizing hormone releasing hormone- agonist (LHRHa) with or without anti-androgen after diagnosis of advanced or metastatic prostate cancer or after relapsing from primary local therapy with curative intent. Hormonal treatment was continued at least until disease progression, based on serum PSA levels, imaging, and clinical findings. The primary endpoint was resistance to ADT, defined as the time from ADT initiation to two consecutive rises of PSA (1 week apart) greater than the PSA nadir (defined as biochemical progression) or progression of bone lesions (new or size increase, soft tissue metastasis, or at least 2 new metastatic spots in bone scintigraphy), despite at least two consecutive hormonal manipulations [221,222]. The secondary endpoints 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), after diagnosis.

Candidate genes involved in adipokine pathways known to affect oncogenesis were selected, including 3 SNPs in *VEGF* and 1 SNP in *KDR* (mentioned in Table 8 of Experimental study 2). A total of 27 literature-defined putative functional SNPs in 17 different genes were chosen, corresponding to 9 adipokine pathways. We also examined combinations of SNPs by adipokine pathway according to their functional implications (Table 10).

Table 10. VEGF/KDR pathway SNPs included in experimental study 3 and the rationale for combined analysis

Pathway	SNPs	Genotypes	Functional outcomes	SNP functional combinations
VEGF/KDR	*KDR* -604	TT	↑signaling	Expression *
		[256]	↑activation	(-460/+405)
	VEGF-460	C carrier	↑expression	Expression **
		[257,258]	↑activation	(-460/+405/+936)
	VEGF+405	GG	↑expression	Activation ***
		[257,258]	↑activation	(-460/+405/KDR)
	VEGF+936	CC	↑expression	Activation ****
		[259]	↑activation	(-460/+405/+936/KDR)

* Expression 2 *VEGF* SNPs (-460/+405, according to ref [257]): *low* vs. *high.*
** Expression 3 *VEGF* SNPs: *high*, -460/+405 high/936 CC; *intermediate*, -460/+405 high/936 T carrier and -460/+405 low/936 CC; *low*, -460/+405 low/936 T carrier.
*** Activation 2 *VEGF* SNPs: *high*, -460/+405 high/*KDR* TT; *intermediate*, -460/+405 high/*KDR* Ccarrier and -460/+405 low/*KDR* TT; *low*, -460/+405 low/*KDR* Ccarrier.
**** Activation 3 *VEGF* SNPs: *high*, high or intermediate expression/*KDR* TT; *intermediate*, high expression/ *KDR* Ccarrier and low expression/ *KDR* TT; *low*, low or intermediate expression/ *KDR* Ccarrier.

Allelic discrimination through Taqman genotyping (Applied Biosystems) or polymerase chain reaction, followed by restriction fragment length polymorphism analysis was used for genotyping.

3.3.3. Results

The median duration between ADT initiation and disease progression was 91.8 months, while the median follow-up from ADT initiation to death or last visit was 126.9 months. Empirical analysis using Cox regression was then performed to evaluate the association of SNPs and their functional combinations with the outcomes of interest.

The genotypes *ADIPOQ* +276 TT/TG, *IL6R* Asp358Ala CC and *ADIPOQ* +45 GG, and the high expression *ADIPOQ* haplotype, low TNFa expression and low/intermediate TNFa activation genetic profiles were significantly associated with biochemical progression under hormonal castration. However, the VEGF and KDR SNPs, either individually or combined, were not associated with resistance to ADT (Table 11).

Table 11. Association of SNPs in genes of adipokine pathways with resistance to ADT

SNPs and combined SNPs	MGF (%)	Resistance to ADT			
		Model	No.	LR	aHR (95%CI)
KDR-604	48	Dominant	463	2.07	1.13 (0.84-1.52)
VEGF+405	38	Dominant	463	3.24	1.20 (0.92-1.58)
VEGF-460	44	Recessive	457	3.90	1.30 (0.95-1.78)
VEGF+936	13	Recessive	440	2.68	1.80 (0.58-5.65)
VEGF expression 2 SNP [1]	---	---	457	2.35	1.16 (0.86-1.55)
VEGF expression, L/I vs H [1]	---	---	436	2.51	1.18 (0.89-1.55)
VEGF activation, L vs I/H [1]	---	---	457	3.77	1.33 (0.93-1.90)
VEGF activation, L/I vs H [1]	---	---	436	2.69	1.19 (0.89-1.59)

ADT, androgen deprivation therapy; No., number of subjects; MGF, minor genotype frequency in the cohort; LR, likelihood ratio; aHR (95%CI), age-adjusted hazard ratio and respective 95% confidence interval; SNP, single nucleotide polymorphism. *KDR*, vascular endothelial receptor 2; *VEGF*, vascular endothelial growth factor. L, low; I, intermediate; H, high.

Moreover, the *IL6R* Asp358Ala CC and *ADIPOQ* +45 GG, *IL6*-572 C carriers and high VEGF activation 2SNPs were associated with shorter time to ACM following ADT (Table 12). A 62% higher risk for all-cause mortality was associated with carrying high/intermediate activation of VEGF/KDR pathway (combined *VEGF*-460/*VEGF*+405/*KDR*-604).

Table 12. Association of SNPs in genes of adipokine pathways with all-cause mortality

SNPs and combined SNPs	MGF (%)	All-cause mortality			
		Model	No.	LR	aHR (95%CI)
KDR-604	48	Recessive	468	16.29	1.16 (0.80-1.68)
VEGF+405	38	Recessive	468	16.87	1.28 (0.83-1.96)
VEGF-460	44	Recessive	462	13.85	1.14 (0.77-1.68)
VEGF+936	13	Dominant	445	18.24	1.23 (0.83-1.83)
VEGF expression 2 SNP	---	---	462	13.43	1.02 (0.72-1.45)
VEGF expression, L/I vs H	---	---	441	15.93	1.07 (0.77-1.49)
VEGF activation, L vs I/H	---	---	462	18.51	1.62 (1.09-2.41)
VEGF activation, L/I vs H	---	---	441	17.24	1.28 (0.87-1.88)

ADT, androgen deprivation therapy; No., number of subjects; MGF, minor genotype frequency in the cohort; LR, likelihood ratio; aHR (95%CI), age-adjusted hazard ratio and respective 95% confidence interval; SNP, single nucleotide polymorphism. *KDR*, vascular endothelial receptor 2; *VEGF*, vascular endothelial growth factor. L, low; I, intermediate; H, high.

A significant relation with increased risk for developing distant metastasis was observed in the *LEPR* Gln223Arg G carriers, *LEPR* Lys109Arg homozygous G carriers, *TNFRSF1A* -329 G carriers, and for the high/intermediate LEPR signaling genetic profiles, but not with *VEGF* or *KDR* genetic polymorphisms.
The predictive effects of SNPs on time to biochemical progression under hormonal castration and ACM were then evaluated in presence of significant clinicopathological predictors (from Table 1) using Cox regression. Only the effect of *ADIPOQ* +45 and +276 SNPs and of the TNFa activation genetic profile on the response to ADT remained strong after adjustment for clinical factors. Analysis of the secondary endpoint ACM after adjusting for other predictors showed that *ADIPOQ* +45 T>G and *IL6* -572 G>C remained significant predictors. On multivariate logistic regression, patients with the combined high/intermediate LEPR signaling genetic profile remained associated with greater risk of developing distant metastatic disease (OR=3.41, 95%CI: 1.71-6.79).

3.3.4. Discussion

We examined whether germline polymorphisms in adipokine pathways are determinants of the response to ADT. The time to biochemical progression under hormonal castration 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 endpoint ACM, together with *IL6*-572 genetic polymorphism. Additionally, our results also suggest an association of the combined LEPR genetic profile with development of distant metastasis. The combined VEGF/KDR activation genetic profile yielded prognostic relevance only on univariate analysis, thereby revealing lower robustness within the whole adipokine pathway analysis.
Androgen deprivation therapy remains the mainstay treatment for advanced and recurrent prostate cancer [260,221]. The mechanisms responsible for castration-resistant prostate cancer development are not clearly established. Despite obvious interest in AR-dependent pathways, other independent pathways have been described [250,261], in which androgen-refractory cells use alternative survival pathways to overcome the growth inhibition imposed by ADT [250,262]. Adipokine pathways, have been implicated in intracellular signals such as those activated in hormonal castration resistance [263]. Furthermore, mitogenic and anti-apoptoptic

effects of some adipokines (e.g. leptin, IL-6, IGF-1) seem to be limited to androgen-refractory prostate cancer cells [264-266].

Inherited genetic markers have been fairly explored as predictors of prostate cancer outcomes. Although 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, our study has some limitations. Although we included only functional SNPs from genes in adipokine pathways, our SNP panel and SNP combinations could be incomplete. 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 (46.4% for disease progression under ADT; 32.2% for mortality; 44.9% for metastasis).

3.4. Clinical study 4

[Fraga A, Ribeiro R, Coelho A, Vizcaíno JR, Coutinho H, Lopes JM, Príncipe P, Lobato C, Lopes C, Medeiros R. Putative functional genetic polymorphisms in key hypoxia-regulated downstream molecules and phenotypic correlation in prostate cancer. Submitted]

3.4.1. Summary

In this study we sought if, in their quest to handle hypoxia, prostate tumors express target hypoxia-associated molecules and their correlation with putative functional genetic polymorphisms.

Representative areas of prostate carcinoma (n=51) and of nodular prostate hyperplasia (BPH) (n=20) were analysed for HIF-1α, CAIX, LOX and VEGFR2 immunohistochemistry expression using a tissue microarray. DNA was isolated from peripheral blood and used to genotype functional polymorphisms at the corresponding genes (*HIF1A* +1772 C>T, rs11549465; *CA9* +201 A>G; rs2071676; *LOX* +473 G>A, rs1800449; *KDR* – 604 T>C, rs2071559).

Immunohistochemistry disclosed predominance of positive CAIX and VEGFR2 expression in epithelial cells of prostate carcinomas compared to BPH (P=0.043 and P=0.035, respectively). In addition, the VEGFR2 expression score in prostate epithelial cells was higher in organ-confined and extra prostatic carcinoma compare to BPH (P=0.031 and P=0.004, respectively). Notably, for LOX protein the immunoreactivity score was significantly higher in organ-confined carcinomas compare to BPH (P=0.015). The genotype-phenotype analyses showed higher LOX staining intensity for carriers of the homozygous *LOX +473* G-allele (P=0.011), and that *KDR* -604 T-allele carriers were more prone to have higher VEGFR2 expression in prostate epithelial cells (P<0.006).

The expression on prostate epithelial cells of target molecules in hypoxia pathways analysed here (VEGFR2, CAIX and LOX) allowed differentiating malignant from benign prostate disease. Two of the genetic polymorphisms (*LOX* +473 G>A and *KDR* – 604 T>C), account for a potential gene-environment effect in the activation of hypoxia-driven pathways in prostate carcinoma. Further research in larger series is warranted to validate present findings.

3.4.2. Overview and methods

During tumor growth, the oxygen supply and nutrients scarcity urges malignant cells to signal to the microenvironment their needs. The hypoxia inducible factor 1 alpha (HIF-1α) is a key factor by which tumors regulate the

response to hypoxia, triggering cascades with pro-tumoral effects [138,147]. HIF-1α mechanism implies targeting hypoxia response elements in promoters of downstream target genes, notably vascular endothelial growth factor (*VEGF)*, carbonic anhydrase IX (*CA9)*, and lysyl oxidase (*LOX)* promoters, resulting in more aggressive, treatment resistant phenotype [138,147,10]. In prostate carcinoma, a large study has demonstrated the relevance of intrinsic markers of tumor hypoxia for localized disease and outcome of radical treatment [2].

Recent findings indicate that genetic variants may modulate the predisposition for prostate carcinoma and associate with clinical outcome [214,267]. Single nucleotide polymorphisms (SNPs) in genes coding for molecules involved in the response to hypoxia, particularly a functional polymorphism in *HIF1A* gene at locus +1772 C>T [9,231,218,219,226,224,220], has been studied in association with prostate carcinoma with controversial results. However, we are not aware of studies implicating SNPs in other genes (e.g. *LOX*, *CA9*, *KDR)* of HIF-1α-mediated hypoxia downstream pathways.

Based on the role of hypoxia-associated molecules in cancer, we hypothesized an association, at the genetic and protein level, between *HIF1A*, *LOX*, *CA9* and *KDR* genetic variants, the protein expression and prostate carcinoma.

Seventy-one patients with prostate pathology (n=51 with carcinoma, and n=20 with nodular hyperplasia, BPH) were included, after informed consent and approval by hospitals' ethical committees. Patient's clinicopathological data (Table 13) was collected from clinical files and pathological staging determined as organ-confined (T1-T2) (OCPCa) or extra prostatic (T3-T4) (EPCa) disease.

Table 13. Descriptive clinicopathological data of participating patients

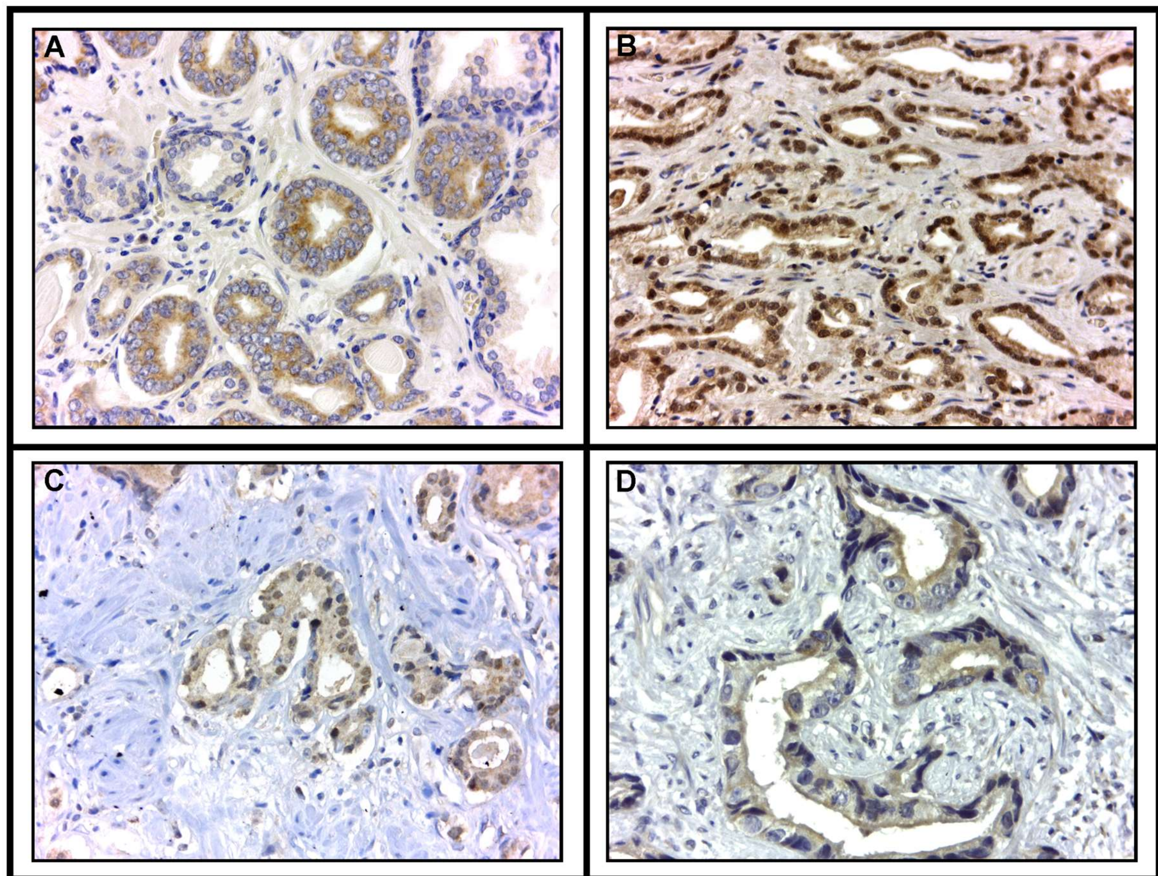
	BPH	OCPCa	EPCa
Age at diagnosis, yrs	67.8 ± 8.4	61.3 ± 6.4	63.3 ± 6.3
PSA at diagnosis, ng/mL	5.5 ± 5.1	6.6 ± 2.4	11.9 ± 5.6
Weight of the prostate, g	94.8 ± 32.1	45.9 ± 14.3	56.6 ± 22.7
Gleason Score			
< 7	-	14 (43.8)	0 (0.0)
≥ 7	-	18 (56.3)	19 (100)
Percentage of tumor *, %	-	15.0 (6.3 – 20.0)	57.0 (28.8 - 78.8)

Descriptive data of continuous variables is presented as mean ± standard deviation, except for percentage of tumor [data shown as median (interquartile range)]. Categorical variable is depicted as number of observations and respective frequencies. BPH, prostate nodular hyperplasia; EPCa, extra prostatic cancer; OCPCa, organ-confined prostate carcinoma; PSA, prostate specific antigen. * on prostatectomy specimens.

The white cell fraction from peripheral blood was used to extract DNA (QIAmp DNA Blood Mini Kit, Qiagen). Four putative functional SNPs (3 non-synonymous and 1 in the promoter region) in 4 candidate genes involved in key hypoxia pathways were selected (*HIF1A* +1772 C>T, rs11549465; *CA9* +201 A>G, rs2071676; *LOX* +473 G>A, rs1800449; *KDR* -604 T>C, rs2071559). Genotyping was done by Real-Time PCR using Taqman ssays (Applied Biosystems).

Representative areas of carcinoma and of nodular hyperplasia were selected and included into tissue microarray as previously described [268]. Slides were stained with anti-HIF-1α (Novus Biologicals), anti-LOX, (Abcam), anti-VEGFR2 (Abcam) and anti-CAIX, (Novus Biologicals) and immunohistochemical evaluation was independently reviewed by two pathologists. Qualitative and quantitative measurements were made for VEGFR2 expression in vasculature and prostate epithelial cells, and HIF-1α, LOX and CAIX in prostate epithelial cells, for both carcinoma and nodular hyperplasia. VEGFR2 intensity was multiplied by the percentage of tumor cells at that intensity level (VEGFR2 H-score); for LOX the score was calculated by multiplicating the percentage of positive cells with staining intensity (LOX immunoreactivity score, IRS). A representative image of the expression of each aforementioned protein is shown in Figure 8.

Figure 8. Representative microscopy images of staining for hypoxia markers in prostate tissues (MO, 400x)

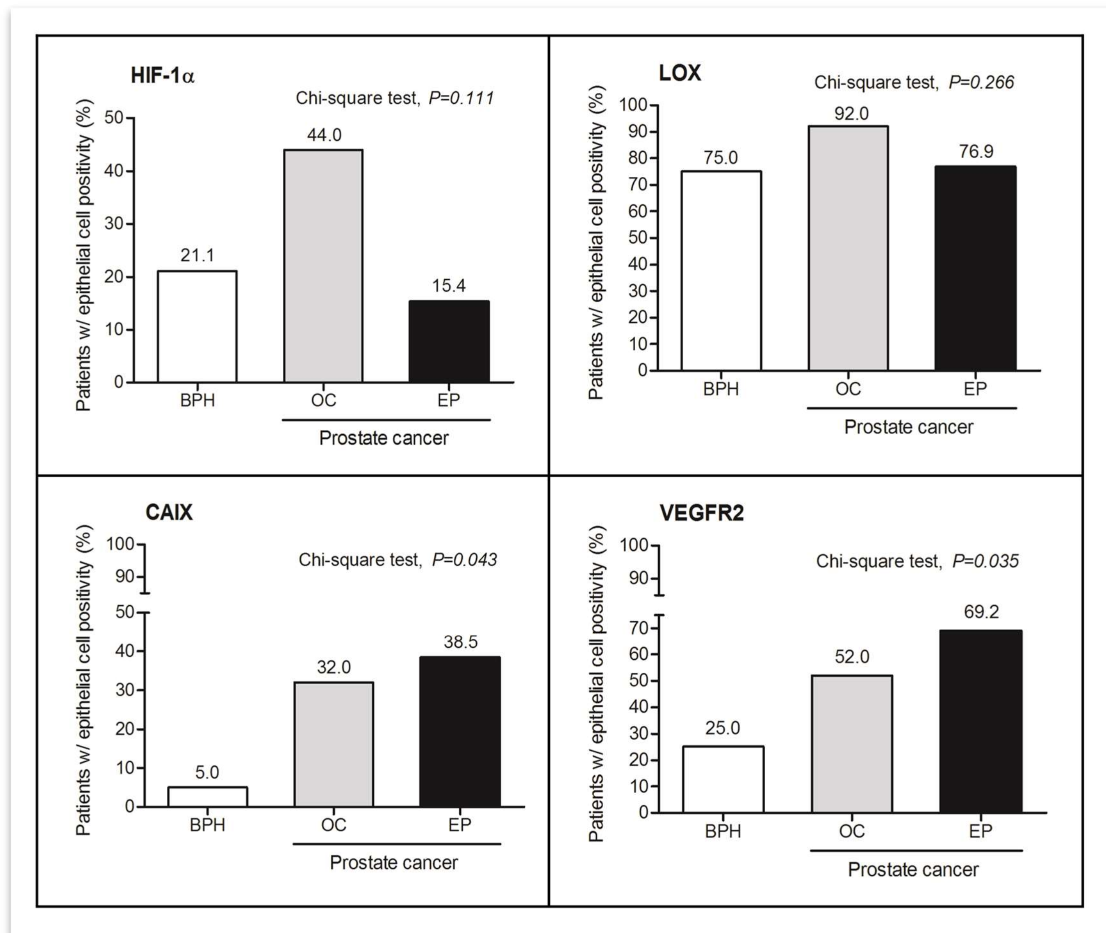


A) HIF-1α - notice the granular cytoplasmic immunoreactivity of the malignant epithelial cells. In this case, more than 50% of the glands stained. B) LOX - strong and diffuse nuclear immunoreactivity of the epithelial cells. C) CAIX - note a focal apical cytoplasmic immunoreactivity in epithelial cells. D) VEGFR2 - moderate nuclear and weak cytoplasmic expression of the epithelial cells

3.4.3. Results

Epithelial cells staining positivity for CAIX and VEGFR was significantly higher in prostate carcinomas compared with BPH (P=0.043 and P=0.035, respectively) (Figure 9). Concurrently, despite non-significant, both HIF-1α and LOX immunoreactivities had a tendency to be elevated in carcinomas (P=0.111 and P=0.266, respectively) (Figure 9).

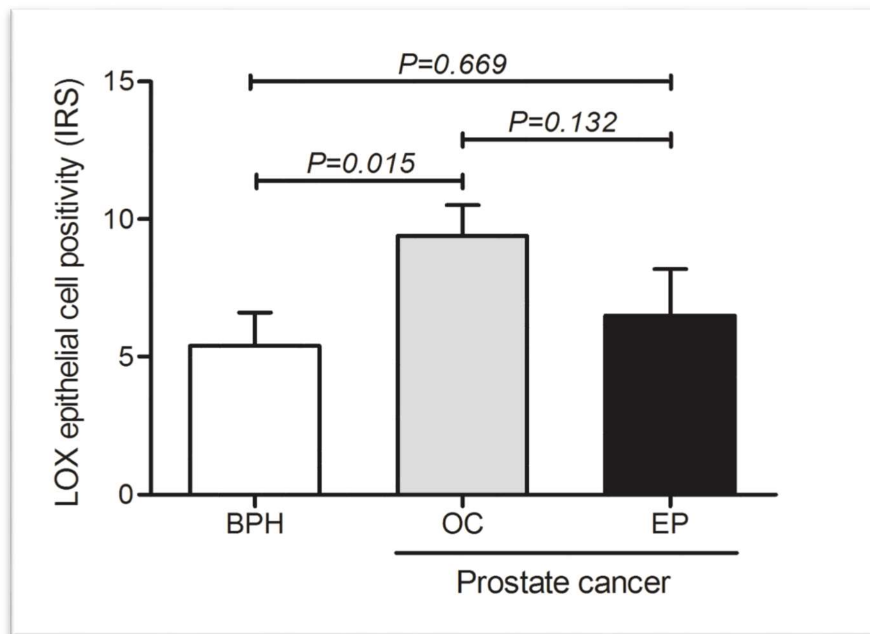
Figure 9. Frequency of patients with positive staining in benign and malignant epithelial cells



CAIX, carbonic anhydrase IX; HIF-1α, hypoxia inducible factor - 1 alpha; LOX, lysyl oxidase; VEGFR2, vascular endothelial growth factor receptor 2. BPH, nodular prostate hyperplasia; EP, extra prostatic disease; OC, organ-confined disease.

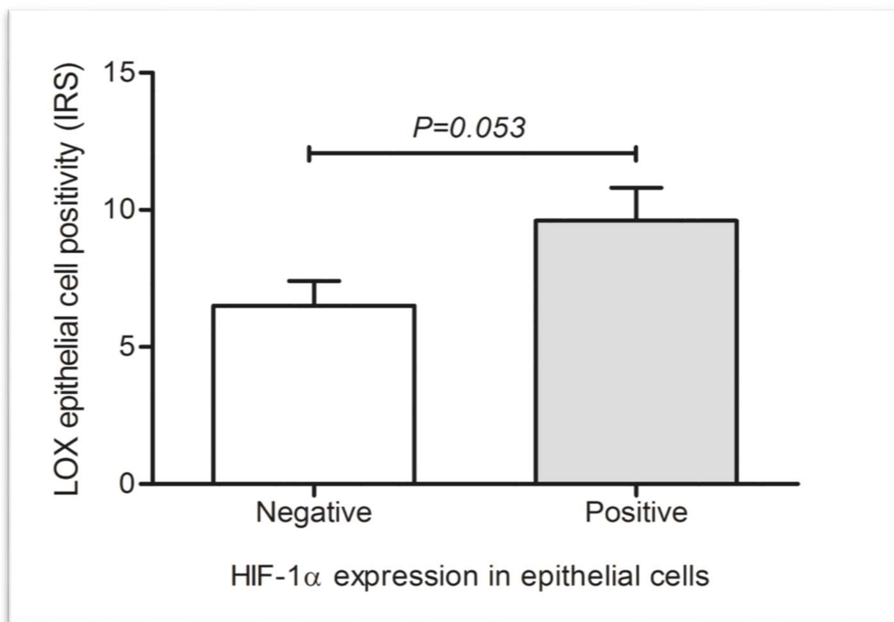
Notably, although not significantly more expressed in prostate carcinomas, the LOX IRS, was significantly more elevated in organ-confined carcinomas than BPH (P=0.015) (Figure 10), and higher in patients with positive HIF-1α expression (P=0.053) (Figure 11).

Figure 10. Comparison of LOX immunoreactivity score in prostate epithelial cells of benign and malignant patients.



BPH, nodular prostate hyperplasia; EP, extra prostatic disease; OC, organ-confined disease. LOX, lysyl oxidase; IRS, immunoreactivity score. Kruskall-Wallis followed by Mann-Whitney non-parametric tests were used to calculate differences between prostatic pathologies.

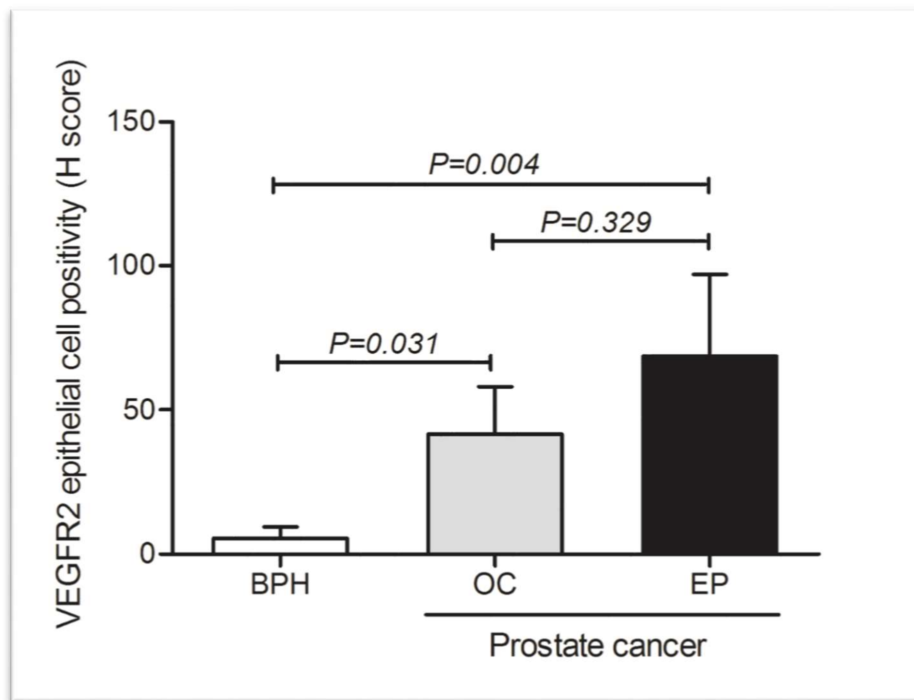
Figure 11. LOX immunoreactivity score by HIF-1α positivity in epithelial cells



Patients with positive HIF-1α expression are prone to higher LOX IRS. HIF-1α, hypoxia inducible factor – 1 alpha; LOX, lysyl oxidase. IRS, immunoreactivity score. Mann-Whitney non-parametric test was used to calculate differences between positive and negative HIF-1α expression.

VEGFR2 immunoreactivity was observed in vascular endothelial cells (only in 20% of all samples) and epithelial cells (70% of patients with extra prostatic carcinomas and approximately half of organ-confined carcinomas). Noteworthy, the VEGFR2 H-score in epithelial cells was statistically distinct between BPH and organ-confined or extra prostatic carcinomas (P=0.031 and P=0.004, respectively) (Figure 12).

Figure 12. Expression of VEGFR2 (H score) in prostate epithelial cells according to prostatic diseases



BPH, nodular prostate hyperplasia; EP, extra prostatic disease; OC, organ-confined disease. VEGFR2, vascular endothelial growth factor receptor 2. Kruskall-Wallis followed by Mann-Whitney non-parametric tests were used to calculate differences between prostatic pathologies.

The genotypic distribution in polymorphisms *HIF1A* +1772 C>T, *LOX* +473 G>A, *CA9* +201 A>G and *KDR* -604 T>C is shown in Table 14. There was no over-represented genotype in disease groups.

Table 14. Genotypic distribution of functional SNPs in genes of hypoxia pathways by disease status using additive and recessive models analyses

	Prostatic disease status			
HIF1A +1772 C>T genotypes	BPH	OCPCa	EPCa	P *
Additive model				
CC	10 (0.59)	23 (0.82)	14 (0.78)	
CT	5 (0.29)	5 (0.18)	4 (0.22)	
TT	2 (0.12)	0 (0.0)	0 (0.0)	0.144
Recessive model				
CC	10 (0.59)	23 (0.82)	14 (0.78)	
TT/CT	7 (0.41)	5 (0.18)	4 (0.22)	0.205
LOX +473 G>A genotypes				
Additive model				
GG	6 (0.71)	16 (0.55)	13 (0.72)	
GA	2 (0.29)	11 (0.38)	4 (0.22)	
AA	0 (0.0)	2 (0.07)	1 (0.06)	0.740
Recessive model				
GG	6 (0.71)	16 (0.55)	13 (0.72)	
AA/GA	2 (0.29)	13 (0.45)	5 (0.28)	0.442
CA9 +201 A>G genotypes				
Additive model				
GG	3 (0.38)	9 (0.31)	5 (0.29)	
GA	5 (0.62)	18 (0.62)	10 (0.59)	
AA	0 (0.0)	2 (0.07)	2 (0.12)	0.882
Recessive model				
GG	3 (0.38)	9 (0.31)	5 (0.29)	
GA/AA	5 (0.62)	20 (0.69)	12 (0.71)	0.918
KDR -604 T>C genotypes				
Additive model				
CC	6 (0.33)	8 (0.26)	3 (0.17)	
CT	8 (0.45)	15 (0.48)	13 (0.72)	
TT	4 (0.22)	8 (0.26)	2 (0.11)	0.436
Recessive model				
CC	6 (0.33)	8 (0.26)	3 (0.17)	
TT/CT	12 (0.67)	23 (0.74)	15 (0.83)	0.515

* Fisher exact test. BPH, nodular prostate hyperplasia; OCPCa, organ-confined prostate carcinoma; EPCa, extra prostatic carcinoma. *CA9*, carbonic anhydrase IX gene; *HIF1A*, hypoxia inducible factor 1 alpha gene; *KDR*, vascular endothelial growth factor receptor 2 gene; *LOX*, lysyl oxidase gene.

Regarding genotype-phenotype relation, there was lack of association between *HIF1A* +1772 C>T and *CA9* +201 A>G genotypes with HIF-1α and CAIX protein expression (Table 15).

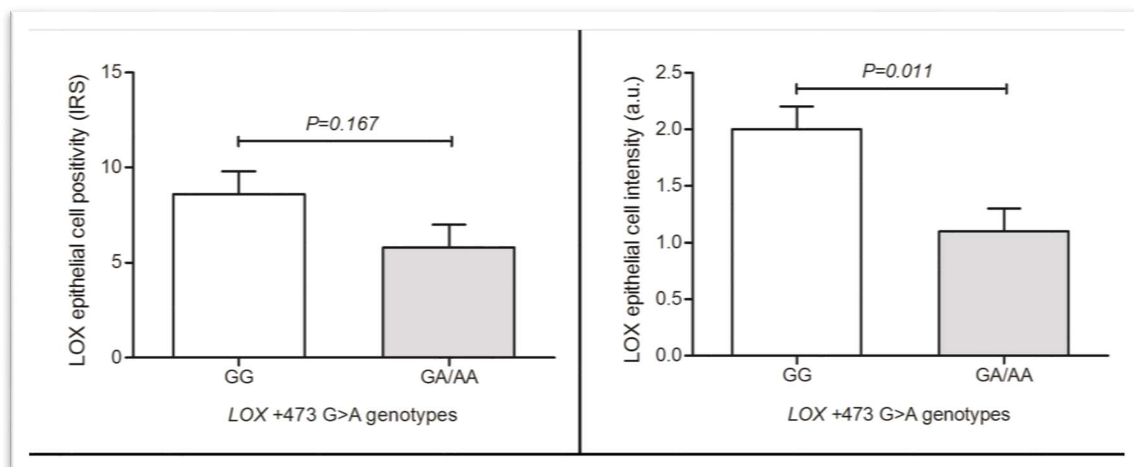
Table 15. Association of the genetic polymorphisms in *HIF1A* +1772 C>T and *CA9* +201 A>G with HIF-1α and CAIX immunoreactivity in prostatic epithelial cells

	Recessive models (*HIF1A* and *CA9*)		
HIF-1α expression	CC	TT/CT	P *
Negative	28 (0.76)	9 (0.24)	
Positive	10 (0.77)	3 (0.23)	0.928
< 50%	32 (0.74)	11 (0.26)	
≥ 50%	6 (0.86)	1 (0.14)	0.516
CAIX expression	GG	GA/AA	
Negative	9 (0.75)	20 (0.69)	
Positive	3 (0.25)	9 (0.31)	0.699

* Fisher exact test

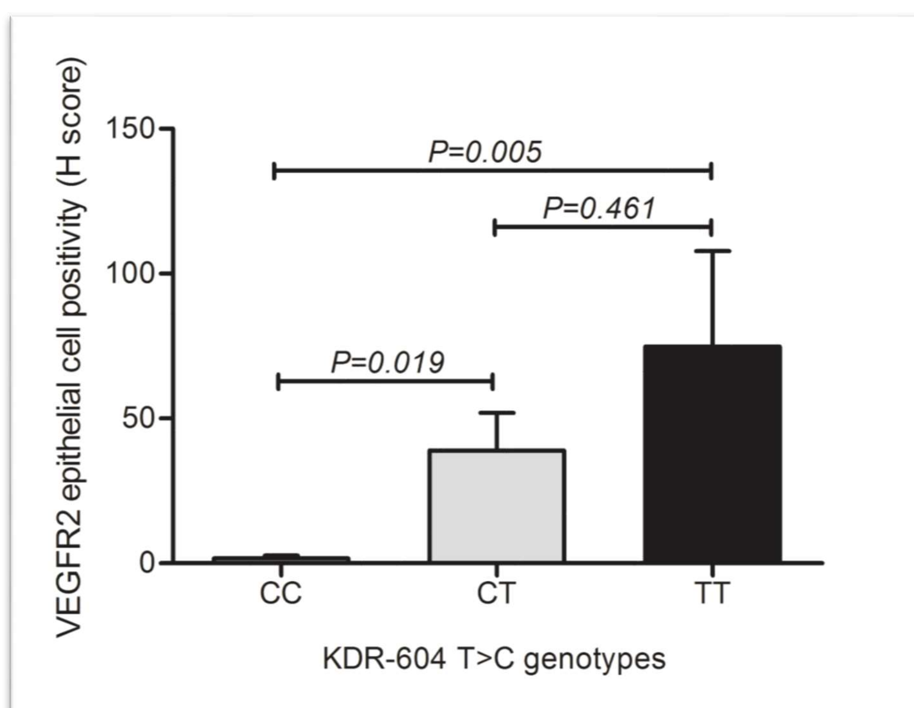
In contrast, LOX expression was significantly more intense in carriers of the *LOX +473* homozygous G allele compared to AA/AG (P=0.011), despite no significance was achieved for IRS (but with similar trend) (Figure 13). Alongside, *KDR* -604 T-allele carriers were more prone to have VEGFR2 expression in prostate epithelial cells but not in vessels (Table 16). The VEGFR2 H-score was significantly higher in T-allele carriers compared to homozygous C (Figure 14).

Figure 13. LOX protein expression (both for immunoreactivity score and staining intensity) according to *LOX* +473 G>A polymorphism



IRS, immunoreactivity score; *LOX*, lysy oxidase; a.u., arbitrary units.

Figure 14. VEGFR2 protein expression (H score) according to *KDR* -604 T>C polymorphism. *KDR*, gene coding for VEGFR2 protein



VEGFR2, vascular endothelial growth factor receptor 2.

Table 16. Association of the *KDR-604 T>C* genetic polymorphism with VEGFR2 immunoreactivity in vessels and in prostatic epithelial cells

	Additive model				Recessive model		
VEGFR⁺ cells	CC	CT	TT	P *	CC	TT/CT	P *
Vessels							
Negative	11 (0.3)	22 (0.5)	9 (0.2)		11 (0.3)	31 (0.7)	
Positive	3 (0.3)	5 (0.4)	4 (0.3)	0.681	3 (0.3)	9 (0.7)	0.626
Epithelial							
Negative	11 (0.4)	13 (0.5)	4 (0.1)		11 (0.4)	17 (0.6)	
Positive	3 (0.1)	14 (0.5)	9 (0.4)	0.039	3 (0.1)	23 (0.9)	0.030

* Fisher exact test

Only data from prostate carcinomas was used to evaluate if hypoxia proteins associated with Gleason score or PSA>10 ng/mL (Table 17). Statistical trends were observed for higher VEGFR2 H-score expression in more undifferentiated carcinomas (Gleason ≥7) (P=0.099) and in patients with prostate specific antigen (PSA) ≥ 10 (P=0.085), and for positive CAIX expression in prostate carcinomas from patients with PSA above 10 (P=0.078).

Table 17. Expression of proteins from hypoxia pathways in prostate cancer patients, by Gleason grade and PSA value

	Gleason grade (n=38)			PSA at diagnosis (n=36)		
	<7	≥7	P	<10	≥10	P
VEGFR2 H-score [a]	30.9±24.7	60.1±17.9	0.099	30.2±1.2	80.0±33.5	0.085
LOX IRS score [a]	10.2±1.6	7.6±1.1	0.184	9.2±1.1	6.6±1.8	0.242
HIF-1α expression [b]						
Negative	6 (0.50)	19 (0.73)		17 (0.65)	8 (0.80)	
Positive	6 (0.50)	7 (0.27)	0.163	9 (0.35)	2 (0.20)	0.335*
CAIX expression [b]						
Negative	10 (0.83)	15 (0.58)		19 (0.73)	5 (0.50)	
Positive	2 (0.17)	11 (0.42)	0.117*	7 (0.27)	5 (0.50)	0.078

PSA, prostate specific antigen; VEGFR2, vascular endothelial growth factor receptor 2; LOX, lysyl oxidase; HIF1a, hypoxia inducible factor 1 alpha; CAIX, carbonic anhydrase IX. [a] Kruskal Wallis and Mann-Whitney U tests for VEGFR2 H-score in epithelial cells; [b] Chi-square test.* Fisher exact test.

3.4.4. Discussion

The hypoxia-driven HIF-1α upregulation activates downstream pathways involved in metabolism (e.g. CAIX), angiogenesis (e.g. VEGF/VEGFR2 pathway) and extracellular matrix activity (e.g. LOX), which can modulate cancer behavior [269]. Experimental and clinical studies in prostate carcinoma demonstrated that HIF-1α overexpression was associated with malignancy, progression and metastatic potential [126] [2]. Here, we found a non-significant statistical trend for higher HIF-1α protein expression in prostate carcinomas compared to BPH, which may be due to the limited number of samples. Besides vascular endothelial cells also prostate epithelial cells express VEGFR2, which were shown to signal through the AKT/mTOR/P70S6K pathway [270]. We found that VEGFR2 was expressed in the epithelium and endothelial cells, though more frequently expressed in epithelial tumor cells of organ confined or extra prostatic carcinomas than in BPH. Hence, in the prostate VEGFR2 expression is mainly expressed in malignant epithelium where its ligand VEGF may exert a direct effect in tumor cell growth. Previous immunohistochemistry studies reported VEGFR2 expression in high-grade prostate intra-epithelial neoplasia and carcinomas of the prostate [271], whereas gene expression findings in prostate cancer cell lines evidenced suppressive growth and promotion of apoptosis with *KDR* antisense oligonucleotide [272]. Taken together

with present data, these findings indicate that VEGFR2 expression in epithelial prostate carcinoma cells supports a function for VEGF that is not limited to angiogenesis. Thus, abrogation of VEGFR2 signalling in malignant epithelial cells may prove an effective therapeutic modality for the treatment of prostate cancer. At present, two anti-angiogenic drugs are being tested in the phase III setting for men with prostate cancer, carbozantinib (a dual VEGFR2/MET inhibitor) and tasquinimod (down-regulator of HIF-1α), that showed beneficial and encouraging results on phase II trials [273].

Tumor cells have to adapt to the hypoxia-driven switch in metabolism, with consequent acidosis, in order to survive. CAIX is a membrane-bound protein crucial for pH regulation in the highly metabolically active malignant cells. In agreement, carbonic anhydrase IX gene (*CA9*) is a target of HIF-1α and is up-regulated in response to hypoxia [274]. *CA9* mRNA expression increases reliably following hypoxia incubation of PC-3 cells [275], although no significant differences on mRNA expression were found when comparing BPH with prostate carcinomas [10]. Other studies described lack of CAIX expression in primary prostate carcinoma and hypothesized alternate pathways for maintaining pH balance [276,277]. Conversely, our results disclosed increased frequency of cases with epithelial cell positivity for CAIX expression in organ confined and extra prostatic carcinomas compared to BPH. Our findings taken together with reports of CAIX expression in epithelial prostate carcinoma cells [275,10] sustain the need for reconsidering CAIX role in prostate carcinoma.

The lysyl oxidase gene (*LOX*), was shown to be directly regulated by HIF-1α transcription factor and essential for hypoxia-induced metastasis and cancer cell proliferation [192]. In the prostate we found that LOX immunoreactivity score was associated with HIF-1α positivity, thus supporting the regulatory nature of HIF-1α in LOX expression. Furthermore, although the number of cases with positive LOX expression in carcinomas was similar to BPH, the LOX IRS was significantly higher in organ confined prostate carcinomas compared with BPH. Interestingly, increased expression of *LOX* mRNA in prostate carcinomas compared with BPH was previously observed [10]. LOX biological functions that include effects in cell growth, migration and polarity agrees with the increased LOX expression found in our carcinoma samples.

In this study, evaluation of protein expression according to SNPs in their coding genes disclosed a genotype-phenotype effect for the *LOX* and *KDR* SNPs, but no functional validation at the protein level was observed for the studied *HIF1A* and

CA9 SNPs. The C-to-T substitution at locus +1772 (rs11549465) in *HIF1A* gene localizes in the oxygen-dependent domain of the gene where the variant allele was shown to stabilize *HIF1A* mRNA and enhance *HIF1A* transcriptional activity [215]. Notwithstanding the functional rationale, association of this SNP with prostate carcinoma risk and with microvessel density, yielded conflicting results [9,218,220,224]. In our study, the lack of statistical differences in *HIF1A* +1772 C>T genotypes for HIF-1α protein expression, agrees with a previous report in prostatic carcinoma [218]. However, the low frequency of TT carriers in our sample (only 2 cases) may have influenced statistical power, since the HIF-1α protein and mRNA overexpression have been associated with the TT genotype [231,278].
A functional genetic variant on *KDR* gene that codifies for VEGFR2 is located in the promoter region (-604 T>C, rs2071559), where the C-allele has been associated with lower transcription activity, and decreased serum VEGFR2 level [256]. Interestingly, we found that T carriers had a significantly higher VEGFR2 expression in prostate epithelial cells, thereby suggesting that this SNP might prove useful for predictive and/or prognostic evaluations in prostate carcinoma, warranting future studies.
A SNP in exon 1 of *CA9* gene is located at locus +201 (rs2071676), where an A-to-G substitution leads to a change of valine-by-methionine in codon 33. Even though we observed an over representation of CAIX positive immunoreactivity in prostate carcinoma compared to BPH, the nonsynonymous SNP in *CA9* +201 was unable to explain variations in the levels of CAIX protein expression in the prostatic tissue, suggesting lack of influence in protein expression, even though the impact of this nonsynonymous substitution (valine to methionine) in CAIX protein activity remains to be confirmed.
The *LOX* gene is translated and secreted as a proenzyme (Pro-LOX), and then processed to a functional enzyme (LOX) and a propeptide (LOX-PP) . We studied a SNP in *LOX* gene that has been identified at locus +473 G>A (rs1800449), that cause an aminoacid substitution (Arg158Glu). This SNP locates at a highly conserved region within LOX-PP, where the A-allele was found to decrease the protective capacity of LOX-PP, while increasing the Pro-LOX-associated invasive ability of tumor cells [279]. When evaluating LOX immunoreactivity and expression intensity by immunohistochemistry in prostate tissues, we found it significantly lower in carriers of the *LOX* +473 A-allele. In the present study, we found that LOX was primarily present at the nucleus of epithelial cells, which fits with other reports asserting that this enzyme may have important functions in secretory cells, as

catalyser of histones in the nucleus [280]. Thus, our findings seem to suggest a wider variety of functions for LOX in prostate epithelial cells, beyond those related to cross-link formation in collagen and elastin, which merit further research. We hypothesize that the trafficking of LOX towards inside the cell or a specific cell compartment may be subordinated to the structural molecular characteristics and folding of the protein, which could be determined by *LOX* +473 G>A polymorphism. Our endeavour to study the genotype-phenotype correlation in key hypoxia markers and its association with prostate cancer yielded encouraging findings, even though results should be interpreted in the context of potential limitations. The lack of statistical significance for genotypic frequencies between disease groups on the putative functional target SNPs in *HIF1A*, *LOX*, *CA9* and *KDR* likely reflects underpowered sample size. This was a major issue as conclusions were impracticable for genetic association analysis and limited for genotype-phenotype inferences. Further limitations arisen from stratification of carcinomas by stage, Gleason score or PSA level, showing at most only statistical trends for increased expression of VEGFR2 and CAIX in more aggressive phenotypes. Nevertheless, considering the hypothesis-generating nature of this study, we report findings that provide important clues to further work in larger samples. Another issue may be related with raised concern over similar hypoxic dysregulation for both prostate carcinoma and benign hyperproliferative diseases. However, inclusion of BPH patients as controls arranged for age-matching with elderly prostate cancer patients, similar clinical and diagnostic procedures (including prostate biopsy) making the possibility of crossover remote; and this group represents the normality in men at that age, since most men of that age carry benign prostate hyperplasia.

CONCLUSIONS AND FUTURE PERSPECTIVES

4.1. General conclusion

Hypoxia is usually found in large solid tumors and is a known inducer of metastasis, being strongly correlated to poorer outcomes. In prostate cancer, as in angiogenesis dependent tumors, there is now evidence of intratumoral hypoxia's profound effect in cancer progression through HIF-mediated regulation of molecules that mediate functional interactions with key aspects of angiogenesis and metastasis.

We have thoroughly revised the state of the art of HIF-mediated molecular mechanisms in cancer in a HIF-1α centric perspective where HIF-1α is crucial for the initiation of angiogenesis, tumor growth, progression, and metastasis. In prostate cancer, the combination of insightful studies on cancer hypoxia suggests the existence of a regulatory circuit between molecules or pathways (such as VEGF, LOX, or CAIX) downstream of HIF-1a, which synergistically modulate tumor microenvironment and promote prostate cancer aggressiveness.

The orchestrator role attributed to HIF as a master regulator of the transcription of genes encoding factors involved in these processes provides the rationale for including HIF inhibitors in prostate cancer therapy regimens, particularly in patients with localized or locally advanced disease, with elevated expression of hypoxia driven molecules in primary tumors.

Additional studies are needed to clarify the cross-talk between cellular players in the hypoxic prostate tumor microenvironment, whereas further insight from translational and clinical data connecting prostate tumor hypoxia with metastasis and mortality will definitively contribute toward novel, personalized therapies.

We have shown here that a *HIF1A* SNP was able to predict disease progression and aggressiveness in a large series of prostate cancer patients. The *VEGF* and *KDR* SNPs had a low predictive and prognostic value for prostate cancer. However, the study concerning the relationship between *HIF1A* and other genetic markers such as *CA9*, *LOX* and *VEGF/KDR* with prostate cancer aggressiveness remains incomplete yet running in the laboratory. We hypothesize that synergistic influence of these SNPs (and eventually others) combined according to their phenotypic effect, will add predictive and prognostic value to clinicopathological variables in the context of prostate cancer.

Prostate carcinoma growth triggers hypoxia, which regulates *HIF1A* expression that in turn impacts the expression of downstream pathways including LOX, CAIX and VEGFR2 in tumor cells. From the putatively functional SNPs included here we observed that only the genetic variants in *LOX* and *KDR* were functionally associated with differential protein expression in malignant epithelial cells. Thereby, no functional confirmation was observed for the SNP in *HIF1A*, even though this SNP was associated with resistance to ADT and bone metastasis development in the genetic epidemiology study. Conversely, for the *KDR* SNP we analysed, albeit there was overexpression of VEGFR2 in prostate tumor cells for the variant genotype, no association was found with PCa risk, or with endpoint analysis in patients undergoing ADT. These seemingly controversial findings might rely on the heterogeneity of patients and diseases for the molecular epidemiology and histochemical studies. Therefore, future immunohistochemical studies should use prostate tumor samples from ADT patients in order to inform more appropriately about a rapidly growing cancer that upwardly impacts hypoxia. In these tumors, higher concordance between germline DNA-to-gene/protein expressions would be expected. Results presented here warrant further research in larger samples in order to evaluate the predictive and prognostic value of *KDR* and *LOX* SNPs in prostate carcinoma.

4.2. Future perspectives

With advances in genotyping and sequencing technologies, continued discovery of novel genetic markers associated with disease initiation, progression and response or resistance to treatment is expected. Furthermore, multicentric large scale studies are required in order to generate novel markers with increased precision. Therefore, we expect to foster investigation by increasing the number of SNPs, performing "GWAS local" in hypoxia markers, and always correlating with phenotypic expression. From combining markers from key pathways involved in prostate cancer hypoxia to the participation/implementation of collaborative studies, we anticipate a shorter route towards the translation of these discoveries into the development of novel tests to assist clinical decision making reasoning and to assist patient stratification in clinical trials. Large clinical trials involving multi institutional collaborations will be required to prospectively validate the utility of these markers for clinical decision making.

In the framework of the line of research presented here, we are aware that information from gene expression analysis (from both *in vitro* and FFPE tissues) will be required for validation of results. In addition, analysing expression profiles of microRNAs might also reveal a hypoxia-associated microRNA predictive or prognostic role that can independently forecast outcome.

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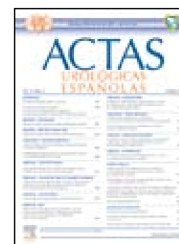

Review – Oncology

Tumor hypoxia. The role of HIF

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Solid tumors usually occur and progress in a hypoxic environment, suggesting that tumor cells are resistant to apoptosis and are associated to an increased angiogenesis, which makes them more aggressive, with invasive capacity and resistant to treatment.

The genetic and biological mechanisms underlying this phenomenon are still unclear, but many studies suggest a role of HIF in this process. Under hypoxic conditions, the alpha subunit is not destroyed, and will activate transcription of a set of genes contributing to tumor aggressiveness. Its expression is associated to an increased metastatic potential that has been shown in both animal studies and human tumors.

Tumor hypoxia has emerged as a key factor in tumor progression and is associated to a poor prognosis, particularly in kidney and prostate tumors. The purpose of this study was to review the significance of hypoxia in carcinogeneses and tumor progression by reviewing the current knowledge on the subject and the mechanisms of action and activation of HIF-1α.

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Hipoxia tumoral. Papel del factor inducible por hipoxia

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Los tumores sólidos, por lo general, existen y progresan en un ambiente de hipoxia; así se observa que las células tumorales son resistentes a la apoptosis y se acompañan de un aumento de la angiogénesis, volviéndose más agresivas, con capacidad invasora y resistentes al tratamiento.

La genética y los mecanismos biológicos subyacentes a este fenómeno son todavía poco claros, pero muchos estudios sugieren un papel del factor inducible por hipoxia *(hipoxia inducible factor* [HIF]) en este proceso. En condiciones de hipoxia, la subunidad alfa no es destruida y activará la transcripción de un conjunto de genes que contribuyen a la agresividad del tumor. Su expresión está asociada a un aumento del potencial metastásico que se verifica tanto en estudios animales, como en tumores humanos.

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La hipoxia tumoral se ha convertido en un factor clave en la progresión tumoral y se asocia a un mal pronóstico, sobre todo en tumores de riñón y próstata. Este trabajo tiene por objetivo revisar la importancia de la hipoxia en la carcinogénesis y en la progresión tumoral, presentando una revisión de los conocimientos actuales sobre el tema, mecanismos de acción y la activación del HIF-1α.

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Introduction

Hypoxia-inducible factor (HIF) is a transcription factor that regulates cells' response to hypoxia and acts as a regulator of oxygen homeostasis[1-3]. Wang and Semenza's[4] identification of the HIF transcription system is crucial for understanding the physiology of O_2; we now know that HIF and hypoxia are the main determinants of angiogenesis and that, for instance, they regulate the processes of invasion and metastasis that determine the tumor's aggressiveness.

The transcription factor activates genes that codify proteins that increase the availability of oxygen and permit metabolic adaptation in the absence of oxygen; it controls the expression of dozens of genes and protein products involved in angiogenesis, erythropoiesis, glycolysis, invasion, apoptosis, vascular tone, pH regulation, epithelial homeostasis, and drug resistance.

More than 60 target genes induced by HIF have been identified[2]; others are suppressed[7]; many functions are HIF-dependent[7].

Molecular structure of HIF-1α

The *HIF1A* gene, which codifies HIF-1α, is located in the 14q21-q24 *locus*[9], which contains 15 exons[10]. It is a heterodimer composed of alpha chains (regulated by O_2) and beta chains, arranged in a helix-loop-helix (bHLH); it belongs to a family of transcription factors consisting of three alpha subunits (HIF-1α, HIF-2α, HIF-3α) and one beta subunit (HIF 1α), also known as aryl hydrocarbon nuclear translocator (ARNT)[4,11,15].

There are two nuclear localization signals (NLS), located on the C-terminal (aminoacids 718-721) and on the N-terminal (aminoacids 17-33), but only the C-terminal is responsible for the nuclear accumulation of HIF-1α[16]. It is also known that HIF contains two transactivation domains (TAD) in the C-terminal (aminoacids 531-575 and 786-826), separated by a sequence of aminoacids (575-786) that inhibit transactivation[17] (Fig. 1).

The N-terminal of the molecule (aminoacid 1-390) contains the bHLH-PAS domain, necessary for dimerization and binding to DNA[18]. The interaction between the bHLH domains of the two subunits regulates their dimerization[19].

The C-terminal domain's function is to signal the translocation of HIF-1α for the nucleus, protein stabilization, and interaction with coactivator p300[17]. In the domain of oxygen-dependent degradation (ODD) domain of HIF-1α, proline residues in positions 402 and 564 have an important effect on the stability of the protein in normoxic conditions, as they permit, when hydroxylated, recognition by the von Hippel-Lindau protein (pVHL) and subsequent activation of the ubiquitin degradation pathway[20-25]. The hydroxylation of proline residues in the ODD domain of HIF-1α is the critical point that regulates the protein's stability[26,27] (Fig. 2). The transcription activity of HIF1A genes is thus regulated by the cellular oxygen tension.

Molecular mechanisms of HIF and of HIF1A activation

In the presence of O_2, the proline hydroxylation domains (PHD1, 2, 3) provoke specific hydroxylation in two proline residues (P402 y P564) in the HIF-1α ODD, which allows pVHL to recognize HIF-1α; the E3-ubiquitin complex is formed, which will transform HIF-1α into a degradation target[30-33]. Jaakkola et al[32] showed that the interaction between pVHL and the specific HIF-1α domain is regulated by the hydroxylation of the proline residue (HIF-1α P564) by an enzyme called HIF-1α prolyl hydroxylase (HIF-PH), which requires iron and oxygen.

Another O_2 sensor is the factor inhibiting HIF-1 (FIH-1), which hydroxylates HIF-1α in the presence of O_2, at the asparagine residue 803 in the transcription activation domain of the C-terminal (C-TAD), and is inactive in hypoxia, which permits interaction with co-activators CBP/p300[34,35] (fig. 2).

In hypoxic conditions, molecular O_2 is not available, and thus the enzymes are inactive, which implies elevated levels of HIF-1α[36]. HIF-1α is not hydroxylated, and therefore not degraded; this causes it to accumulate in heterodimerized form with the beta subunit (HIF-β). This heterodimer migrates toward the nucleus, where it binds to the specific DNA sequences, and activates genes involved in the adaptation to hypoxia, cell survival, angiogenesis, and metastasis, such as, for instance, vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGF-α), glucose transporter 1 (GLUT-1), and carbonic anhydrase IX (CA9), among many others known to be involved in tumor development and aggressiveness[37,38].

Therefore, the main regulator of HIF is oxygen[22,39]. The second in order of importance are oncogenes, which may contribute to stabilize or degrade protein. For example, protein p53, the product of the tumor suppressor gene TP53, inhibits the activity of HIF-1α and becomes a target for proteasomal degradation[40]. However, TP53 deletions or mutations may facilitate the accumulation of HIF-1α in conditions of hypoxia, increasing the expression of VEGF in tumor cells.

The product of the tumor suppressor gene VHL also regulates the stability of HIF-1α[42], since in the presence of oxygen pVHL can bind to the HIF-1α subunit and become

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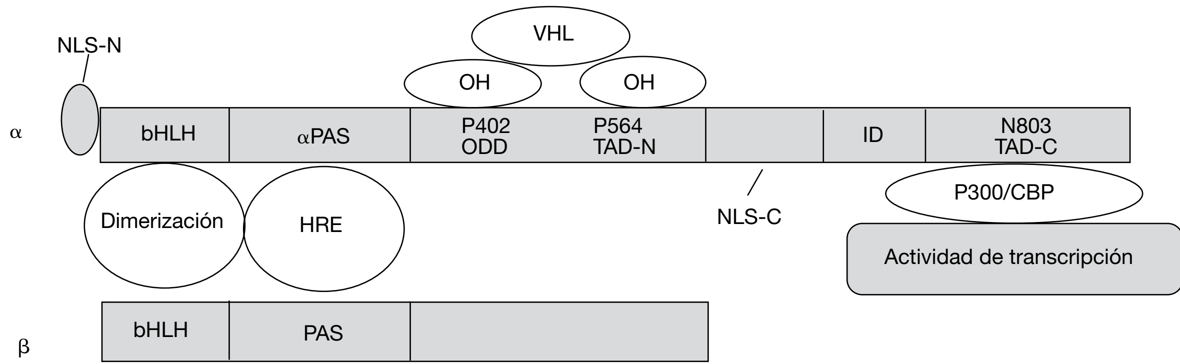

Figure 1 – Molecular structure of HIF-1α. Adapted from Shi YH[55].

Figure 2 - Stability and activity of the hypoxia-inducible factor. Adapted from Brahimi-Horn and Pouyssegur[94].

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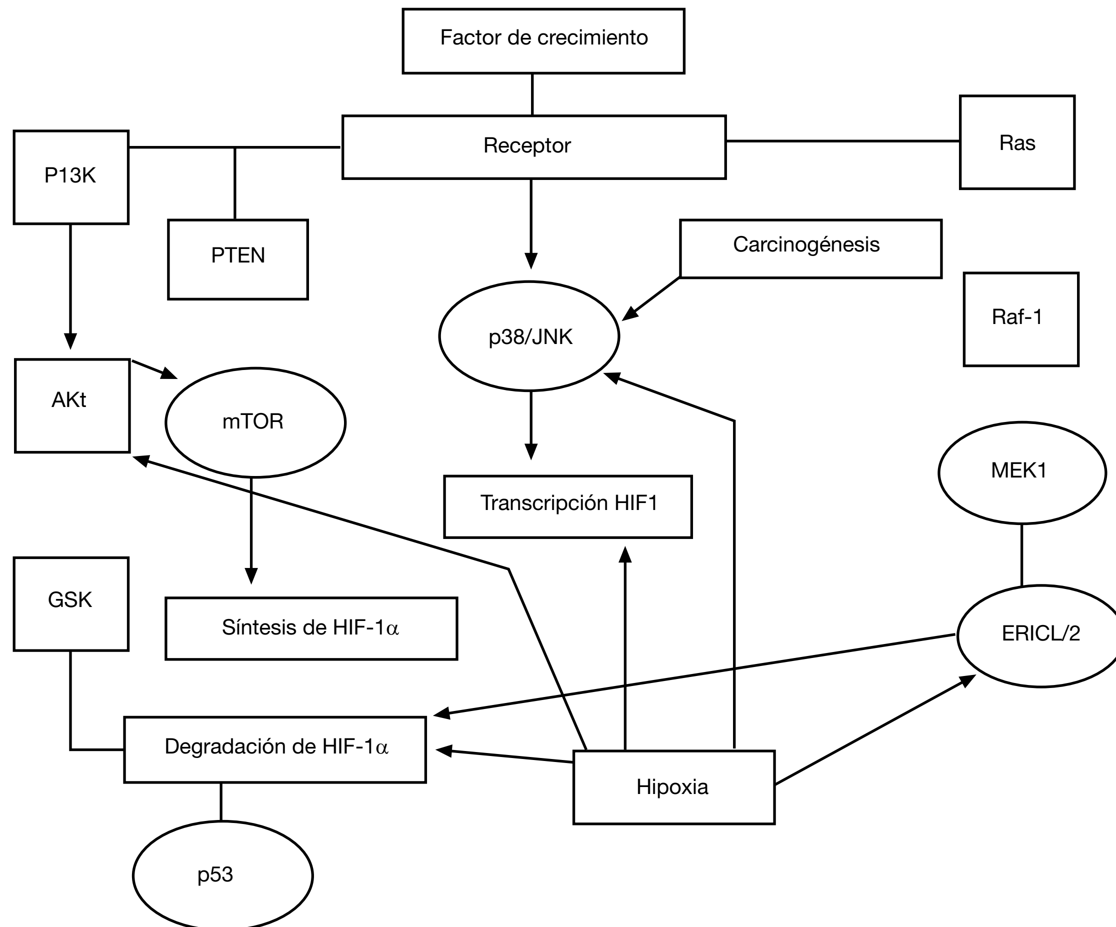

Figure 3 – HIF-1α signaling and regulation pathways: oncogenes, growth factors, and hypoxia. Adapted from Shi YH et al[4].

a target for prolyl-hydroxylation[25-27]. Additionally, other oncogenes (v-Src or RasV12) inhibit prolyl-hydroxylation, which implies stabilization of HIF-1α[39-42].

We also know that the expression of the *HIF1A* gene can be regulated through other pathways, mainly the those of intracellular signaling, such as protein-kinase B (Akt) and phosphatidylinositol 3'-kinase (PI3K), although its role in these regulation pathways is not yet clear.

Other HIF1A-regulating molecules have been described, such as the oxygen-reactive species (ROS) involved in carcinogenesis, or cytokines like the tumor necrosis factor (TNF-α) and angiotensin[49-53], which signal pathways such as RAS/RAF1/MEK1/ERK1/2 and/or p53/JNK, activated as a response to oncogenes, growth factors, or hypoxia (Fig. 3).

General functions of the HIF1A gene

Hypoxia is a diminished oxygen tension, defined in clinical terms as a reduction of the availability of oxygen to critical levels (tension under 7%)[53].

HIF-1α is involved in the response to hypoxia, in oxygen homeostasis, and in myocardial, brain and retinal ischemia, pulmonary hypertension, preeclampsia, intrauterine growth retardation, and cancer. It plays a crucial role in physiological homeostatic and etiopathological mechanisms. It acts on target genes because its function is regulated by growth factors and genetic abnormalities involved in tumor progression[54,55].

Aberrant blood vessels can disappear at any time, but they can sometimes be reutilized, causing local reoxygenation, stimulating sudden changes of hypoxia and reoxygenation as a result of local angiogenesis[56-59].

The tumor's environment is well characterized; it is understood as a fluctuation between hypoxia and nutrient deprivation that leads to genetic and epigenetic adaptation of cell clones, which increases its invasion and metastatic capacity.

Additionally, these adaptations to hypoxia make tumors more difficult to treat and more resistant to therapies. An important part of this process is the adaptation of gene products as a response to hypoxia, and the fact that many of these hypoxia-regulated genes are mediated by HIF1A[60]; approximately 1% of the genome is estimated to be regulated by hypoxia.

Tumor hypoxia by itself is an important epigenetic factor in the regulation of the HIF-1α protein. In addition to inhibiting PSDs and HIF-1α, hypoxia generates oxygen free radicals capable of stabilizing the HIF-1α protein and of inducing the *HIF* and *VEGF* genes[61,62].

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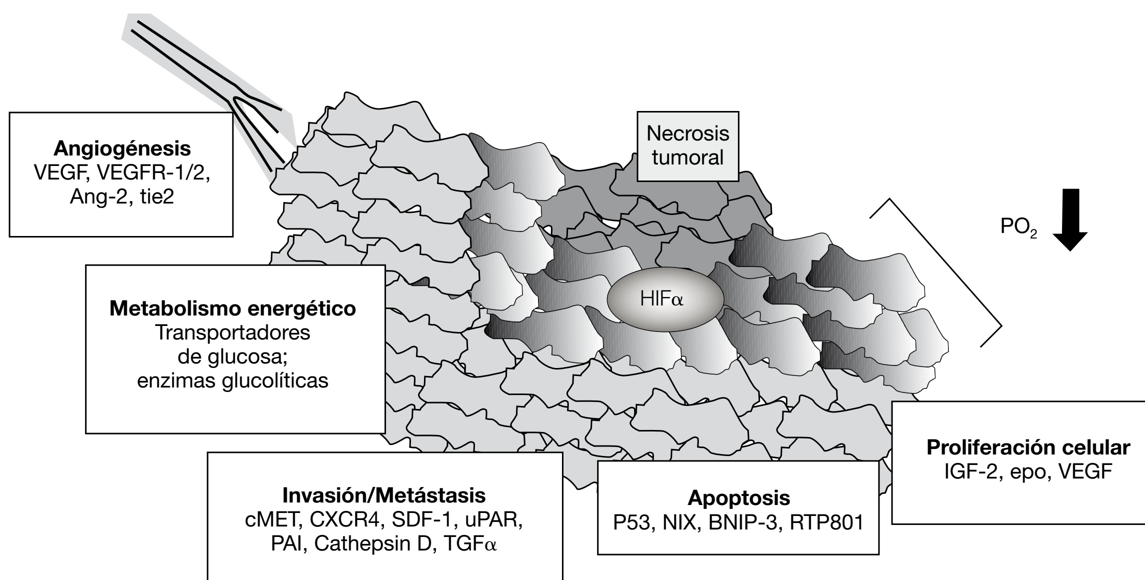

Figure 4 – Responses determined by the hypoxia-inducible factor: it acts as the main physiologic regulator of hypoxia. Adapted from Acker and Plate[93].

When hypoxia is established, there is a cell response to prevent apoptosis[63], and the HIF-1α transcription factor is activated, which generates a heterodimer with HIF-1 (ARNT) in the hypoxia response element (HRE), which leads to a multiple cell response and the activation of oncogenes[64], increased vascularization with the production of VEGF, increased glucose transport (GLUT1), increased activity of carbonic anhydrase (CA9), and even the induction of several apoptotic genes[65-67]. HIF is known to act on genes that codify erythropoietin, transferrin, endothelin-1, inducible nitric oxide synthase (iNOS), hemoxygenase 1, insulin growth factor-2 (IGF-2), insulin-like growth factor-binding proteins 1, 2 and 3 (IGFBP 1, 2, 3), glucose transporters (GLUT), and glycolytic enzymes[18,28,68] (Fig. 4). This promotes metabolic adaptation to hypoxia, and is also regulated by O_2 tension, depending on the expression of the HIF-1α subuinit[69]. Malignant cells' ability to adapt to hypoxia is fundamental for tumor growth (Table 1).

Table 1 – Molecules regulated by HIF-1# and their pathophysiologic action

Molecule	Function	References
VEGF	Angiogenesis	5-7, 16, 37, 38, 66, 68, 71-78
Erythropoietin	Erythropoiesis	5-7, 16, 66, 68, 77, 78
GLUT-1	Glycolysis	5-7, 16, 37, 38, 66, 68, 77, 78
TGF-α	Invasion and metastasis	5-7, 37, 38, 78
Transferrin	Apoptosis	5-7, 16, 68, 77, 78
Endothelin	Vascular tone	5-7, 16, 68, 77, 78
CA 9	pH regulator	5-7, 37, 38, 66, 77, 78
iNOS	Drug resistance	5-7, 16, 68, 77, 78 8
IGFBP-1, 2, 3	Homeostasis	5-7, 16, 68, 77, 78

Hypoxia, hypoxia inducible factor, and cancer

Hypoxia is significantly less in tumors in which the average O_2 tension exceeds 1.5%[53,79,80].

In order to survive, tumor cells must adapt to a low pO_2; many genomic products are involved in tumor neoangiogenesis. These adaptations contribute to phenotypic survival and clinical aggressiveness[81]. Tumor hypoxia has been associated with poor prognosis in many kinds of cancer[82].

Tumor cell clones can adapt to hypoxic microenvironments in both primary and metastatic sites. The genetic and epigenetic mechanisms of adaptation to hypoxia (genetic instability, aerobic glycolysis, loss of control of the cell cycle, loss of apoptosis signaling) are characteristic of malignancy[60] (Fig. 5).

There is evidence that hypoxia may control and maintain genetic instability. This genetic instability may reduce DNA repair and increase the rate of mutation[66].

Intratumor hypoxia is a factor of poor prognosis observed in prostate, breast, musculoskeletal, head and neck, and cervical cancer[83-85]; it is associated with a higher rate of failure of radiotherapy, chemotherapy, and with increased metastases[66].

We know that the activation of aerobic glycolysis represents an initial event in the process of neoplastic transformation, probably as a response to increased cell proliferation[86], since rapidly proliferating cells consume more oxygen. Tumors have increased glycolysis, and we know that the concentration of glucose and of components of the glycolytic pathway have an effect on HIF[87,88]. The tumor pH is more acidic due to an increased production of lactate and CO_2. In order to survive,

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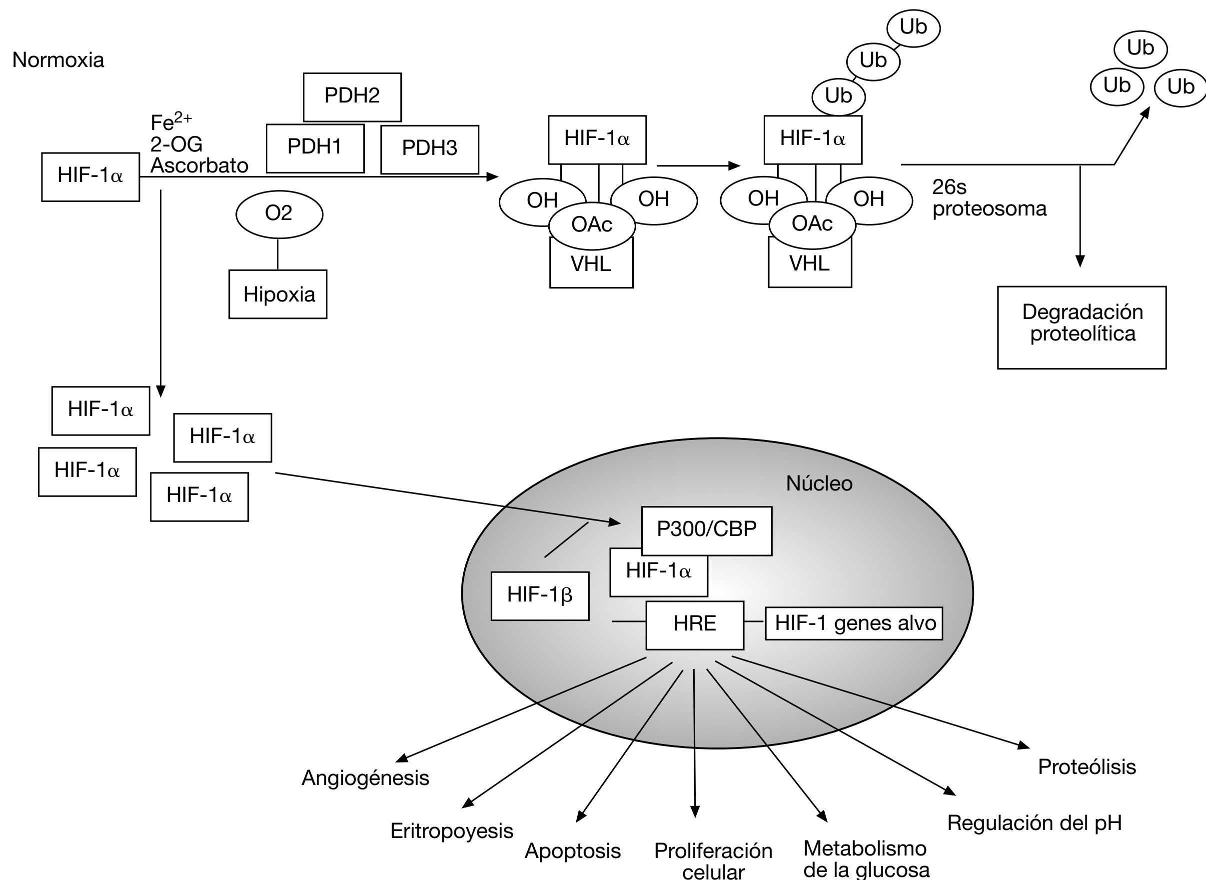

Figure 5 – HIF-1α regulation.

cells must maintain a balance between the intracellular and the extracellular pH; this is achieved thanks to several transporters. Carbonic anhydrase IX (CA9) plays a fundamental role in this balance; several studies have shown a correlation between hypoxia, angiogenesis, HIF-1α, and CA9[89].

Therefore, HIF levels are adapted for cells to maintain a high rate of proliferation; on the other hand, the increased cell proliferation may induce an increased expression of HIF[28]. In conditions of hypoxia, where the action of growth factors leads to an increased cell proliferation and thus to an increased oxygen requirement, HIF-1α is more expressed and activated, inducing the expression of genes that codify the pro-angiogenic molecules that permit metabolic adaptation to hypoxia; this is the most powerful activator of genes that codify glycolytic enzymes and pro-angiogenic growth factors[28,90-93], since tumors cannot thrive without angiogenesis that allows the diffusion of oxygen, glucose, and other nutrients[77,78].

Angiogenesis is the development of new blood vessels from the preexisting vessel network, and plays a preponderant role in various pathophysiologic mechanisms, both benign (cicatrization, wounds, ischemia, diabetic retinopathy) and malignant (tumor growth and metastasis); VEGF plays a fundamental role in angiogenesis, and is regulated by HIF[94-96].

Currently, there is evidence that tumor blood vessels are disorganized and lack an adequate structure for circulation, which often leads to collapse. Since tumor development requires oxygen, nutrients, and an adequate metabolic function, it is necessary to promote angiogenesis factors in order to inhibit the apoptosis of tumor cells triggered by hypoxia. Therefore, angiogenesis as a response to tumor hypoxia is mediated by HIF-1α[55].

HIF-1α has been considered a key factor in the regulation of VEGF and its receptor (VEGRF), as well as of other angiogenic factors. Several immunohistochemical studies conducted on various tumor models[71] show that the expression of HIF-1α is associated with an increase in VEGF and of vascularization and metastasis, which imply a worse prognosis[72,76]. There seems to be a direct relationship between angiogenesis and metastasis in several kinds of tumors, such as melanoma, glyoma, lung, breast, ovary, bladder, and prostate cancers[97,98]. It has been proven that HIF-1α target proteins are implicated in the proliferation, survival, adhesion, and mobility of cancer cells.

On the other hand, an increased expression of HIF-1α, in combination with inactivated mutations in suppressor genes such as VHL, p53, PTEN or the amplification of the oncogenes Akt, RAS, ERK1/2, has often been observed in cancer patients; these abnormalities are associated with tumor growth, invasion, and metastasis.

Zhong et al[99] have demonstrated an increased expression of HIF-1α in approximately 53% of tumors, including cancer

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of colon, stomach, pancreas, lung, ovary, prostate, kidney, melanoma, and glyoblastoma. The increased expression of HIF-1α is associated with a shorter survival in breast and uterine cancer, and with poor response to treatment in nasopharyngeal cancer, highlighting the role of tumor hypoxia in prognosis[72,100-104] (Table 2).

In prostate cancer, it is expressed in the initial stages of carcinogenesis, and this expression is associated with diagnostic and prognostic indicators of early relapse and metastasis; HIF-1 may be a potential poor-prognosis biomarker. Its importance in tumor progression becomes a potential target in chemoprevention strategies and in the ability to inhibit angiogenesis[60]. Experimental studies with mice prostate cancer cells show that an overexpression of HIF-1α is associated with more growth and metastatic potential[108]. Similarly, a greater expression of HIF-1α has been found in human prostate tumors[48,99]. The *VEGF* gene, induced mainly by HIF-1α, has been frequently found to be overexpressed in prostate cancer, especially in patients with metastatic or hormone-resistant cancer; this suggests a central action of this molecule in this process[105,106].

The activation of oncogenes and growth factors can induce the HIF system in non-hypoxidating cells, or amplify the response to hypoxia. In fact, several growth factors and cytokines of the stroma and parenchyma also act as regulators and are capable of inducing the expression of HIF-1α, its binding and transactivation capacity, such as the epidermal growth factor (EGF)[46], TGFα[92,107], factors IGF-1 and IGF-2[109], and interleukin 1b[110]. Additionally, recent studies show that HIF may play an important role in resistance to treatment[111-113].

The HIF system acts as the main regulator of the response to hypoxia, triggering the cascade of mechanisms that permit the tumor to adapt to a hostile environment, and emerges as an important transcription factor in the biology of cancer.

Conclusion

The activation of HIF is regulated by several mechanisms that arise from the stabilization of the HIF-1α subunits, which involves multiple signals and pathways.

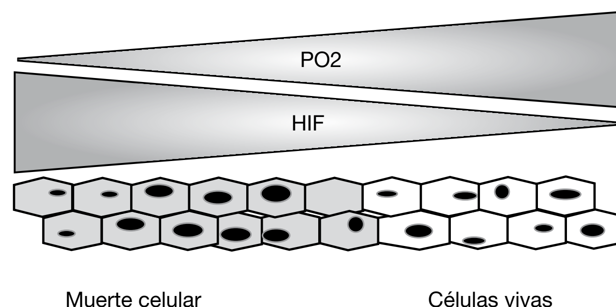

Figure 6 - Role of HIF in cell survival and death. Adapted from Acker and Plate[93].

Hypoxia, some tumor suppressor genes, growth factors, and cytokines increase the stability and/or transactivation of HIF1A, which results in an increased production of HIF-1α and consequently, tumor angiogenesis, metabolic adaptation to hypoxia, and a prolonged cell survival, due to the action on several target genes. HIF-1α is crucial for the initiation of angiogenesis, tumor growth, progression, and metastasis.

Thus, it seems critical to develop techniques to block or inhibit angiogenesis and the HIF1α factor to reduce the chances of it becoming a more aggressive cancer. This would reduce cancer morbidity and mortality.

HIF-1α may be an early marker for carcinogenesis, valuable for predicting tumor progression and prognosis.

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Table 2 – Tumors that show overexpression of HIF assessed with immunohistochemistry

References	99	83-85	97, 98	72, 100-104	48, 60, 105-108
Colon	X				
Stomach	X				
Pancreas	X				
Lung	X		X		
Ovary	X		X		
Uterus		X		X	
Prostate	X	X	X		X
Kidney	X				
Glioma	X		X		
Breast		X	X	X	
Head and neck		X			
Melanoma	X		X		

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Hypoxia and Prostate Cancer Aggressiveness: A Tale With Many Endings

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Abstract

Angiogenesis, increased glycolysis, and cellular adaptation to hypoxic microenvironment are characteristic of solid tumors, including prostate cancer. These representative features are the cornerstone of cancer biology, which are well correlated with invasion, metastasis, and lethality, as well as likely with the success of prostate cancer treatment (eg, tumor hypoxia has been associated with resistance to chemotherapy and radiotherapy). It is well established that prostate cancer cells also metabolically depend on enhanced glucose transport and glycolysis for expansion, whereas growth is contingent with neovascularization to permit diffusion of oxygen and glucose. While hypoxia inducible factor 1 alpha (HIF-1α) remains the central player, the succeeding activated molecules and pathways track distinct branches, all positively correlated with the degree of intratumoral hypoxia. Among these, the vascular endothelial growth factor axis as well as the lysyl oxidase and carbonic anhydrase IX activities are notable in prostate cancer and merit further study. Here, we demonstrate their linkage with HIF-1α as a tentative explanatory mechanism of prostate cancer aggressiveness. Hypoxia drives a tale where HIF-1α-dependent effects lead to many influences in distinct key cancer biology features, rendering targeted therapies toward targets at the endings less efficient. The most appropriate approach will be to inhibit the upstream common driver (HIF-1α) activity. Additional translational and clinical research initiatives in prostate cancer are required to prove its usefulness.

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Introduction

Neoangiogenesis is a characteristic of progressing solid tumors. During tumor growth, malignant cells become progressively distant from the vasculature, oxygen supply, and nutrients, urging tumor cells to signal to the microenvironment the requirement to form new blood vessels. Tumors have been reported to possess extensive regions of hypoxia relative to the corresponding normal tissue.[1,2] At least partially, this is due to the rapid proliferation of tumor mass that distances cells from the oxygen carrying vasculature, but is also the consequence of distorted and irregular characteristics of newly formed vessels, ultimately leading to inefficient oxygen transport. It is well established that solid tumors, like prostate cancer, exist under fluctuating oxygen tensions and are exposed to both acute and chronic hypoxia.[3-5]

The hypoxic tumor microenvironment correlates with increased tumor invasiveness, metastasis, and resistance to radiotherapy and chemotherapy.[2,6-8] Hypoxia has a detrimental effect on the efficacy of treatment and consequently in the clinical outcomes of patients with prostate cancer, being an independent poor prognostic indicator for patients with prostate and other cancers.[2,3]

Over 1% of the genome is transcriptionally responsive to hypoxia, although this varies according to cell type.[9] A large number of endogenous markers of hypoxia which are up-regulated under hypoxic conditions include the vascular endothelial growth factor A (VEGF-A), prolyl hydroxylase 2 (PHD2), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), carbonic anhydrase IX (CAIX), lysyl oxidase (LOX), hypoxia inducible factor 1α (HIF-1α), hypoxia inducible factor 2α (HIF-2α),

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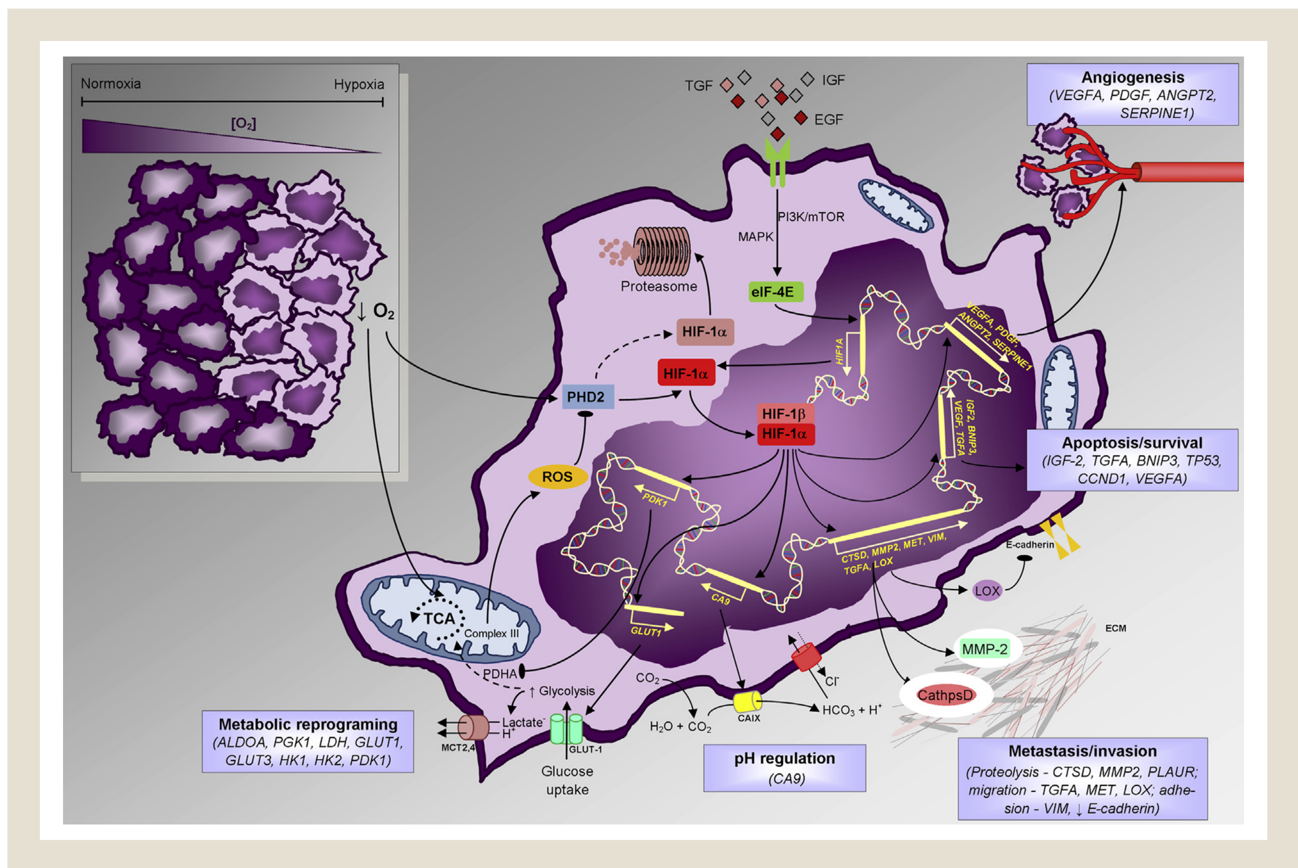
glucose transporter 1 (GLUT-1), erythropoietin (EPO), E-cadherin, and angiopoietin 2 (Ang2), among others (Figure 1).[10-12] Most of these genes have previously been shown to be up-regulated by hypoxia in in vitro and in vivo tumor models, resulting in a more aggressive, treatment-resistant phenotype.[13-16] Nonetheless, of all these hypoxia biomarkers, none could adequately predict tumor hypoxia,[1] even though a biomarker that could reliably and easily identify a man's prostate cancer oxygen status would be useful for personalized medicine. Current knowledge suggests that rather than considering individual genes, a panel of genes may provide a more accurate reflection of tumor hypoxia.[17,18] Moreover, tumor hypoxia is cyclical, with a mix of acute and chronic hypoxia in a constantly changing environment due to a changeable microvascular supply.[1]

A recent large study identified HIF-1α and VEGF as intrinsic markers of tumor hypoxia and angiogenesis, which were associated with risk of biochemical failure in patients with localized prostate cancer.[19] In another study, it was shown that LOX and GLUT-1 were significantly overexpressed in malignant compared to benign prostate tissue and were correlated with Gleason score.[20] LOX and GLUT-1 have been previously reported as hypoxia-associated genes, respectively involved in matrix remodeling and glucose transport,[21,22] which are key features of cancer aggressiveness. Hypoxic cancer cells overexpress GLUT-1 to accelerate glucose intake mainly

Figure 1 Hypoxia-Induced HIF-1α-Driven Modulation of Key Genes and Resulting Biological Effect



During tumor growth, the unavoidable low availability of oxygen in some areas triggers oxygen-sensing mechanisms, notably prolyl hydrolases (PHDs), which regulate HIF-1α activity (if down-regulated, or alternatively proteasomal degradation). In addition, mitochondria-mediated use of oxygen produces reactive oxygen species that suppress PHD2 activity, further stabilizing HIF-1α. Alternative hypoxia-independent or -dependent pathways for HIF-1α up-regulation include binding of growth factors (IGF, EGF, TGF) to tyrosine kinase receptors that signal *HIF1A* transcription through MAPK and PI3K/Akt/mTOR pathways (by up-regulating the transcription factor eIF-4E). Stabilized and active HIF-1α protein enters the nucleus and binds to HIF-1β to form a complex that regulates the expression of key genes that code for proteins with relevant functions in prostate cancer development and progression. Regulation of genes encoding proteins responsible for metabolic reprograming (eg, *GLUT1, ALDOA, PGK1, LDH, PDK1, HK1*, and *HK2* that switch tumor cell toward glycogenolysis as the main source of energy); genes responsible for pH regulation (eg, *MCT1, MCT4*, and *CA9* that alkalinize the intracellular environment); genes involved in tumor cell apoptosis and survival (eg, *IGF2, TGFA, BNIP3, CCND1, TP53*, and *VEGFA*, which down-regulate apoptosis while inducing survival); genes accounting for neoangiogenesis (eg, *VEGF, PDGF, ANGPT2*, and *SERPINE1* that up-regulate sprouting of new tumor vessel; and genes coding for modulators of invasion and metastasis (eg, the proteolytic *CTSD, MMP2*, and *PLAUR*, migration inducers *TGFA, MET*, and *LOX*, and adhesion molecules E-cadherin and vimentin).

Abbreviations: *ALDOA* = aldolase A gene; *ANGPT2* = angiopoietin 2 gene; *BNIP3* = bcl2/adenovirus e1b 19 kDa protein-interacting protein 3 gene; *CA9* = carbonic anhydrase 9 gene; CAIX = carbonic anhydrase IX; CathpsD = cathepsin D gene; *CCND1* = cyclin D1 gene; *CTSD* = cathepsin D gene; ECM = extracellular matrix; EGF = epidermal growth factor; eIF-4E = eukaryotic translation initiation factor 4E; *GLUT1* = solute carrier family 2 (facilitated glucose transporter) member 1 or *SLC2A1* gene; HIF-1α = hypoxia-inducible factor 1 alpha; HIF-1β = hypoxia-inducible factor 1 beta; *HK1* = hexokinase 1 gene; *HK2* = hexokinase 2 gene; IGF = insulin growth factor; *IGF2* = insulin growth factor 2 gene; *LDH* = lactate dehydrogenase A gene; LOX = lysyl oxidase; *LOX* = lysyl oxidase gene; MAPK = mitogen activated protein kinase pathway; *MCT1* = solute carrier family 16 (monocarboxylic acid transporter) member 1 or SLC16A1 gene; *MCT4* = solute carrier family 16 (monocarboxylic acid transporter) member 1 or SLC16A3 gene; *MET* = met protooncogene gene; *MMP2* = matrix metalloproteinase 2 gene; O_2 = molecular oxygen; *PDGF* = platelet-derived growth factor gene; PDHA = pyruvate dehydrogenase A; *PDK1* = pyruvate dehydrogenase kinase isoenzyme 1 gene; *PGK1* = phosphoglycerate kinase 1 gene; PHD2 = prolyl hydrolase 2; PI3K/mTOR = phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway; *PLAUR* = plasminogen activator receptor urokinase-type gene; ROS = reactive oxygen species; *SERPINE1* = serpin peptidase inhibitor member 1 or plasminogen activator inhibitor type 1 gene; TCA = tricarboxylic acid cycle; TGF = transforming growth factor; *TGFA* = transforming growth factor alpha gene; *TP53* = tumor protein p53 gene; *VEGFA* = vascular endothelial growth factor A gene.

for anaerobic respiration, preventing death due to oxygen deficiency.[21] LOX is an extracellular matrix protein that is consistently overexpressed in hypoxic human tumor cells[9,22] and is also a useful marker of the hypoxia response in vitro.[20,22] Nevertheless, further studies at the protein level are needed to confirm LOX and GLUT-1 as useful hypoxia markers in prostate cancer.

A Common Tumor Hypoxia-Driven Mechanism (Through HIF-1α) With Many Paths and Therapeutic Implications

The hypoxia-inducible factor induces the transcription of numerous genes involved in multiple functions on hypoxia conditions.[5,23,24] HIF-1α is a heterodimeric transcription factor that is the prototypical hypoxia-associated molecule.[25] Is the key of master regulator in the hypoxic response of cells by the activity of PHD (prolyl hydroxylase domain) and orchestrates the hypoxic response (Figure 2). Usually HIF-1α has cytoplasmic localization, but under hypoxic conditions it is detected and localized in the nucleus, where it binds to HIF-1β and induces transcription causing up-regulation of effector genes by binding to the hypoxia response element within their promoter regions (Figure 2).[26,27] Under hypoxic conditions, HIF-1α induces expression of pro-angiogenic factors and endothelial cell mitogens, eg, vascular endothelial growth factor A (VEGF-A), thus inducing proliferation, sprouting and tube formation of endothelial cells and sustained angiogenesis.[28] Unlike HIF-1α, HIF-2α protein is expressed only in some cell types, can escape degradation, and is transcriptionally active at near-normoxic conditions.[29,30] Still, HIF-2α contributes as HIF-1α to the development of tumor aggressiveness.[30,31] In the prostate, focal HIF-2α expression has been detected in benign neuroendocrine-like and malignant cells,[32] being more pronounced in larger prostate tumors.[33] Thus, the role of HIF-2α in hypoxia-associated tumors, particularly prostate cancer, warrants further investigation.

HIF-1α protein has been shown to be increased in prostate cancer tissue sections compared to benign prostatic hypertrophy (BPH) and to be associated with higher risk for biochemical failure.[19,26] One study reported a trend for higher HIF-1α mRNA expression in prostate cancer versus BPH samples.[20] However, this finding agrees with previous studies showing that HIF-1α is decisively regulated at the posttranslational level.[33,34] Additionally, a direct link between androgen receptors and pro-angiogenic factors may exist, as HIF-1α expression is increased with androgens[33] and decreased in prostatectomy specimen treated with preoperative androgen deprivation therapy.[19,35]

Neovascularization is essential for physiologic processes, including in the cancer pathophysiology. In fact, it is well established that tumor growth is associated with increased vascularity.[14,36,37] Mounting evidence from in vitro and in vivo models indicates vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis through an effect in endothelial cell growth and proliferation.[37] VEGF binds 2 highly related receptor tyrosine kinases, VEGFR-1 and VEGFR-2. VEGFR-1 expression is up-regulated by hypoxia via an HIF-1α dependent mechanism, thereby favoring the activation of VEGF/VEGFR-1 and -2 signaling pathways due to increased availability of both ligand and receptors.[38]

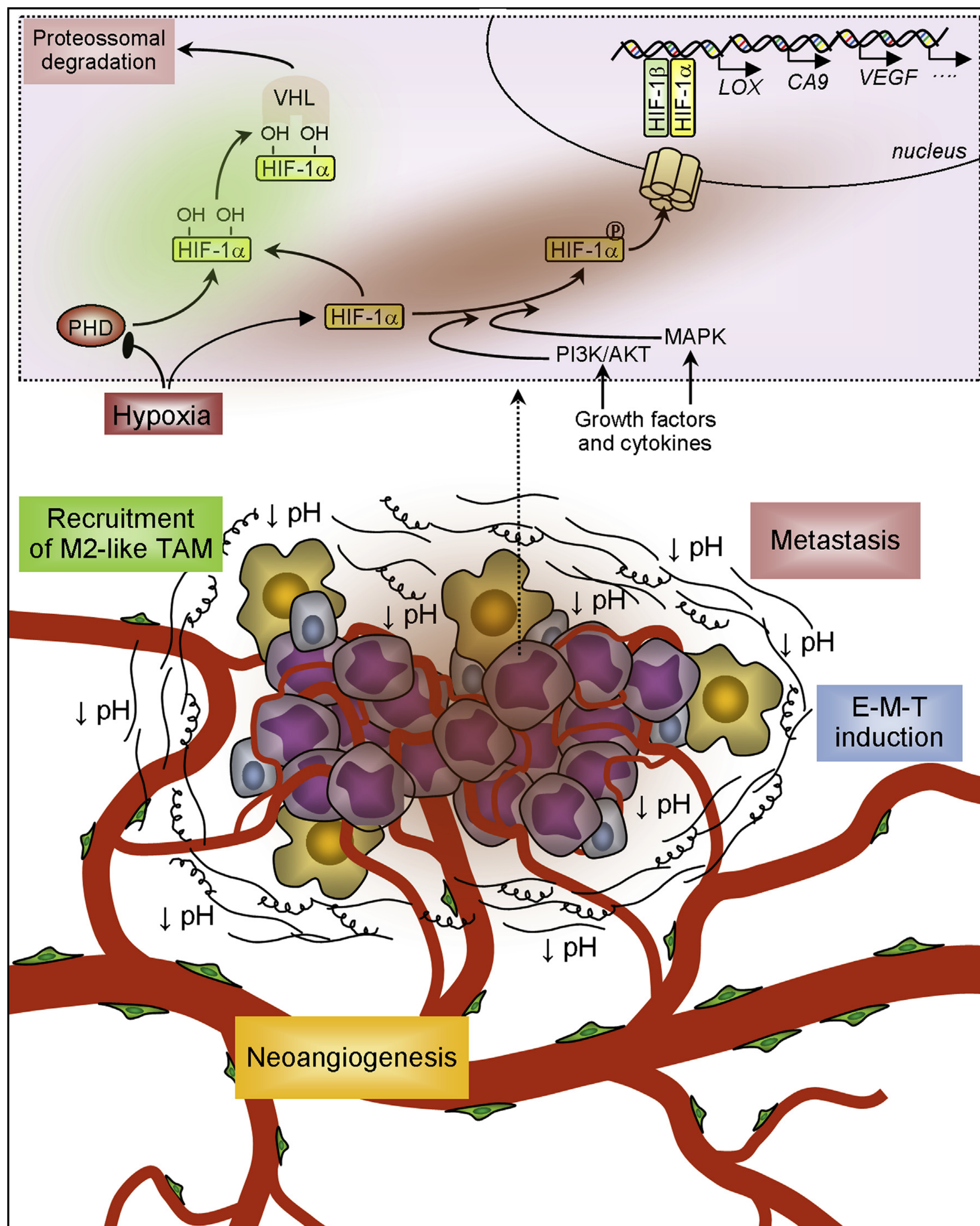
It is known that oxygen tension plays a key role in regulating the expression of VEGF,[39] whereas VEGF inhibition suppresses pathologic angiogenesis in a wide variety of preclinical models. More specifically, hypoxia may trigger vascular endothelial growth factor (VEGF) expression via the transcription complex of hypoxia-inducible factor HIF-1α (Figure 1). Hypoxia and the consequential angiogenesis may play a major role in prostate cancer progression,[40] as VEGF and HIF-1α is increased in prostate cancer compared to BPH.[26,41]

Tumor cells usually have a high rate of glucose uptake accompanied by elevated glucose consumption through the preferential activation of the glycolytic pathway.[42] Several genes involved in glucose uptake and glycolysis (eg, *GLUT1* and most genes coding for enzymes in the glycolytic pathway) have been shown to be targets of HIF-1α.[43] Additionally, HIF-1α activation inhibits mitochondrial metabolism by promoting the expression of pyruvate dehydrogenase kinase 1 to inhibit pyruvate dehydrogenase activity,[44] thereby diverting pyruvate to lactate. Noteworthy, despite the decreased flux of glucose-derived pyruvate into the mitochondria, in place of oxidative metabolism, cancers rely on reductive reactions from glutamine carbon.[45] Enhanced lactate production and the production of CO_2 induced by anaerobic conditions contributes to the major acid load in tumor environment. The production of CO_2 induced by anaerobic conditions further contributes to the major acid load in the tumor environment. One of the striking features of cancer cells is their ability to acidify their environment, and the orientation of CAIX suggests that it may serve as one of the mechanisms by which cancer cells regulate extracellular pH and induce cytoplasmic alkalization, playing a role in the adaptation of tumors to hypoxic conditions by regulating the pH of the intracellular and extracellular compartment (Figure 1).[46,47]

The membrane-bound enzyme CAIX catalyzes the reversible conversion of CO_2 to carbonic acid and contributes to the modulation of pH in tumor cells.[48] The CAIX is HIF dependent and has been shown to be up-regulated in multiple human cancers.[48] A correlation between hypoxia, angiogenesis, HIF-1α, and CAIX in tumors and metastasis has been reported,[49] although the involvement of cancer-associated antigen in prostate tumor progression and metastasis through the modulation of pH remains elusive.

Despite being normally expressed in normal tissues, CAIX becomes highly expressed when tumor cell hypoxia occurs in malignancies.[50] CAIX is up-regulated by hypoxia,[51] and its gene is a target of HIF-1α (Figure 1).[52] Interestingly, the degree of CAIX expression was found to be a prognostic factor of poor survival in many cancer types.[53-58] Prostate cancer cell lines can express CAIX during severe hypoxic,[59] which is a good marker of hypoxia particularly for androgen-independent cell lines, with reliable increases in *CA9* mRNA expression after hypoxia exposure.[20] Even though initial findings showed an absence of CAIX expression in primary prostate cancers,[59,60] others have observed moderate expression in both BPH and malignant prostatic tissue.[20] Thus, the clinical usefulness of CAIX as a diagnostic tool with implications for therapy and patient outcome remains to be elucidated.

Figure 2 Integration of Hypoxia With HIF-1α-Associated Mechanisms in Prostate Cancer, Specifically Downstream-Activated LOX, VEGF, and CAIX Pathways, and Emergence of Metastatic Traits



The hypoxic environment at the growing prostate tumor primary site conducts HIF-1α toward phosphorylation and translocation to the nucleus instead of the usual proteosomal degradation in normoxia. Here, both the stimulus to increase HIF-1α availability and the suppression of PHD activity concur to hamper HIF-1α degradation. Within the nucleus of the malignant cell, this transcription factor initiates the expression of genes (eg, *VEGF, LOX, CA9*) notable for their role in driving prostate cancer progression and metastasis. Taken together, these molecules are responsible for modulating the tumor microenvironment through recruitment of TAMs, promoting angiogenesis (neoangiogenesis with loss of pericytes, contributing to tortuous and permeable vessels), inducing E-M-T and metastasis, thus promoting prostate cancer aggressiveness.

Abbreviations: CA9 = carbonic anhydrase IX; E-M-T = epithelial-to-mesenchymal transition; HIF-1α = hypoxia inducible factor subunit 1 alpha; HIF-1β = hypoxia inducible factor subunit 1 beta; LOX = lysyl oxidase; MAPK = mitogen activated protein kinase; PHD = prolyl hydrolases; PI3K = phosphoinositol-3-kinase; TAM = tumor-associated macrophages; VEGF = vascular endothelial growth factor; VHL = von Hippel-Lindau.

The clinical and pathologic heterogeneity found in cancers highly depends on reciprocal interactions between malignant cells and their dynamic microenvironment.[61] The cross-talk between cells and with extracellular matrix (ECM) in tumor microenvironment seems to be critical in many aspects of cancer development, including maintenance of cancer cell dormancy, cancer progression and metastasis, and drug resistance.[61] The ECM of solid tumors is composed of a complex meshwork of fibrillar collagens, glycoproteins, and proteoglycans,[62,63] which affect metastasis, proliferation, angiogenesis, adhesion, migration, invasion, and drug delivery.[64,65]

Hypoxia is an important microenvironment factor in the development of cancer, and while HIF-1α has been shown to be the key regulator of the cellular response to hypoxia,[61,66] the relationship between tumor hypoxia and components of ECM is far less known. The role of ECM components and remodeling in cancer has only been a focus of research during the last years. Recent findings suggest that hypoxia mediates collagen 1 fiber remodeling in the ECM of tumors, which may impact delivery of macromolecular agents and the dissemination of cells.[67] Collagen type I is the major structural ECM component in prostate tumors,[64,68,69] with cancer cell invasion occurring radially along its fibers.[67] Moreover, cells of myofibroblast phenotype in the reactive stroma of Gleason 3–scored prostate cancers exhibited elevated collagen type 1 synthesis, which was first observed in activated periacinar fibroblasts adjacent to prostatic intraepithelial neoplasia.[64]

In a previously described hypoxia gene signature,[70] LOX was shown to be directly regulated by HIF-1α and essential for hypoxia-induced metastasis in several cancer models.[66,71] In agreement with this finding, hypoxia-induced cancer cell invasion was severely impaired through inhibition of LOX expression.[72,73] Cancer cell proliferation was stimulated by LOX in a HIF-1α-dependent manner both in vitro and in vivo.[73] Thus, the regulatory circuit between LOX and HIF-1α act in synergy to foster tumor formation in the adaptation of tumor cells to hypoxia (Figure 1).

The LOX family of oxidases oxidizes lysine residues in collagens and elastin, resulting in the covalent cross-linking and stabilization of these ECM structural components, thus providing collagen and elastic fibers with most of their tensile strength and structural integrity.[74] The accurately regulated expression and activity of the LOX family of oxidases are a prerequisite for them to exert critical functions in connective tissue homeostasis. LOX mRNA level is highly up-regulated under hypoxic conditions mediated by HIF-1α at the transcriptional level.[63] In addition to the well-documented roles in connective tissue homeostasis, the LOX family of oxidases participates in other critical biological functions, including cell migration, cell polarity, epithelial-to-mesenchymal transition (EMT), and angiogenesis.[75-79]

LOX is synthesized as a pro-enzyme (Pro-LOX) from stromal cells, from normal epithelial cells, or from tumor cells under hypoxic conditions, and is secreted where it undergoes extracellular proteolytic processing by pro-collagen C-proteinases to a functional enzyme and a pro-peptide (LOX-PP).[80,81] Levels of Pro-LOX production in prostate cancer epithelium are decreased as a function of prostate cancer progression.[82] A recent study proposed that Pro-LOX, but not LOX-PP, is a tumor suppressor.[83] Further studies showed that LOX-PP is an active inhibitor of prostate cancer and other tumor cells growth and of RAS-dependent signaling.[73,84,85]

Although LOX was initially implicated as a tumor suppressor, now it is accepted as a poor prognosis marker, particularly in promoting metastasis in breast, lung, prostatic, head and neck, and bronchogenic carcinomas.[23,66,71,82,86,87] Cancer invasion is facilitated by stromal collagen reorganization, and this behavior is significantly increased in collagen-dense tissues (Figure 1).[88] Many ECM modifying enzymes, including matrix metalloproteinases and LOX family oxidases, are aberrantly expressed during malignant transformation, progression, and metastasis of cancers.[66]

Lysyl oxidase-like 2 (LOXL2), a LOX oxidase family member, accumulates in the endothelial ECM and regulates sprouting angiogenesis through assembling type IV collagen in the endothelial basement membrane.[89] Therefore, oxidases of the LOX family play roles in cancer progression and metastasis, promoting not only cancer cell migration and invasion but also angiogenesis in concert with pro-angiogenic factors under hypoxia. Furthermore, inhibition of LOXL2 resulted in a marked reduction in activated fibroblasts and endothelial cells, as well as decreased production of growth factors and cytokines.[90] In agreement, a recent report in advanced renal cell carcinoma patients receiving therapy with angiogenesis inhibitors (pazopanib and sunitinib) disclosed an association of a *LOXL2* intronic single nucleotide polymorphism (rs4872122) with overall survival, suggesting its potential role as a predictive biomarker for antiangiogenic drugs and as a therapeutic target in cancer.[91]

LOX is a potent chemokine inducing directional migration of varied cell types; when it is present, it strongly induces directional migration of cells,[66] and it regulates cell polarity and the EMT process (Figure 1).[66,73] Hypoxia represses E-cadherin expression and promotes EMT.[77,79] HIF-1α enhanced EMT in vitro and induced epithelial cell migration through up-regulation of LOX.[77-79,92] The up-regulated expression of LOX and LOXL2 under hypoxia is required and sufficient for hypoxic repression of E-cadherin, possibly through stabilization of the SNAIL transcription factor.[77,78] Further studies are warranted to investigate the contribution of individual LOX family members to the induction of EMT in the context of dynamic microenvironment during cancer cell invasion and metastasis.

Conclusion

Hypoxia is usually found in large solid tumors and is a known inducer of metastasis, being strongly correlated to poorer outcomes. In prostate cancer, as in angiogenesis-dependent tumors, there is now evidence of intratumoral hypoxia's profound effect in cancer progression through HIF-mediated regulation of molecules that mediate functional interactions with key aspects of angiogenesis and metastasis. So far, the combination of insightful studies on cancer hypoxia suggests the existence of a regulatory circuit between molecules or pathways (such as VEGF, LOX, or CAIX) downstream of HIF-1α, which synergistically modulate tumor microenvironment and promote prostate cancer aggressiveness.

The orchestrator role attributed to HIF as a master regulator of the transcription of genes encoding factors involved in these

processes provides the rationale for including HIF inhibitors in prostate cancer therapy regimens, particularly in patients with localized or locally advanced disease, with elevated expression of hypoxia-driven molecules in primary tumors. Additional studies are needed to clarify the cross-talk between cellular players in the hypoxic prostate tumor microenvironment, whereas insight from further translational and clinical data connecting prostate tumor hypoxia with metastasis and mortality will definitively contribute toward novel, personalized therapies.

Clinical Practice Points

- Hypoxia is an important factor in the development of cancer, and HIF1alpha has been shown to be a central molecule in the progression of prostate cancer. However, the relation between HIF1alpha and other factors such as CAIX, LOX and VEGF in prostate cancer is yet to be investigated. It is understood that there is some relation, but no interrelation, between the factors in the context of prostate cancer.
- In this review, we demonstrate the straight relation between these factors, and we believe that these factors are the same processes determinated by hypoxia.
- Further studies on the relation between HIF1alpha, CAIX, LOX, and VEGFR2 and the different mechanisms and proteins that determinate the progression of prostate cancer are required in an effort to find biomarkers.

Disclosure

The authors have stated that they have no conflicts of interest.

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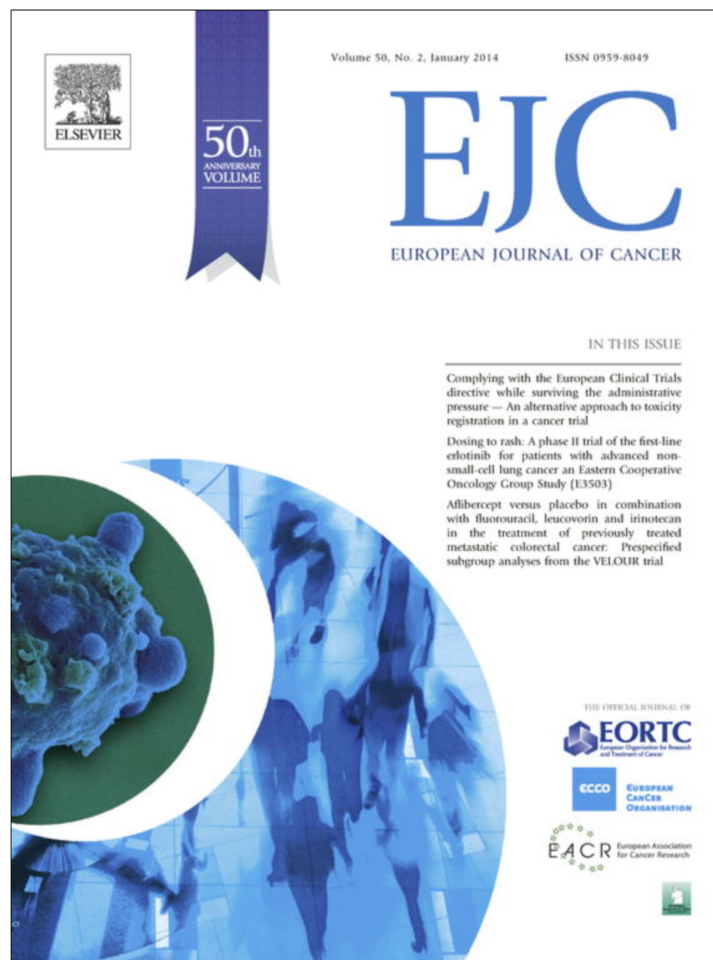

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The *HIF1A* functional genetic polymorphism at locus +1772 associates with progression to metastatic prostate cancer and refractoriness to hormonal castration

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Abstract The hypoxia inducible factor 1 alpha (HIF1a) is a key regulator of tumour cell response to hypoxia, orchestrating mechanisms known to be involved in cancer aggressiveness and metastatic behaviour. In this study we sought to evaluate the association of a functional genetic polymorphism in *HIF1A* with overall and metastatic prostate cancer (PCa) risk and with response to androgen deprivation therapy (ADT).

The *HIF1A* +1772 C>T (rs11549465) polymorphism was genotyped, using DNA isolated from peripheral blood, in 1490 male subjects (754 with prostate cancer and 736 controls cancer-free) through Real-Time PCR. A nested group of cancer patients who were eligible for androgen deprivation therapy was followed up. Univariate and multivariate models were used to analyse the response to hormonal treatment and the risk for developing distant metastasis. Age-adjusted odds ratios were calculated to evaluate prostate cancer risk.

Our results showed that patients under ADT carrying the *HIF1A* +1772 T-allele have increased risk for developing distant metastasis (OR, 2.0; 95%CI, 1.1–3.9) and an independent 6-fold increased risk for resistance to ADT after multivariate analysis (OR, 6.0; 95%CI, 2.2–16.8). This polymorphism was not associated with increased risk for being diagnosed with prostate cancer (OR, 0.9; 95%CI, 0.7–1.2).

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The *HIF1A* +1772 genetic polymorphism predicts a more aggressive prostate cancer behaviour, supporting the involvement of HIF1a in prostate cancer biological progression and ADT resistance. Molecular profiles using hypoxia markers may help predict clinically relevant prostate cancer and response to ADT.

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1. Introduction

Prostate cancer (PCa) remains a major public health concern because it is the most common malignant neoplasia and the second leading cause of cancer death in men [1].

Clinically, it is a heterogeneous disease, with aggressiveness risk differing greatly among individuals despite similar clinical and pathological characteristics. Currently, only incipient but scarce markers help to predict whether PCa will be an aggressive, fast growing disease or an indolent slow growing type of cancer [2]. Therefore, new strategies to help clinicians distinguish between lethal and indolent prostate cancer are needed. Recent findings indicate that genetic variants may predispose to more aggressive prostate cancer [3–5], which is supported by epidemiological studies that propose genetic background influences cancer prognosis [6–8]. Recent genome-wide association studies (GWAS) revealed numerous genetic variants associated with prostate cancer risk, although only little discriminatory ability was shown for fatal forms of the disease [9].

Intratumoural hypoxia is a hallmark of solid neoplasias. It is well established that hypoxic tumoural microenvironment initiates multiple cellular responses, ultimately resulting in cancer progression [10,11]. The hypoxia inducible factor 1 alpha (HIF1a) is a transcription factor coded by the *HIF1A* gene that regulates cellular response to hypoxia [12,13], inducing cancer progression through activation of many genes involved in regulatory cancer biology (angiogenesis, cell metabolism, cell survival, and epithelial-to-mesenchymal transition) [14]. The *HIF1A* gene harbours several SNPs, including a C-to-T substitution at locus +1772 that result in aminoacid modification (proline by serine). Previous *in vitro* studies showed higher transcriptional activity of the variant allele under both normoxic and hypoxic conditions [12,14], whereas additional research associated this SNP with increased tumour microvessel density [12,14,15].

Recent studies yielded conflicting results regarding the involvement of *HIF1A* +1772 C>T genetic polymorphism in cancer, albeit a significant positive association remained after meta-analysis in Caucasian women specific cancers [16,17]. In prostate cancer, the few studies were conducted in distinct ethnic populations and clinicopathological characteristics leading to conflicting results [16,18,19]. Furthermore, the association of *HIF1A* +1772 C>T SNP with prostate cancer progression, metastasis and refractoriness to androgen deprivation therapy (ADT) merits further evaluation in larger series of patients. In the present study we sought to analyse the association of the functional SNP +1772 C>T in *HIF1A* with PCa using prostatic biopsy-proven controls, and to predict the response to treatment in men receiving ADT.

2. Patients and methods

2.1. Patients

Subjects with histological confirmation, whether on biopsy or surgical specimen, of prostate cancer ($n = 754$) or absence of malignancy ($n = 736$) were included in a case-control study. Patients were recruited from five Hospitals in Portugal between 1990 and 2009: Portuguese Institute of Oncology – Porto Centre, S. João Hospital, Porto Military Hospital, Porto Hospital Centre, and Central Lisbon Hospital Centre. The study was approved by hospital's research ethics committees and consent obtained from participants.

The non-PCa control group comprises men referred for prostate biopsy (8–13 cores) on the basis of abnormal digital rectal examination and/or single baseline PSA levels over 2.5 ng/ml, but with normal or benign prostatic histology. Subjects without malignancy at biopsy (BPH or chronic prostatitis) were considered controls since (1) diagnosis was contemporary, (2) were age matched with elderly cancer patients, (3) all were submitted to digital rectal examination, PSA estimate and prostatic biopsy, making remote the possibility of crossover, (4) most men have benign diseases of the prostate by the 7th–8th decades of life, making it normal in men of that age, (5) bias would be expectable if only men without prostatic disease were eligible, because of the much younger range of ages. Patients with high-grade prostatic intraepithelial neoplasia or a biopsy suspicious of cancer were excluded.

A nested sample of subjects from the group of PCa patients (those eligible for androgen deprivation therapy, ADT, ($n = 429$) was followed up for several years. These patients were submitted to orchiectomy or luteinising hormone releasing hormone agonist (LHRHa) (with or without anti-androgen) immediately after diagnosis or after relapsing from surgery/radiotherapy. Resistance to ADT was defined as the time from ADT initiation to two consecutive rises of PSA greater than the PSA nadir or progression of bone lesions [20,21].

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The time intervals between visits to the clinic were those routinely in use and determined by international, namely European, guidelines [20,22]. Information was collected through chart review.

2.2. Genotyping

A venous blood sample (6 ml) was obtained by forearm venipuncture and the white cell fraction used to extract DNA (QIAmp DNA Blood Mini Kit, Qiagen). Blood samples for genetic analysis were collected independent of treatment initiation. The *HIF1A* +1772 C>T (rs11549465) genetic polymorphism was genotyped by Real-Time PCR using a pre-designed validated Taqman assay (Applied Biosystems). Procedures implemented for quality control included double sampling in about 5% of samples and the use of negative controls in every run.

2.3. Statistical analysis

The Kolmogorov–Smirnov test was used to assess departure from normality of continuous variables, while medians and interquartile ranges were used as descriptive statistics. The Mean differences between groups for data not normally distributed was compared by Mann–Whitney or Kruskal–Wallis tests. The departure from Hardy-Weinberg equilibrium for *HIF1A* +1772 C>T polymorphism in the non-prostate cancer group was tested by Pearson's chi-square.

Unconditional logistic regression was used to estimate age-adjusted odds ratios (aORs) and 95% confidence intervals (95%CIs) for the associations between the polymorphism and development of prostate cancer based on additive, recessive and dominant genetic models (additive, CC versus Ct versus tt, and based on the minor allele: dominant, CC versus Ct + tt; recessive, CC + Ct versus tt). We examined the association of *HIF1A* +1772 C>T genetic polymorphism with overall prostate cancer and restricted to high-grade prostate cancer (combined Gleason score $\geqslant 7$) in comparison with controls non-cancers.

Serum PSA at diagnosis was stratified according to a 20 ng/ml cutoff, the combined Gleason score was stratified into two groups (<7 versus $\geqslant 7$), whereas clinical stage was further stratified as localised (T1–T2) or advanced (defined as a tumour invading and extending beyond the prostate capsule and/or extending into adjacent tissue, involving regional lymph nodes and/or distant metastatic sites). The time-to-resistance to ADT was calculated as the interval (in months) since the beginning of ADT until the date of resistance to ADT or last visit.

Empirical analyses were conducted to determine covariates for multivariate models. For time-to-event analyses, age-adjusted Cox regression models were used to assess risk of ADT resistance, whereas age-adjusted logistic regression models were used to evaluate the risk for metastasis. Then, multivariate analysis included relevant clinical variables from empirical evaluation and genetic models. A multivariate Cox proportional hazards model was derived to identify the independent predictive risks for biochemical progression under hormonal castration, while a multivariate logistic regression model was performed to evaluate clinical and genetic predictive factors for prostate cancer metastasis. Statistical analyses were done using STATA version 10.0 (StataCorp, College Station, Texas).

3. Results

One-thousand four hundred ninety individuals were included in this study, 736 cancer-free controls and 754 with a positive biopsy for prostate cancer (median age, 66.8 and 68.0 years old, respectively, $p = 0.001$). Biopsy findings in the control cancer-free group revealed normal histology (10.9%), benign prostatic hyperplasia (33.4%), chronic prostatitis (55.2%) and atrophy (0.5%). As expected, PCa patients presented significantly higher serum PSA levels at diagnosis ($p < 0.0001$).

HIF1A +1772 (rs11549465) genotype distribution by group and risk analysis is shown in Table 1. Both additive and dominant genetic models were not associated with prostate cancer risk or high grade disease. The distribution of *HIF1A* +1772 C>T genotypes among the non-cancer control subjects were in agreement with Wardy–Weinberg equilibrium ($p = 0.988$). Furthermore, we found that this SNP was not associated to earlier onset of disease, using Kaplan–Meier plots and functions (data not shown).

In the group of prostate cancer patients, analyses of the association between *HIF1A* +1772 genetic variants and patient's clinicopathological characteristics showed over-representation of T-allele in the group of patients not treated with definitive therapy ($p = 0.05$) and who developed metastasis at any time during the course of malignant disease (Table 2).

From the group of 754 patients with prostate cancer, 429 were eligible for androgen deprivation therapy, either due to advanced disease at diagnosis or due to disease progression. The clinicopathological characteristics of this nested group are shown in Table 3. From the group of patients on ADT, 194 (45.2%) developed resistance to hormonal therapy. The median (95%CI) follow-up time was 91.8 (79.8–103.7) months.

Univariate age-adjusted empirical time-to-ADT resistance analysis on clinical covariates showed that Gleason grade $\geqslant 7$ (HR, 2.8; 95%CI, 2.0–4.1), advanced clinical stage (HR, 3.7; 95%CI, 2.5–5.3), definitive treatment (HR, 0.6; 95%CI, 0.4–0.8), PSA $\geqslant$ 20 ng/ml (HR, 1.9; 95%CI, 1.5–2.6) and presence of metastasis at ADT initiation (HR, 2.9; 95%CI, 2.1–3.9) were all

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Table 1
HIF1A +1772 genotype distribution and risk for prostate cancer.

	Control	Prostate cancer			
		All		High-grade (Gleason ⩾7)	
HIF1A genotypes	*N*	*N*	aOR (95%CI)	*N*	aOR (95%CI)
Additive model					
CC	566	579	Referent	333	Referent
CT	156	164	1.0 (0.8–1.3)	83	0.9 (0.7–1.2)
TT	14	11	0.9 (0.4–2.1)	7	1.0 (0.4–2.5)
Dominant model					
CC	566	579	Referent	333	Referent
T carriers	170	175	1.0 (0.8–1.3)	90	0.9 (0.7–1.2)

aOR(95%CI), age-adjusted odds ratios and the respective 95% confidence intervals.

Table 2
Genotype distribution in PCa subjects ($n = 754$) according to clinicopathological characteristics.

	HIF1A +1772 C>T genotypes			
	CC ($n = 579$)	CT ($n = 164$)	TT ($n = 11$)	*p*
Definitive therapy				
No	228 (75.0)	69 (22.7)	7 (2.3)	
Yes	281 (78.5)	76 (21.2)	1 (0.3)	0.05*
Clinical stage				
Localised	262 (78.9)	67 (20.2)	3 (0.9)	
Advanced	222 (76.0)	66 (22.6)	4 (1.4)	0.639*
Gleason score				
<7	177 (75.0)	56 (23.7)	3 (1.3)	
⩾7	333 (78.7)	83 (19.6)	7 (1.7)	0.443*
Tumour percent[a]	17.0 (6.0–40.0)	20.0 (5.0–38.5)	65.0 (50.0–80.0)	0.185**

Data are presented as number of cases and respective percentage.
[a] Median (interquartile range).
* Chi-square test.
** Kruskal–Wallis test. Columns do not sum up because of missing data.

significantly associated with resistance to ADT. The associations between *HIF1A* +1772 C>T genotypes and the time-to-event age-adjusted univariate and multivariate analyses are shown in Table 4. Although we have not found association of *HIF1A* +1772 C>T polymorphism with resistance to ADT on univariate analysis, in the recessive model the T homozygous genotype was associated with a 6-fold higher risk for developing resistance to ADT, after adjustment for relevant clinicopathological variables (Gleason grade, clinical stage, PSA ⩾ 20 ng/ml, definitive therapy and existence of metastases at the time of hormonal castration initiation) (Table 4). The risk of developing metastasis at any time during the course of disease in patients under ADT was significantly higher for T-allele carriers, still after adjustment for other clinical covariates (Gleason grade, clinical stage and PSA ⩾ 20 ng/ml) (Table 5).

4. Discussion

Hypoxia is a frequent event during prostate cancer progression, while the hypoxia-responsive gene *HIF1A* codes for a key transcription factor that has been proposed as a modulator of PCa initiation and progression [23–25]. We analysed a functional SNP (+1772 C>T) in the *HIF1A* gene in prostate cancer patients and controls and found lack of association, although a relatively large population with approximately 1500 men was analysed. Concordantly, two large case-control studies from the United States of America and China also observed no risk for having PCa in carriers of this polymorphism [19,26], even though opposite results have been also reported [16,27]. The C-by-T substitution in the +1772 locus at the oxygen-dependent domain of the *HIF1A* gene results in a proline-to-serine substitution and was shown to stabilise *HIF1A* and enhance its activity as a transcription factor in both normoxia and hypoxia [12,28]. In agreement, albeit we hypothesised those carriers of T allele were more susceptible to have cancer, our data, together with other, suggest no influence in earlier stages of prostate cancer development. As PCa natural history usually reveals slow growing indolent tumours, the initial steps of carcinogenesis are not likely to be relevant sources of hypoxia, thereby inducing the

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Table 3
Clinicopathological characteristics features of the group of patients under that received ADT ($n = 429$).

	n (%)
Age at diagnosis, yrs	
Median (IQR)	70.0 (64.9–75.4)
PSA at diagnosis, ng/ml	
Median (IQR)	19.0 (8.9–51.6)
Gleason score	
<7	128 (32.2)
⩾7	269 (67.8)
Clinical stage	
Localised	156 (38.7)
Advanced	247 (61.3)
Metastasis at ADT initiation	
No	286 (75.9)
Yes	91 (24.1)
Definitive therapy	
No	299 (69.7)
RP/RT	130 (30.3)
ADT pharmacological group	
aLHRH alone	91 (21.2)
aLHRH + antiandrogen	338 (78.8)

ADT, androgen deprivation therapy; aLHRH, luteinising hormone releasing hormone agonist; RP/RT, radical prostatectomy/radiotherapy; IQR, interquartile range.

activation of other than the HIF1a pathway. Actually, a previous report found that *HIF1A* +1772 C>T genotypes were not correlated with HIF1a and VEGF expression in localised prostatic tumours [16]. However, HIF1a overexpression has been reported in cancer precursor lesions, high grade prostate intraepithelial neoplasia, and early stage PCa, compared with normal prostate epithelium [24].

Previous studies have shown overexpression of HIF1a in many tumours with advanced grade, implying HIF1a as an independent prognostic factor in cancer [15]. In addition, increasing evidence suggests that genetic markers may be independent predictors of outcome in PCa with various SNPs predicting decreased progression-free and overall survival [3–6]. Data presented here show that the homozygous T genotype T-allele of *HIF1A* +1772 C>T is associated with increased relapsing after ADT, whereas the T allele is prone to higher risk for having distant metastasis, still after adjustment for empirical covariates (adjusted by Gleason grade, clinical stage and PSA ⩾ 20 ng/ml for the risk of metastasis; and by Gleason grade, clinical stage, PSA ⩾ 20 ng/ml, definitive therapy and existence of metastases at the time of hormonal castration initiation for the risk of disease recurrence after ADT). While the recessive model (TT versus CT/CC) was significantly associated with resistance to ADT, the dominant (TT/CT versus CC) and additive models were significant for metastasis development under ADT. A recently published meta-analysis suggests that both the T allele and TT genotype were significantly associated with increased cancer risk [17]. Experimental data also support a functional role for the C-by-T substitution at the allele and homozygous genotype level [12,28,29]. We found that additivity was better fitted for metastasis but not to ADT resistance, even though the low number of patients carrying the TT genotype in metastasis analyses yielded a very wide CI, hence deserving careful interpretation.

Our findings in a large cohort of patients that received ADT, support a role for HIF1a in the pathophysiology of castration resistance and the *HIF1A* +1772 C>T polymorphism as a potential pharmacogenomic predictor of the response to ADT. Concordantly, a recent study demonstrated that HIF1a expression contributed both to metastasis and chemo-resistance of castration resistant prostate cancer [30]. A study comparing *HIF1A* +1772 C>T genotypes between castration-resistant PCa and non-cancer men showed that the T-allele was overrepresented in the cancer group, although it was not associated with survival [18]. Noteworthy, this report presents data from 196 castration-resistant

Table 4
Association of *HIF1A* +1772 C>T polymorphism with resistance to ADT.

		Resistance to ADT			
		Univariate		Multivariate*	
HIF1A +1772 C>T	LR	HR (95%CI)	*p*	HR (95%CI)	*p*
Additive model	2.24				
CC		Referent		Referent	
CT		0.8 (0.6–1.2)	0.288	1.0 (0.7–1.5)	0.918
TT		1.8 (0.7–4.6)	0.183	6.1 (2.2–17.0)	0.001
Dominant model	2.70				
CC		Referent			
T carriers		0.9 (0.6–1.2)	0.460	1.1 (0.8–1.7)	0.536
Recessive model	3.86				
C carriers		Referent		Referent	
TT		1.9 (0.8–4.8)	0.149	6.0 (2.2–16.8)	0.001

LR, likelihood ratio. ADT, androgen deprivation therapy. HR, hazard ratio; 95%CI, 95% confidence interval.
* Cox regression using as covariates: Gleason grade, clinical stage, PSA ⩾ 20 ng/ml, definitive therapy and existence of metastases at the time of hormonal castration initiation.

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Table 5
Risk for metastasis in patients receiving androgen deprivation therapy

	Univariate analysis*			Multivariate analysis**		
HIF1A +1772 C>T	*N*	OR (95%CI)	*p*	*N*	OR (95%CI)	*p*
Additive model	380			323		
CC		Referent			Referent	
CT		1.7 (1.0–2.7)			1.9 (1.0–3.6)	
TT		3.5 (0.6–19.4)	0.055[a]		14.9 (1.0–223.1)	0.031[a]
Dominant model						
CC		Referent			Referent	
T carriers	380	1.7 (1.1–2.8)	0.023	323	2.0 (1.1–3.9)	0.027
Recessive model	380			323		
C carriers		Referent			Referent	
TT		3.1 (0.6–17.1)	0.199		12.9 (0.9–190.1)	0.063

[a] *p* for trend. OR (95%CI), odds ratio with 95% confidence interval.
* Age-adjusted ORs.
** Multivariate logistic regression analysis using Gleason grade, clinical stage and PSA with cut-off 20 ng/ml as covariates.

patients using univariate analysis. Another study observed a somatic rare mutation at the same locus in 1/15 androgen-independent prostate tumours, whereas functional studies demonstrated in androgen-independent prostate cancer cells that the T-allele is associated with increased transcriptional activity and protein expression [28]. Therefore, we hypothesise that carrying the T-allele, which stabilises HIF1a protein and upregulates the *HIF1A1* gene expression, may offer a selective advantage to androgen-independent tumour cells through the upregulation of several genes involved in metastasis, angiogenesis, epithelial-to-mesenchymal transition or in other cancer-associated mechanisms [10,23,31–33]. The SNP in *HIF1A* at locus +1772 represents a germline variant, suggesting a cumulative impact of higher HIF1a expression since birth. However, we hypothesise that *HIF1A*+1772 functional SNP repercussion when combined with hypoxic environmental events or with other genetic risk factors is triggered to higher extent in response to hypoxia-inductive treatments such as ADT. When confirmed in larger and independent samples, additional therapeutic schemes (such as CYP17A1 inhibitors or chemotherapy) could be offered to carriers of the poor responder TT genotype as alternative to ADT. These patients could also be enrolled in clinical trials with drugs that target HIF1a function (e.g. tasquinimod and other agents that target HIF1a or its downstream products) [34–37].

Present findings should be further extended and replicated by future studies focusing on genetic polymorphisms as predictors of treatment response to allow tailored therapy in PCa patients. Using this focused candidate gene approach to evaluate the *HIF1A* +1772 C>T SNP gives us an incomplete analysis of hypoxia mechanism. Other hypoxia-related SNPs were not included in this study. However, our study has several strengths such as the selection of the candidate gene based on biological evidence of functional importance; statistical analyses accounted for relevant clinical and pathological factors. In this study all men (including the controls) 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 or surgical sample, thus making outcome misclassification unlikely.

Our findings suggest that the *HIF1A* +1772 C>T might be a useful marker of aggressive PCa, particularly a predictor of the response to ADT, thus a plausible candidate to include in a panel of risk prediction SNPs in combination with clinical and pathologic features.

5. Conflict of interest statement

None declared.

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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 (Gleason≥7), whereas three variants were associated with high metastatic-risk prostate cancer (PSA≥20 ng/mL and/or Gleason≥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.

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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 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 *SPP1*-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, *SPP1*-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, *SPP1*-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).

The inclusive (age and PSA added to the multi-locus genetic set) linear risk scores computed on the basis of the above logistic regression models were tested as overall risk predictors categorized in tertiles based on the distribution in the non-prostate cancer group. As shown in Table 3, the risk for prostate cancer and high-grade prostate cancer increased according to the tertile of risk score (P_{trend} <0.0001 for both outcome categories). The age-adjusted ORs for unit changes in the inclusive risk score were 2.52 (95%CI: 2.0–3.2) and 2.77 (95%CI: 2.2–3.5) for all prostate cancers and high-grade prostate cancers, respectively. The goodness of fit for the logistic regression models based on the inclusive score were significantly greater than for the models based on the restricted age plus PSA score, for all prostate cancers (P = 0.0002) and high-grade prostate cancers (P = 0.0001), after likelihood ratio test.

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.

Table 1. Age and hormonal variables by disease status.

	Disease Status						
	Non-Prostate cancer			Prostate cancer			
	N[a]	Mean	Median	N[a]	Mean	Median	P[b]
Age, years	553	66.2	66.2	447	68.1	69.0	<0.0001
PSA, ng/mL	540	7.5	5.9	437	26.9	8.2	<0.0001
Free PSA, ng/mL	485	1.6	1.2	373	2.4	1.1	0.373
Free/Total PSA ratio	482	0.22	0.20	372	0.16	0.14	<0.0001
Serum Testosterone, ng/mL	494	478.0	444.5	381	471.5	443.0	0.690

[a]Number of evaluable patients for each variable;
[b]Differences between groups, Mann-Whitney test. PSA, prostate specific antigen.
doi:10.1371/journal.pone.0039236.t001

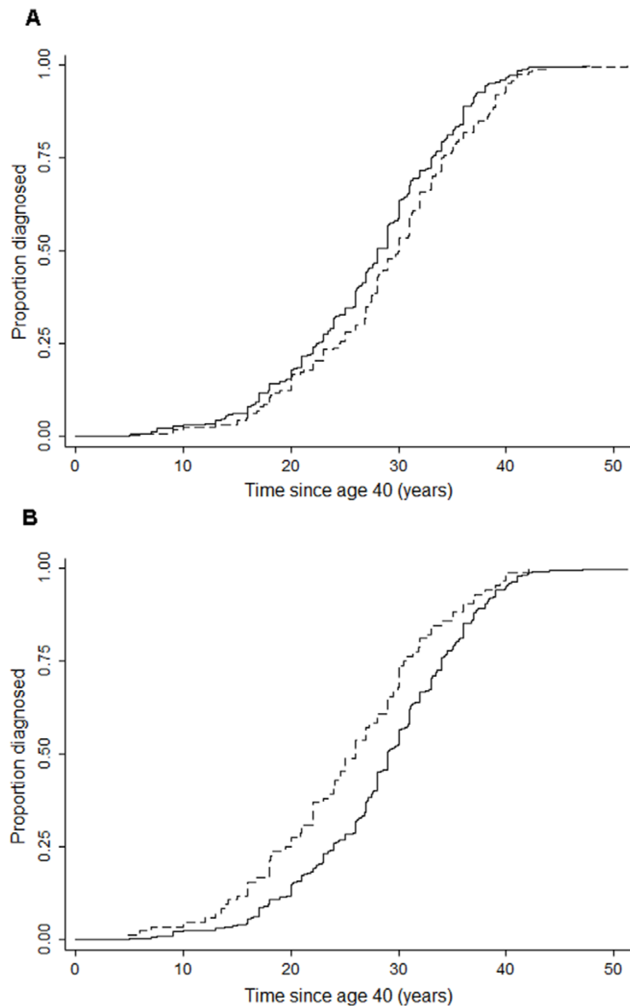

Figure 1. Kaplan Meier analyses plots of significant genetic polymorphisms. (A) *IL6R* D358A A>C and (B) *IGF1R*+3174 G>A. In figure 1A the dashed line corresponds to AA and the dotted line to CC/CA genotype. In figure 1B the dashed line represents AA, whereas the solid corresponds to GG/GA genotype. The Log Rank test was used to compare genotypes in *IL6R* D358A A>C (P = 0.026) and *IGF1R*+3174 G>A (P = 0.002).
doi:10.1371/journal.pone.0039236.g001

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 SNP risk score (excluding the 3 SNPs that were not in equilibrium). Findings showed that the predictive and discriminative ability of the inclusive risk score based on 4 SNPs remained significant (data not shown). 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 *SPP1*-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 *SPP1* 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

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]
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)
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)	1.07 (1.04–1.09)	1.14 (1.09–1.19)
LEPR Gln223Arg	G carriers	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)	1.50 (0.91–2.45)	1.55 (0.93–2.58)
SPP1-66 T>G	T carriers	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)	2.64 (1.16–6.01)	2.52 (1.12–5.64)
IGF1R+3174 G>A	G carriers	Referent	Referent	Referent	Referent		
	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		
	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
	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)	2.20 (1.01–4.78)	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).
doi:10.1371/journal.pone.0039236.t002

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, IL6ST. 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 *ADIPOQ*+276, *VEGF*-460, *VEGF*+405, *VEGF*+936, *PPARG* Pro12Ala and *TNF*-308, are in agreement with other studies [14,17,46,47]. To our knowledge, there have been no prior reports

Table 3. Tertiles of inclusive genetic risk score (GRS) and age-adjusted OR (CI 95%) for prostate cancer.

Inclusive Risk Score	Non-prostate cancer	All prostate cancer		High-grade prostate cancer	
Tertiles	N	N	aOR (95%CI)	N	aOR (95%CI)
T1	185	78	Referent	46	Referent
T2	186	101	1.2 (0.9–1.8)	85	1.7 (1.1–2.6)
T3	186	270	3.2 (2.3–4.6)	243	4.8 (3.2–7.2)

Tertiles for all prostate cancer: T1 (<2.74897), T2 (2.74897–3.15913), T3 (≥3.15913). Tertiles for high-grade prostate cancer: T1 (<2.85839), T2 (2.85839–3.30669), T3 (≥3.30669). The genetic risk scores were computed separately derived for overall and high-grade prostate cancer. aOR, age-adjusted ORs (95%CI).
doi:10.1371/journal.pone.0039236.t003

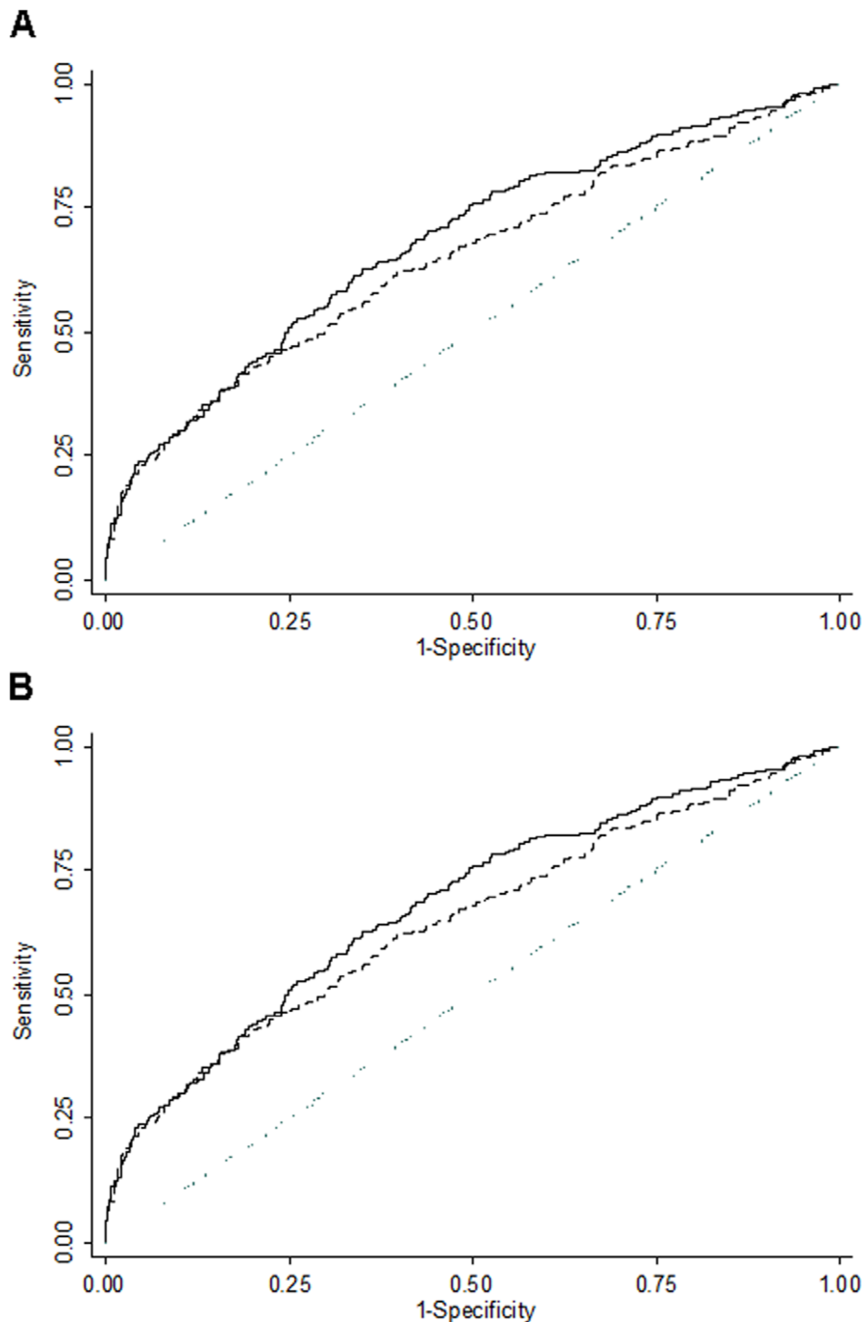

Figure 2. ROC curves and AUC for the inclusive risk score and PSA plus age alone. (A) All prostate cancer and (B) restricted to high-grade prostate cancer. Solid line corresponds to the all inclusive score, whereas dashed line represents the PSA and age risk score. The dotted line indicates the behavior of a hypothetical random score. The Likelihood ratio test was used to estimate the superiority of the inclusive risk score relative to that of the age+PSA score for all prostate cancer (inclusive: AUC = 0.6806, PSA and age: AUC = 0.6476, P = 0.0002) and high-grade prostate cancer (inclusive: AUC = 0.7119, PSA and age: AUC = 0.6808, P = 0.0001). PSA, prostate specific antigen.
doi:10.1371/journal.pone.0039236.g002

of null associations of *KDR*-604, *PPARD*-87, *PPARGC1A* Gly482-Ser, *TNFRSF1A*-329, *ADIPOQ*+45, *ADIPOQ*-11426, *IL6ST* Gly148Arg, *IL6*-6331, and *TNF*-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 ≥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 predictive value of the score for 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 this study population with an exploratory intent, without having the opportunity to validate our findings in a separate sample of patients undergoing prostate cancer screening. Therefore, 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 we did not have data on heredity information in a large set of subjects, only 2.2% were actually younger than 55 years of age, suggesting that hereditary prostate cancers were rare in our sample; 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, thus making outcome misclassification unlikely.

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 strategies 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 consisted of 1099 consecutively-admitted Caucasian men who had histological evaluation and consented for genotyping.

We selected a control group of patients with non-prostate cancer (benign prostate hyperplasia [BPH] or chronic prostatitis) from the prospectively enrolled men undergoing prostate biopsy. Our choice of this control group was based on the following reasons: (i) diagnosis was contemporary with that of cancers; (ii) their advanced age at diagnosis allowed matching with elderly cancer patients; (iii) all patients underwent digital rectal examination, PSA testing and prostate needle biopsy, making the possibility of crossover remote. Most men develop BPH or chronic prostatitis by the 7th–8th decades of life, making it normal in men of that age to carry benign prostatic disease. This permitted our control group subjects to have comparable ages to those of our prostate cancer patients, thus minimizing the likelihood of outcome misclassification. Had we restricted controls to men without prostatic disease there would have been a severe imbalance in age distributions, which would 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*, *PPARGC1A*, *PPARD*, *SPP1*, *IGF1R*, *IGFBP3*, *IRS1*, *FGF2*, *FGFR2*, *TNF*, *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 *TNF*-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

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/BPH 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 ≤0.7 or aOR ≥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 $= \Sigma\ \beta i \times Xi + \gamma \times PSA + \alpha \times Age$; where Xi = SNPs scaled for risk, βi = 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 two 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].

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].

Supporting Information

Table S1 Characteristics of candidate Single Nucleotide Polymorphisms (SNPs) involved in adipokine pathways potentially associated with cancer. HW-E, Hardy-Weinberg Equilibrium; *ADIPOQ*, adiponectin gene; *IL6*, interleukin-6 gene; *IL6R*, interleukin-6 receptor gene; *IL6ST*, 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; *SPP1*, 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; *TNF*, tumoral necrosis factor alpha gene; *TNFRSF1A*, tumoral necrosis factor receptor 1 gene. [a] The percentage of successfully genotyped DNA samples from the 1006 participants.
(DOC)

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

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

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. *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, signle 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%.
(DOC)

Author Contributions

Conceived and designed the experiments: RJTR FMP CMSL RMM ELF. Performed the experiments: RJTR CPDM ASMA VFMC AMF FMP. Analyzed the data: RJTR AVR ELF. Contributed reagents/materials/analysis tools: RJTR CMSL RMM. Wrote the paper: RJTR ELF.

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Functionality of genetic polymorphisms in key hypoxia-regulated downstream molecules and phenotypic correlation in prostate cancer

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ABSTRACT

Purpose: In this study we sought if, in their quest to handle hypoxia, prostate tumors express target hypoxia-associated molecules and their correlation with putative functional genetic polymorphisms.

Methods: Representative areas of prostate carcinoma (n=51) and of nodular prostate hyperplasia (BPH) (n=20) were analysed for HIF-1α, CAIX, LOX and VEGFR2 immunohistochemistry expression using a tissue microarray. DNA was isolated from peripheral blood and used to genotype functional polymorphisms at the corresponding genes (*HIF1A* +1772 C>T, rs11549465; *CA9* +201 A>G; rs2071676; *LOX* +473 G>A, rs1800449; *KDR* – 604 T>C, rs2071559).

Results: Immunohistochemistry disclosed predominance of positive CAIX and VEGFR2 expression in epithelial cells of prostate carcinomas compared to BPH (P=0.043 and P=0.035, respectively). In addition, the VEGFR2 expression score in prostate epithelial cells was higher in organ-confined and extra prostatic carcinoma compare to BPH (P=0.031 and P=0.004, respectively). Notably, for LOX protein the immunoreactivity score was significantly higher in organ-confined carcinomas compare to BPH (P=0.015). The genotype-phenotype analyses showed higher LOX staining intensity for carriers of the homozygous *LOX +473* G-allele (P=0.011), and that *KDR* -604 T-allele carriers were more prone to have higher VEGFR2 expression in prostate epithelial cells (P<0.006).

Conclusions: The expression on prostate epithelial cells of VEGFR2, CAIX and LOX allowed differentiating malignant from benign prostate disease. Two of the genetic polymorphisms (*LOX* +473 G>A and *KDR* – 604 T>C), account for a potential gene-environment effect in the activation of hypoxia-driven pathways in prostate carcinoma. Further research in larger series is warranted to validate present findings.

Keywords: genetic polymorphism; hypoxia; immunohistochemistry; prostate cancer;

INTRODUCTION

Prostate carcinoma is a common and heterogeneous malignant neoplasia, with aggressiveness differing among individuals despite similar clinicopathological characteristics.

During tumor growth, the oxygen supply and nutrients scarcity urges malignant cells to signal to the microenvironment their needs. The hypoxia inducible factor 1 alpha (HIF-1α) is a key factor by which tumors regulate the response to hypoxia, triggering cascades with pro-tumoral effects [1,2]. HIF-1α mechanism implies targeting hypoxia response elements in promoters of downstream target genes, notably vascular endothelial growth factor (*VEGF)*, carbonic anhydrase IX (*CAIX)*, and lysyl oxidase (*LOX)* promoters, resulting in more aggressive, treatment resistant phenotype [1-3]. In prostate carcinoma, a large study has demonstrated the relevance of intrinsic markers of tumor hypoxia for localized disease and outcome of radical treatment [4].

Recent findings indicate that genetic variants may modulate the predisposition for prostate carcinoma and associate with clinical outcome [5,6]. Single nucleotide polymorphisms (SNPs) in genes coding for molecules involved in the response to hypoxia, particularly a functional polymorphism in *HIF1A* gene at locus +1772 C>T [7-13], has been studied in association with prostate carcinoma with controversial results. However, we are not aware of studies implicating SNPs in other genes (e.g. *LOX*, *CA9*, *KDR)* of HIF-1α-mediated hypoxia downstream pathways.

Based on the role of hypoxia-associated molecules in cancer, we hypothesized an association, at the genetic and protein level, between *HIF1A*, *LOX*, *CA9* and *KDR* genetic variants, the protein expression and prostate carcinoma. Hence, if these polymorphisms modulate the protein expression, then the knowledge of the genotype could help to identify patients at higher risk for prostate carcinoma and eventually more aggressive disease.

MATERIAL AND METHODS

Seventy-one patients with prostate pathology (n=51 with carcinoma, and n=20 with nodular hyperplasia, BPH) and elective for prostatic surgery at the Porto Hospital Centre - Sto. António Hospital and Porto Military Hospital were included, after informed consent ans approval by hospitals' ethical committees. Inclusion criteria were 45-75 years of age and for prostate carcinomas absence of

previous treatments. Patient’s clinicopathological data (Table 1) was collected from clinical files and pathological staging determined as organ-confined (T1-T2) (OCPCa) or extra prostatic (T3-T4) (EPCa) disease.

The white cell fraction from peripheral blood was used to extract DNA (QIAmp DNA Blood Mini Kit, Qiagen). Four putative functional SNPs (3 non-synonymous and 1 in the promoter region) in 4 candidate genes involved in key hypoxia pathways were selected (*HIF1A* +1772 C>T, rs11549465; *CA9* +201 A>G, rs2071676; *LOX* +473 G>A, rs1800449; *KDR* -604 T>C, rs2071559). Genotyping was done by Real-Time PCR using Taqman ssays (Applied Biosystems).

Representative areas of carcinoma and of nodular hyperplasia were selected and included into tissue microarray as previously described [14]. Slides were stained with mouse monoclonal antibody to HIF-1α (dilution 1:100, NB100-105, Novus Biologicals), and rabbit polyclonal antibodies to LOX, (dilution 1:100, ab 31238, Abcam), VEGFR2 (dilution 1:200, ab 2349, Abcam) and CAIX, (dilution 1:1000, NB100-417, Novus Biologicals) using the VENTANA BenchMark XT series slide-staining instrument (with the VENTANA ultraView DAB IHC detection kit, VENTANA, Tucson, AZ, United States). Immunohistochemical evaluation was independently reviewed by two pathologists (JRV and AC) to assess VEGFR2 expression in vasculature and prostate epithelial cells, and HIF-1α, LOX and CAIX in prostate epithelial cells (carcinoma and nodular hyperplasia). Discordant cases were discussed in order to attain a final consensus. Staining positivity was sought for VEGFR2 in vessels and epithelial cells, whereas CAIX, HIF-1α and LOX expression was only performed in prostatic epithelial cells (both in carcinoma and nodular hyperplasia). Briefly, scores were calculated as following: VEGFR2 intensity was multiplied by the percentage of tumor cells at that intensity level (VEGFR2 H-score); for LOX the score was calculated by multiplicating the percentage of positive cells with staining intensity (LOX immunoreactivity score, IRS). A representative image of the expression of each aforementioned protein is shown in Figure 1.

Descriptive statistics included means with respective standard errors, whereas departure from normality was assessed with Shapiro-Wilk test. Groups were compared through Kruskal-Wallis and Mann-Whitney test or Student t-test. Pearson chi-square tests were used to compare frequencies among categorical variables. Analyses were performed in SPSS 17.0.

RESULTS

Epithelial cells staining positivity for CAIX and VEGFR was significantly higher in prostate carcinomas compared with BPH (P=0.043 and P=0.035, respectively) (Figure 2). Concurrently, despite non-significant, both HIF-1α and LOX immunoreactivities had a tendency to be elevated in carcinomas (P=0.111 and P=0.266, respectively) (Figure 2). Notably, although not significantly more expressed in prostate carcinomas, the LOX IRS, was significantly more elevated in organ-confined carcinomas than BPH (P=0.015) (Figure 3), and higher in patients with positive HIF-1α expression (P=0.053) (Figure 4). VEGFR2 immunoreactivity was observed in vascular endothelial cells (only in 20% of all samples) and epithelial cells (70% of patients with extra prostatic carcinomas and approximately half of organ-confined carcinomas). Noteworthy, the VEGFR2 H-score in epithelial cells was statistically distinct between BPH and organ-confined or extra prostatic carcinomas (P=0.031 and P=0.004, respectively) (Figure 5).

The genotypic distribution in polymorphisms *HIF1A* +1772 C>T, *LOX* +473 G>A, *CA9* +201 A>G and *KDR* -604 T>C is shown in supplementary table 1. There was no over-represented genotype in disease groups. Regarding genotype-phenotype relation, there was lack of association between *HIF1A* +1772 C>T and *CA9* +201 A>G genotypes with HIF-1α and CAIX protein expression (Table 2). In contrast, LOX expression was significantly more intense in carriers of the *LOX +473* homozygous G allele compared to AA/AG (P=0.011), despite no significance was achieved for IRS (but with similar trend) (Figure 6). Alongside, *KDR* -604 T-allele carriers were more prone to have VEGFR2 expression in prostate epithelial cells but not in vessels (Table 3). The VEGFR2 H-score was significantly higher in T-allele carriers compared to homozygous C (Figure 7).

Only data from prostate carcinomas was used to evaluate if hypoxia proteins associated with Gleason score or PSA>10 ng/mL (Table 4). Statistical trends were observed for higher VEGFR2 H-score expression in more undifferentiated carcinomas (Gleason $\geq$7) (P=0.099) and in patients with prostate specific antigen (PSA) $\geq$ 10 (P=0.085), and for positive CAIX expression in prostate carcinomas from patients with PSA above 10 (P=0.078).

DISCUSSION

The hypoxia-driven HIF-1α upregulation activates downstream pathways involved in metabolism (e.g. CAIX), angiogenesis (e.g. VEGF/VEGFR2 pathway) and extracellular matrix activity (e.g. LOX), which can modulate cancer behavior [15]. Experimental and clinical studies in prostate carcinoma demonstrated that HIF-1α overexpression was associated with malignancy, progression and metastatic potential [16] [4]. Here, we found a non-significant statistical trend for higher HIF-1α protein expression in prostate carcinomas compared to BPH, which may be due to the limited number of samples.

Besides vascular endothelial cells also prostate epithelial cells express VEGFR2, which were shown to signal through the AKT/mTOR/P70S6K pathway [17]. We found that VEGFR2 was expressed in the epithelium and endothelial cells, though more frequently expressed in epithelial tumor cells of organ confined or extra prostatic carcinomas than in BPH. Hence, in the prostate VEGFR2 expression is mainly expressed in malignant epithelium where its ligand VEGF may exert a direct effect in tumor cell growth. Previous immunohistochemistry studies reported VEGFR2 expression in high-grade prostate intra-epithelial neoplasia and carcinomas of the prostate [18], whereas gene expression findings in prostate cancer cell lines evidenced suppressive growth and promotion of apoptosis with *KDR* antisense oligonucleotide [19]. Taken together with present data, these findings indicate that VEGFR2 expression in epithelial prostate carcinoma cells supports a function for VEGF that is not limited to angiogenesis. Thus, abrogation of VEGFR2 signalling in malignant epithelial cells may prove an effective therapeutic modality for the treatment of prostate cancer. At present, two anti-angiogenic drugs are being tested in the phase III setting for men with prostate cancer, carbozantinib (a dual VEGFR2/MET inhibitor) and tasquinimod (down-regulator of HIF-1α), that showed beneficial and encouraging results on phase II trials [20].

Tumor cells have to adapt to the hypoxia-driven switch in metabolism, with consequent acidosis, in order to survive. CAIX is a membrane-bound protein crucial for pH regulation in the highly metabolically active malignant cells. In agreement, carbonic anhydrase IX gene (*CA9*) is a target of HIF-1α and is up-regulated in response to hypoxia [21]. *CA9* mRNA expression increases reliably following hypoxia incubation of PC-3 cells [22], although no significant differences on mRNA expression were found when comparing BPH with prostate carcinomas [3]. Other studies described

lack of CAIX expression in primary prostate carcinoma and hypothesized alternate pathways for maintaining pH balance [23,24]. Conversely, our results disclosed increased frequency of cases with epithelial cell positivity for CAIX expression in organ confined and extra prostatic carcinomas compared to BPH. Our findings taken together with reports of CAIX expression in epithelial prostate carcinoma cells [22,3] sustain the need for reconsidering CAIX role in prostate carcinoma.

The lysyl oxidase gene (*LOX*), was shown to be directly regulated by HIF-1α transcription factor and essential for hypoxia-induced metastasis and cancer cell proliferation [25]. In the prostate we found that LOX immunoreactivity score was associated with HIF-1α positivity, thus supporting the regulatory nature of HIF-1α in LOX expression. Furthermore, although the number of cases with positive LOX expression in carcinomas was similar to BPH, the LOX IRS was significantly higher in organ confined prostate carcinomas compared with BPH. Interestingly, increased expression of *LOX* mRNA in prostate carcinomas compared with BPH was previously observed [3]. LOX biological functions that include effects in cell growth, migration and polarity agrees with the increased LOX expression found in our carcinoma samples.

In this study, evaluation of protein expression according to SNPs in their coding genes disclosed a genotype-phenotype effect for the *LOX* and *KDR* SNPs, but no functional validation at the protein level was observed for the studied *HIF1A* and *CA9* SNPs. The C-to-T substitution at locus +1772 (rs11549465) in *HIF1A* gene localizes in the oxygen-dependent domain of the gene where the variant allele was shown to stabilize *HIF1A* mRNA and enhance *HIF1A* transcriptional activity [26]. Notwithstanding the functional rationale, association of this SNP with prostate carcinoma risk and with microvessel density, yielded conflicting results [7,9,13,12]. In our study, the lack of statistical differences in *HIF1A* +1772 C>T genotypes for HIF-1α protein expression, agrees with a previous report in prostatic carcinoma [9]. However, the low frequency of TT carriers in our sample (only 2 cases) may have influenced statistical power, since the HIF-1α protein and mRNA overexpression have been associated with the TT genotype [8,27].

A functional genetic variant on *KDR* gene that codifies for VEGFR2 is located in the promoter region (-604 T>C, rs2071559), where the C-allele has been associated with lower transcription activity, and decreased serum VEGFR2 level [28]. Interestingly, we found that T carriers had a significantly higher VEGFR2 expression in prostate epithelial cells, thereby suggesting that this SNP

might prove useful for predictive and/or prognostic evaluations in prostate carcinoma, warranting future studies.

A SNP in exon 1 of *CA9* gene is located at locus +201 (rs2071676), where an A-to-G substitution leads to a change of valine-by-methionine in codon 33. Even though we observed an overrepresentation of CAIX positive immunoreactivity in prostate carcinoma compared to BPH, the nonsynonymous SNP in *CA9* +201 was unable to explain variations in the levels of CAIX protein expression in the prostatic tissue, suggesting lack of influence in protein expression, even though the impact of this nonsynonymous substitution (valine to methionine) in CAIX protein activity remains to be confirmed.

The *LOX* gene is translated and secreted as a proenzyme (Pro-LOX), and then processed to a functional enzyme (LOX) and a propeptide (LOX-PP) . We studied a SNP in *LOX* gene that has been identified at locus +473 G>A (rs1800449), that cause an aminoacid substitution (Arg158Glu). This SNP locates at a highly conserved region within LOX-PP, where the A-allele was found to decrease the protective capacity of LOX-PP, while increasing the Pro-LOX-associated invasive ability of tumor cells [29]. When evaluating LOX immunoreactivity and expression intensity by immunohistochemistry in prostate tissues, we found it significantly lower in carriers of the *LOX* +473 A-allele. In the present study, we found that LOX was primarily present at the nucleus of epithelial cells, which fits with other reports asserting that this enzyme may have important functions in secretory cells, as catalyser of histones in the nucleus [30]. Thus, our findings seem to suggest a wider variety of functions for LOX in prostate epithelial cells, beyond those related to cross-link formation in collagen and elastin, which merit further research. We hypothesize that the trafficking of LOX towards inside the cell or a specific cell compartment may be subordinated to the structural molecular characteristics and folding of the protein, which could be determined by *LOX* +473 G>A polymorphism.

Our endeavour to study the genotype-phenotype correlation in key hypoxia markers and its association with prostate cancer yielded encouraging findings, even though results should be interpreted in the context of potential limitations. The lack of statistical significance for genotypic frequencies between disease groups on the putative functional target SNPs in *HIF1A*, *LOX*, *CA9* and *KDR* likely reflects underpowered sample size. This was a major issue as conclusions were impracticable for genetic association analysis and limited for genotype-phenotype inferences. Further

limitations arisen from stratification of carcinomas by stage, Gleason score or PSA level, showing at most only statistical trends for increased expression of VEGFR2 and CAIX in more aggressive phenotypes. Nevertheless, considering the hypothesis-generating nature of this study, we report findings that provide important clues to further work in larger samples. Another issue may be related with raised concern over similar hypoxic dysregulation for both prostate carcinoma and benign hyperproliferative diseases. However, inclusion of BPH patients as controls arranged for age-matching with elderly prostate cancer patients, similar clinical and diagnostic procedures (including prostate biopsy) making the possibility of crossover remote; and this group represents the normality in men at that age, since most men of that age carry benign prostate hyperplasia.

Prostate carcinoma triggers an increase in hypoxia, which regulates *HIF1A* that in turn impacts downstream the expression of LOX, CAIX and VEGFR2 in tumor cells. In this study we observed that the inherited genetic variants in *LOX* and *KDR* seem to modulate the expression of LOX and VEGFR2 in carcinoma cells, supporting a gene-environment interaction in the activation of hypoxia-driven pathways in prostate carcinoma. Results presented here warrant further research in larger samples in order to evaluate the predictive and prognostic value of *KDR* and *LOX* SNPs in prostate carcinoma.

Conflict of interest: The authors declare that they have no conflict of interest

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LEGENDS

Figure 1. Representative microscopy images of staining for hypoxia markers in prostate tissues (MO, 400x). A) HIF-1α - notice the granular cytoplasmic immunoreactivity of the malignant epithelial cells. In this case, more than 50% of the glands stained. B) LOX - strong and diffuse nuclear immunoreactivity of the epithelial cells. C) CAIX - note a focal apical cytoplasmic immunoreactivity in epithelial cells. D) VEGFR2 - moderate nuclear and weak cytoplasmic expression of the epithelial cells

Figure 2. Frequency of patients with positive staining in benign (BPH) and malignant (organ-confined and extra prostatic disease) epithelial cells. CAIX, carbonic anhydrase IX; HIF-1α, hypoxia inducible factor - 1 alpha; LOX, lysyl oxidase; VEGFR2, vascular endothelial growth factor receptor 2. BPH, nodular prostate hyperplasia; EP, extra prostatic disease; OC, organ-confined disease.

Figure 3. Comparison of LOX immunoreactivity score in prostate epithelial cells of benign and malignant patients. BPH, nodular prostate hyperplasia; EP, extra prostatic disease; OC, organ-confined disease. LOX, lysyl oxidase; IRS, immunoreactivity score. Kruskall-Wallis followed by Mann-Whitney non-parametric tests were used to calculate differences between prostatic pathologies.

Figure 4. LOX immunoreactivity score by HIF-1α positivity in epithelial cells. Patients with positive HIF-1α expression are prone to higher LOX IRS. HIF-1α, hypoxia inducible factor – 1 alpha; LOX, lysyl oxidase. IRS, immunoreactivity score. Mann-Whitney non-parametric test was used to calculate differences between positive and negative HIF-1α expression.

Figure 5. Expression of VEGFR2 (H score) in prostate epithelial cells according to prostatic diseases. BPH, nodular prostate hyperplasia; EP, extra prostatic disease; OC, organ-confined disease. VEGFR2, vascular endothelial growth factor receptor 2. Kruskall-Wallis followed by Mann-Whitney non-parametric tests were used to calculate differences between prostatic pathologies.

Figure 6. LOX protein expression (both for immunoreactivity score and staining intensity) according to *LOX* +473 G>A polymorphism. IRS, immunoreactivity score; *LOX*, lysy oxidase; a.u., arbitrary units.

Figure 7. VEGFR2 protein expression (H score) according to *KDR* -604 T>C polymorphism. *KDR,* gene coding for VEGFR2 protein; VEGFR2, vascular endothelial growth factor receptor 2.

TABLES

Table 1. Descriptive clinicopathological data of participating patients

	BPH	**OCPCa**	**EPCa**
Age at diagnosis, yrs	67.8 ± 8.4	61.3 ± 6.4	63.3 ± 6.3
PSA at diagnosis, ng/mL	5.5 ± 5.1	6.6 ± 2.4	11.9 ± 5.6
Weight of the prostate, g	94.8 ± 32.1	45.9 ± 14.3	56.6 ± 22.7
Gleason Score			
< 7	-	14 (43.8)	0 (0.0)
≥ 7	-	18 (56.3)	19 (100)
Percentage of tumor *, %	-	15.0 (6.3 – 20.0)	57.0 (28.8 - 78.8)

Descriptive data of continuous variables is presented as mean ± standard deviation, except for percentage of tumor [data shown as median (interquartile range)]. Categorical variable is depicted as number of observations and respective frequencies. BPH, prostate nodular hyperplasia; EPCa, extra prostatic cancer; OCPCa, organ-confined prostate carcinoma; PSA, prostate specific antigen. * on prostatectomy specimens.

Table 2. Association of the genetic polymorphisms in *HIF1A* +1772 C>T and *CA9* +201 A>G with HIF-1α and CAIX immunoreactivity in prostatic epithelial cells

	Recessive models (*HIF1A* and *CA9*)		
HIF-1α expression	*CC*	*TT/CT*	P *
Negative	28 (0.76)	9 (0.24)	
Positive	10 (0.77)	3 (0.23)	0.928
< 50%	32 (0.74)	11 (0.26)	
≥ 50%	6 (0.86)	1 (0.14)	0.516
CAIX expression	GG	GA/AA	
Negative	9 (0.75)	20 (0.69)	
Positive	3 (0.25)	9 (0.31)	0.699

* Fisher exact test

Table 3. Association of the *KDR-604 T>C* genetic polymorphism with VEGFR2 immunoreactivity in vessels and in prostatic epithelial cells

	Additive model				Recessive model		
	CC	CT	TT	P *	CC	TT/CT	P *
Vessels VEGFR+							
Negative	11 (0.26)	22 (0.53)	9 (0.21)		11 (0.26)	31 (0.78)	
Positive	3 (0.25)	5 (0.42)	4 (0.33)	0.681	3 (0.25)	9 (0.22)	0.626
Epithelial cells VEGFR+							
Negative	11 (0.39)	13 (0.47)	4 (0.14)		11 (0.39)	17 (0.42)	
Positive	3 (0.11)	14 (0.54)	9 (0.35)	0.039	3 (0.11)	23 (0.58)	0.030

* Fisher exact test

Table 4. Expression of proteins from hypoxia pathways in prostate cancer patients, by Gleason grade and PSA value

	Gleason grade (n=38)			PSA at diagnosis (n=36)		
	<7	≥7	P	<10	≥10	P
VEGFR2 H-score [a]	30.9±24.7	60.1±17.9	0.099	30.2±1.2	80.0±33.5	0.085
LOX immunoreactivity score [a]	10.2±1.6	7.6±1.1	0.184	9.2±1.1	6.6±1.8	0.242
HIF-1α expression [b]						
Negative	6 (0.50)	19 (0.73)		17 (0.65)	8 (0.80)	
Positive	6 (0.50)	7 (0.27)	0.163	9 (0.35)	2 (0.20)	0.335 *
CAIX expression [b]						
Negative	10 (0.83)	15 (0.58)		19 (0.73)	5 (0.50)	
Positive	2 (0.17)	11 (0.42)	0.117 *	7 (0.27)	5 (0.50)	0.078

PSA, prostate specific antigen; VEGFR2, vascular endothelial growth factor receptor 2; LOX, lysyl oxidase; HIF1a, hypoxia inducible factor 1 alpha; CAIX, carbonic anhydrase IX. [a] Kruskal Wallis and Mann-Whitney U tests for VEGFR2 H-score in epithelial cells; [b] Chi-square test.* Fisher exact test.

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Title: Functionality of genetic polymorphisms in key hypoxia-regulated downstream molecules and phenotypic correlation in prostate cancer

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Supplementary table 1. Genotypic distribution of functional SNPs in genes of hypoxia pathways by disease status using additive and recessive models analyses

	Prostatic disease status			
HIF1A +1772 C>T genotypes	BPH	OCPCa	EPCa	P *
Additive model				
CC	10 (0.59)	23 (0.82)	14 (0.78)	
CT	5 (0.29)	5 (0.18)	4 (0.22)	
TT	2 (0.12)	0 (0.0)	0 (0.0)	0.144
Recessive model				
CC	10 (0.59)	23 (0.82)	14 (0.78)	
TT/CT	7 (0.41)	5 (0.18)	4 (0.22)	0.205
LOX +473 G>A genotypes				
Additive model				
GG	6 (0.71)	16 (0.55)	13 (0.72)	
GA	2 (0.29)	11 (0.38)	4 (0.22)	
AA	0 (0.0)	2 (0.07)	1 (0.06)	0.740
Recessive model				
GG	6 (0.71)	16 (0.55)	13 (0.72)	
AA/GA	2 (0.29)	13 (0.45)	5 (0.28)	0.442
CA9 +201 A>G genotypes				
Additive model				
GG	3 (0.38)	9 (0.31)	5 (0.29)	
GA	5 (0.62)	18 (0.62)	10 (0.59)	
AA	0 (0.0)	2 (0.07)	2 (0.12)	0.882
Recessive model				
GG	3 (0.38)	9 (0.31)	5 (0.29)	
GA/AA	5 (0.62)	20 (0.69)	12 (0.71)	0.918
KDR -604 T>C genotypes				
Additive model				
CC	6 (0.33)	8 (0.26)	3 (0.17)	
CT	8 (0.45)	15 (0.48)	13 (0.72)	
TT	4 (0.22)	8 (0.26)	2 (0.11)	0.436
Recessive model				
CC	6 (0.33)	8 (0.26)	3 (0.17)	
TT/CT	12 (0.67)	23 (0.74)	15 (0.83)	0.515

* Fisher exact test. BPH, nodular prostate hyperplasia; OCPCa, organ-confined prostate carcinoma; EPCa, extra prostatic carcinoma. *CA9*, carbonic anhydrase IX gene; *HIF1A*, hypoxia inducible factor 1 alpha gene; *KDR*, vascular endothelial growth factor receptor 2 gene; *LOX*, lysyl oxidase gene.

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 commonly used to treat advanced and recurrent prostate cancer, although prognosis varies widely among individuals. We evaluated whether polymorphisms in adipokine pathway genes may predict clinical outcomes among prostate cancer patients.

Patients and Methods

We enrolled 483 patients who underwent ADT and genotyped them 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 *ADIPOQ* +45 T>G G homozygous carriers were more likely to present biochemical progression (HR=4.1, 95%CI: 1.62-10.54) and to die (HR=5.0, 95%CI: 1.75-14.38) than T-allele carriers. Having the *ADIPOQ* +276 G>T G homozygous genotype and the tumor necrosis factor high activation genetic profile were associated with reduced likelihood of resistance to ADT (HR=0.71, 95%CI: 0.51-0.99 and HR=0.62, 95%CI: 0.41-0.93, respectively). Presence of the *IL6* -572 G>C C-allele was independently associated with all-cause mortality (HR=1.78, 95%CI: 1.01-3.13). The *LEPR* Gln223Arg G-allele variant was associated with a more than twofold increased risk of developing metastasis (OR=2.1, 95%CI: 1.2-3.6).

Conclusion

Genetic polymorphisms in adipokine pathways might have a clinical role in evaluating prognosis among men treated with ADT. In addition, combined targeting of identified adipokine pathways

may represent a therapeutic strategy for castration-resistant prostate cancer, metastasis development, thus improving survival.

INTRODUCTION

In the last decades, depletion or blockage of androgen action has been the standard of care for men with advanced prostate cancer [1]. Androgen deprivation therapy (ADT) results in decreased levels of serum prostate-specific antigen (PSA) as well as waning of androgen receptor (AR)-dependent growth. Response to treatment is not durable since patients become resistant to ADT, leading to castration-resistance status, an invariably fatal condition [2]. Clinical and tumor biology factors that may partially account for disease burden and thus serve as useful prognostic predictors, include Gleason score, serum PSA and distant metastasis [3]. Although mechanisms responsible for prostate cancer cell survival after ADT are not entirely understood, there is evidence that AR-dependent and AR-independent pathways may be implicated [4,5]. Single nucleotide polymorphisms (SNPs) in genes involved in biosynthesis and metabolism of steroids and androgens seem to influence response to ADT [6-9]. Recent findings showed also 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]. Some functional SNPs in genes encoding molecules of these pathways have been shown to be associated with prostate cancer risk [16-19] and a recent study found that obese men were at increased risk of developing castration-resistant prostate cancer and metastasis [20]. We studied a cohort of prostate cancer patients treated with ADT to examine the prognostic significance of 27 functional adipokine pathway SNPs with risk of metastasis, response to chemical/surgical castration, and all-cause mortality (ACM).

MATERIALS AND METHODS

Patients

Patients with histopathologically confirmed prostate cancer and treated with ADT between 1990 and 2009 were included in this study (n=513). 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 signed an informed consent.

ADT consisted of orchiectomy or treatment with luteinizing hormone releasing hormone-agonist (LHRHa) with or without anti-androgen after diagnosis of advanced or metastatic prostate cancer or after relapsing from primary local therapy with curative intent. Patients with adjuvant hormonal therapy for localized disease were excluded (n=24). Hormonal treatment was continued at least until disease progression, based on serum PSA levels, imaging, and clinical findings. The primary endpoint was resistance to ADT, defined as the time from ADT initiation to two consecutive rises of PSA (1 week apart) greater than the PSA nadir (defined as biochemical progression) or progression of bone lesions (new or size increase, soft tissue metastasis, or at least 2 new metastatic spots in bone scintigraphy), despite at least two consecutive hormonal manipulations [21,22]. The secondary endpoints 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), after diagnosis. Information concerning clinical endpoints was collected via standardized chart review (6 patients were excluded due to missing data).

Genetic variants and genotyping

Samples of peripheral blood were used for genotyping. Candidate genes involved in adipokine pathways known to affect oncogenesis were selected. SNPs were selected based on 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). We also examined combinations of SNPs by adipokine pathway according to their functional implications (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, followed by restriction fragment length polymorphism analysis. We have previously referenced these genotyping protocols [23]. Quality control measures included negative controls in all runs and repeated genotyping in more than 5% of the samples.

Statistical analysis

We calculated descriptive statistics of clinicopathological characteristics for all patients. PSA at diagnosis was dichotomized at 20 ng/mL based on its association with micrometastasis[24].

Clinical stage was classified according to TNM as localized (T1-T2, with N0 and M0) or advanced (T3-T4 and/or N+ and/or M+).

For time-to-event analyses, age-adjusted Cox regression models were used to calculate hazard ratios (HR) and 95% confidence intervals (CIs) for the association between clinicopathological characteristics with each of the outcomes of interest, i.e., biochemical progression under hormonal castration and ACM. Age-adjusted logistic regression models were used to evaluate the risk for metastasis after diagnosis. Multivariate analysis was conducted after selecting confounding variables by empirical evaluation for each of clinical and genetic models. We used two different approaches based on the minor allele: a dominant ("aa" + "Aa" genotype versus "AA" genotype) and a recessive ("aa" genotype versus "Aa" + "AA" genotype) model to evaluate the individual association of 27 SNPs with the three outcomes. The model with the highest likelihood ratio was presented as the best-fitting genetic model for each SNP. Functionally combined SNPs in each pathway were dichotomized (low/intermediate vs. high; low vs. intermediate/high) and the one with highest likelihood ratio was retained. For the subset of clinical factors, SNPs and functional combinations of SNPs were selected on the basis of a regression P-value <0.15. A multivariate Cox proportional hazards model was derived by stepwise selection (P-value for retention < 0.05) to identify the independent prognostic factors for biochemical progression. A multivariate logistic regression model was similarly performed using only non metastatic patients at diagnosis, in order 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

Clinical characteristics of the final 483 patients analyzed are presented in Table 1. At diagnosis, 27% of patients presented distant metastasis, 62% had clinically advanced disease (T3-T4 and/or N+ and/or M+), and 51% had a biopsy Gleason score ≥ 7 (4+3). The median duration between ADT initiation and disease progression was 91.8 months, while the median follow-up from ADT initiation to death or last visit was 126.9 months. Several clinical factors were identified to predict biochemical progression under hormonal castration and ACM (Table 1).

Logistic regression analysis showed that definitive therapy (OR=3.51; 95%CI: 1.86-6.61) and advanced clinical stage (T3-T4) (OR=4.08; 95%CI: 2.13-7.79) were associated with risk for distant metastasis on follow-up. Empirical analysis using Cox regression was then performed to evaluate the association of SNPs and their functional combinations with the outcomes of interest. As shown in Supplementary table 3, the genotypes *ADIPOQ* +276 TT/TG, *IL6R* Asp358Ala CC and *ADIPOQ* +45 GG, and the high expression *ADIPOQ* haplotype, low TNFa expression and low/intermediate TNFa activation genetic profiles were associated with biochemical progression under hormonal castration. Notably, the difference in median time to progression during ADT for the genotypes at *ADIPOQ* +45 was greater than 5 years, whereas for *ADIPOQ* +276 and the combined TNFa activation genetic profile the difference was respectively 15 and 24 months (Figure 1). Moreover, the *IL6R* Asp358Ala CC and *ADIPOQ* +45 GG, *IL6*-572 C carriers and high VEGF activation 2SNPs were associated with shorter time to ACM following ADT (Supplementary table 3). The median survival time was significantly lower for *ADIPOQ* +45 GG carriers (by more than 6 years difference) and for *IL6* -572 C-allele carriers (over 2 years of difference compared with -572 GG) (Figure 2). A significant relation with increased risk for

developing distant metastasis was observed in the *LEPR* Gln223Arg G carriers, *LEPR* Lys109Arg homozygous G carriers, *TNFRSF1A* -329 G carriers, and for the high/intermediate LEPR signaling genetic profiles (Supplementary table 4).

The predictive effects of SNPs with P<0.15 (univariate analysis) on time to biochemical progression under hormonal castration and ACM were evaluated in presence of significant clinicopathological predictors (from Table 1) using Cox regression. The effect of *ADIPOQ* +45 and +276 SNPs and of the TNFa activation genetic profile on the response to ADT remained strong after adjustment for clinical factors (Table 2). Analysis of the secondary endpoint ACM after adjusting for other predictors showed that *ADIPOQ* +45 T>G and *IL6* -572 G>C remained significant predictors, together with age at diagnosis, biopsy Gleason score, metastasis at ADT initiation and biochemical progression under hormonal castration (Table 2). On multivariate logistic regression, patients with the combined high/intermediate LEPR signaling genetic profile remained associated with greater risk of developing distant metastatic disease (OR=3.41, 95%CI: 1.71-6.79). In this model men that received definitive therapy and who presented with advanced clinical stage at diagnosis were at increased risk for developing metastasis on follow-up (OR=4.26, 95%CI: 2.24-8.13 and OR=3.29, 95%CI: 1.75-6.18, respectively).

DISCUSSION

We examined whether germline polymorphisms in adipokine pathways are determinants of the response to ADT. The time to biochemical progression under hormonal castration 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 endpoint ACM, together with

IL6-572 genetic polymorphism. Additionally, our results also suggest an association of the combined LEPR genetic profile with development of distant metastasis.

Androgen deprivation therapy remains the mainstay treatment for advanced and recurrent prostate cancer [21,25]. Clinical variables such as stage, biopsy Gleason score, PSA serum levels and metastasis [3], are established factors that influence the response to ADT, albeit SNPs may also be useful for prognosticating the response to ADT. Out of 27 SNPs of 17 genes related with 9 adipokine pathways analyzed, we have identified two SNPs, *ADIPOQ* +45 and *ADIPOQ* +276 and a functional *TNFA*/*TNFRSF1A* combination that are associated with the response to ADT. The mechanisms responsible for castration-resistant prostate cancer development are not clearly established. Despite obvious interest in AR-dependent pathways, other independent pathways have been described [4,26], in which androgen-refractory cells use alternative survival pathways to overcome the growth inhibition imposed by ADT [4,27]. ADT is known to induce changes in adiposity and adipokine levels in circulation [28,29]. Adipokine pathways, have been implicated in intracellular signals such as those activated in hormonal castration resistance [30]. Furthermore, mitogenic and anti-apoptoptic effects of some adipokines (e.g. leptin, IL-6, IGF-1) seem to be limited to androgen-refractory prostate cancer cells [31-33].

In our study, *ADIPOQ* +45 G homozygous and *ADIPOQ* +276 T carriers had higher risks of biochemical progression under ADT. The G-allele in locus +45 and T-allele in locus +276 are associated with higher circulating adiponectin plasma levels [34,35]. Generally, low concentrations have been associated with prostate cancer risk and survival, rendering adiponectin a protective role against cancer [36-38]. 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 levels [29], and eventually modulating

the expression of adiponectin receptors, which have been shown to be receptive to cytokines and steroid hormones stimulus according to tumor cells androgen refractoriness status [39]. Additionally, adiponectin has the capacity to exert stimulatory growth and motile effects in some prostate tumor cells, depending on PTEN status [40,41]. Despite advances in understanding the role of adiponectin in prostate cancer risk, studies on castration-resistant prostate cancer are scarce. Although *ADIPOQ* polymorphisms have been inconsistently associated with prostate cancer risk [16,42,43], our study is the first to evaluate *ADIPOQ* SNPs in association with ADT resistance. 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 increased risks of biochemical progression under hormonal castration. Interestingly, case-control studies have shown lack of association between *TNFA* variants at locus -863 or -308 and prostate cancer risk [44,45]. 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 cells [46]. TNFa-mediated activation of TNFR1 signaling is critical for activating tumor-reactive T cells and arresting multistage carcinogenesis [47,48]. More specifically, the proapoptotic actions of estrogen receptor beta in androgen-refractory prostate cancer cells required TNFa signaling [49]. The very low levels of testosterone as result of ADT, together with increased inflammatory markers [50], 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 results [51-54]. In our cohort, where all patients

received ADT for prostate cancer while taking into consideration previous treatment modalities, *ADIPOQ* +45 T>G and *IL6* -572 G>C, remained independently associated with decreased survival. IL-6 has been implicated with poor prognosis in prostate cancer [55,56]. 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 production [57-59], mechanisms related with resistance to hormonal castration and ultimately mortality. In other studies unrelated to prostate cancer, the *IL6* -572 genetic polymorphism was a predictor of bone mineral density, metabolic syndrome and malignant conditions [60-62]. The -572 C allele has been associated with higher serum levels of IL-6 [60,63]. A glucocorticoid receptor element at position -557 to -552 likely influences steroid binding and regulates IL-6 secretion [61,64]. We observed after multivariate analysis that *IL6* -572 C carriers remained significantly associated with reduced overall survival following ADT, which might be the response to higher bioavailability of IL-6. In our study, the *ADIPOQ* +45 G homozygous carriers, besides being associated with biochemical progression under hormonal castration, showed worst survival compared with carriers of the T-allele. The G-allele is associated with higher circulating adiponectin plasma levels [35]. *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 [65,66], as in this study. Moreover, recent findings suggest that AMPK, which is up-regulated by adiponectin signaling [40,41,67], if activated during energy stress conditions such as androgen deprivation therapy may represent an advantage that promotes tumor cell survival [68,69]. 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 proposed [30].

Leptin's actions in tumor development and progression are mediated by leptin receptor [14,15], which is strongly expressed in prostate tumors [70], where pathway activation induces aggressive cell phenotypes [71,72]. We found that carriers of the combined LEPR high/intermediate signaling genetic profile were at increased risk for developing metastatic disease. This means that carrying 2 or 3 risk alleles out of 3 SNPs in *LEPR* (Lys109Arg, Gln223Arg and Lys656Asn), which may represent higher LEPR signaling capacity, increases the risk for metastasis in patients receiving ADT. Since *LEPR* 109 and *LEPR* 223 were associated with metastasis in univariate analysis, the independent effect of the combined *LEPR* SNPs may rely on the influence of those two. Although the *LEPR* 223 polymorphism yielded mixed results in case-control studies [16,23,73], G carriers have a stronger leptin-binding affinity [74]. In addition, this polymorphism is associated with plasma soluble LEPR concentrations and may influence receptor recycling and degradation [75,76], thereby influencing free leptin levels and receptor availability at cell surface. The G-allele of *LEPR* 109 was also found to be associated with plasma soluble LEPR levels [75] and higher circulating leptin levels [77]. Moreover, recent work evidenced a central role for leptin signaling in tumor-initiating stem cells growth and survival [78].

Inherited genetic markers have been fairly explored as predictors of prostate cancer outcomes. Although 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, our study has some limitations. Testosterone levels were not available in all men to confirm castration; therefore we relied on PSA measurements, clinical and imaging information to define progression under hormonal castration. Although we included only functional SNPs from genes in adipokine pathways, our SNP panel and SNP combinations could be incomplete. We did not explore potential gene-environment interactions due to missing data on body mass,

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 (46.4% for disease progression under ADT; 32.2% for mortality; 44.9% for metastasis).

At a time when alternative therapeutic opportunities arise in advanced prostate cancer [79,80], it is important to validate the use of germline polymorphisms to complement the value of clinical factors to prognosticate clinical course after ADT initiation, thereby providing a more personalized medicine approach to therapy and management. Our findings also underscore the need for examining the effectiveness of personalized therapies targeted towards adiponectin, tumoral necrosis factor and leptin pathways. If confirmed, our findings might help targeting patients with predictable precocious ADT failure and mortality for more aggressive intervention.

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Figure 1. Kaplan-Meier estimates of cumulative incidence of disease progression under ADT by SNPs and functional combinations. The Log-rank test was used to analyze the equality of survival distributions for the different genotypes of *ADIPOQ* haplotype (P=0.010), *ADIPOQ* +45 T>G (P=0.008), *ADIPOQ* +276 G>T (P=0.039), *IL6R* Asp358Ala A>C (P=0.028), TNFa pathway expression (P=0.024), and TNFa pathway activation (P=0.048). ADT, androgen deprivation therapy. *TNFA* expression is defined as the functional combination of *TNFA* -308 and -863 SNPs, whereas TNFa activity includes besides the *TNFRSF1A*, two *TNFA* SNPs (detailed in supplementary table 2).

Figure 2. Kaplan-Meier estimates of survival following ADT, stratified by SNPs and functional combinations. The equality of survival distributions for the different genotypes of *IL6* -572 G>C (P=0.022), *ADIPOQ* +45 T>G (P=0.001), *IL6R* Asp358Ala A>C (P=0.016) and VEGF 2SNPs pathway activation (P=0.009) was tested with Log-rank. ADT, androgen deprivation therapy. VEGF pathway activation 2SNPs relates to the functional combination of two *VEGF* SNPs -460 and +405 plus the *KDR* -604 polymorphism (detailed in supplementary table 2).

Table 1. Clinicopathological characteristics of prostate cancer patients and univariate analysis of factors that predicted resistance to ADT and all-cause mortality

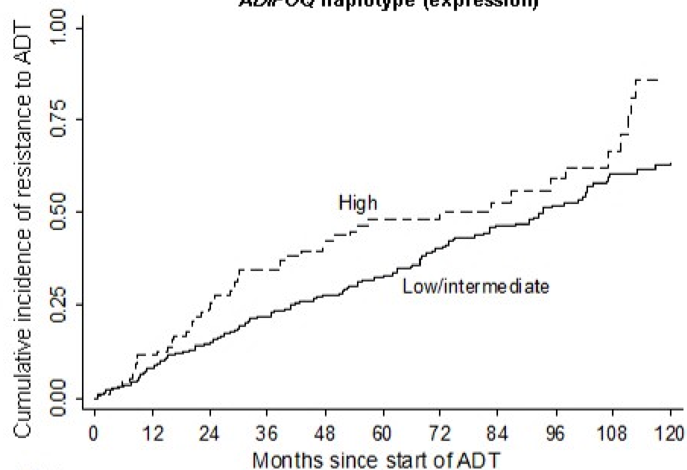
	N (%) *	Resistance to ADT		All-cause mortality	
		No. of Events	aHR (95%CI)	No. of Events	aHR (95%CI)
Age at diagnosis, years [a]	69.5(7.7)[b]		0.99 (0.97-1.01)		1.05 (1.02-1.07)
PSA at diagnosis					
< 20 ng/mL	235 (51)	75	Referent	57	Referent
≥ 20 ng/mL	224 (49)	134	1.94 (1.46-2.59)	89	1.58 (1.13-2.22)
Clinical stage					
Localized (T1-2)	171 (38)	35	Referent	30	Referent
Advanced (T3-4)	282 (62)	173	3.68 (2.53-5.33)	118	2.76 (1.83-4.17)
Biopsy Gleason score					
≤ 7 (3+4)	220 (49)	75	Referent	47	Referent
≥ 7 (4+3)	229 (51)	124	2.51 (1.86-3.39)	85	3.02 (2.08-4.39)
Definitive therapy					
None	327 (68)	168	Referent	120	Referent
Radical prostatectomy/Radiotherapy	156 (32)	56	0.57 (0.40-0.79)	34	0.77 (0.50-1.18)
Hormonal treatment modality					
LHRH-agonist/orchiectomy	102 (21)	42	Referent	16	Referent
Combined ADT	381 (79)	182	1.10 (0.77-1.58)	138	2.57 (1.48-4.48)
Metastases at ADT initiation					
No	297 (69)	111	Referent	79	Referent
Yes	131 (31)	94	3.52 (2.64-4.70)	67	2.96 (2.13-4.12)
Biochemical progression under hormonal castration					
No	259 (54)	--	---	26	Referent
Yes	224 (46)	--	---	126	5.55 (3.63-8.49)

* Column subtotals do not sum to 483 due to missing data. ADT, androgen deprivation therapy; PSA, prostate specific antigen; LHRH-agonist, luteinizing hormone releasing hormone agonist. aHR, age-adjusted hazard ratio; 95%CI, 95% confidence interval. [a] aHR calculated using age as a continuous variable; [b] Mean (standard deviation)

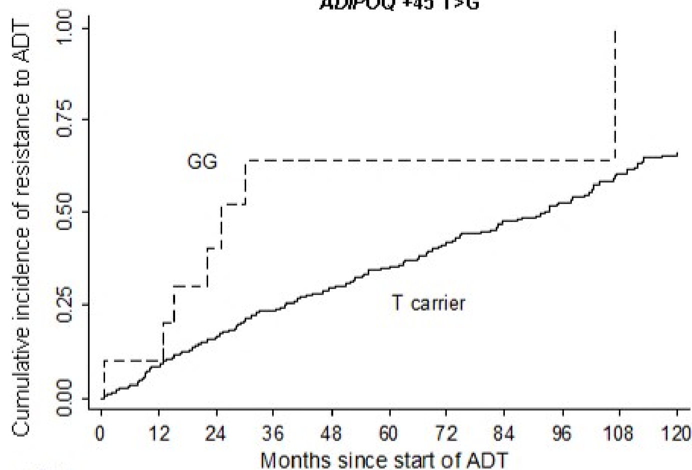
Table 2. Multivariate analysis of the association between adipokine pathway SNPs and resistance to ADT and all-cause mortality

	Resistance to ADT HR (95%CI)	All-cause mortality HR (95%CI)
Age at diagnosis, years	---	1.07 (1.04-1.11)
Metastasis at ADT initiation		
No	Referent	Referent
Yes	2.62 (1.81-3.80)	1.88 (1.23-2.90)
Gleason score		
≤ 7 (3+4)		Referent
≥ 7 (4+3)	---	1.70 (1.11-2.60)
Clinical stage		
Localized	Referent	
Advanced	2.43 (1.55-3.82)	---
Disease progression under ADT		
No		Referent
Yes	---	4.64 (2.75-7.83)
ADIPOQ + 45		
T carrier	Referent	Referent
GG	4.14 (1.62-10.54)	5.02 (1.75-14.38)
IL6 -572 G>C		
GG		Referent
C carrier	---	1.78 (1.01-3.13)
ADIPOQ +276		
T carrier	Referent	
GG	0.71 (0.51-0.99)	---
TNFa activation		
Low/intermediate	Referent	
High	0.62 (0.41-0.93)	---

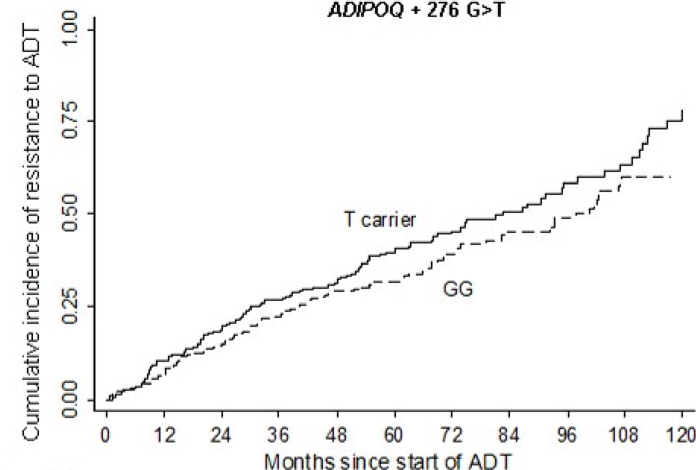
ADT, androgen deprivation therapy; SNP, single nucleotide polymorphism; TNFa, tumor necrosis factor alpha; *ADIPOQ*, adiponectin gene; HR(95%CI), hazard ratio and 95% confidence interval.



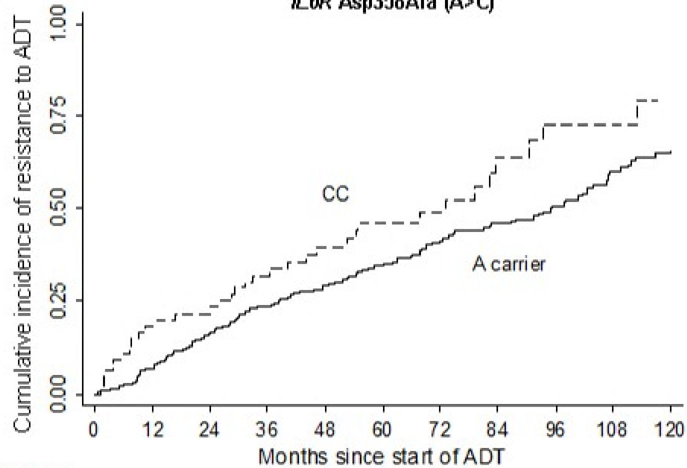
No. at Risk											
Low/Interm	366	330	292	259	210	153	115	81	59	43	32
High	98	85	68	55	42	32	24	18	12	8	2



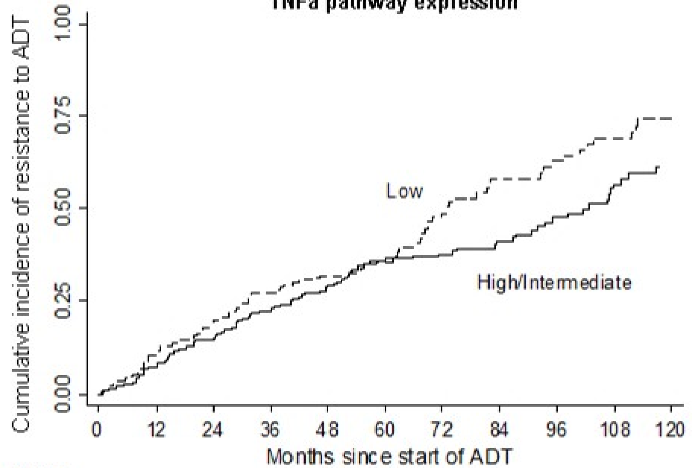
No. at Risk											
T carriers	455	407	356	313	251	184	139	99	71	52	35
GG	10	9	5	2	2	2	1	1	1	0	0



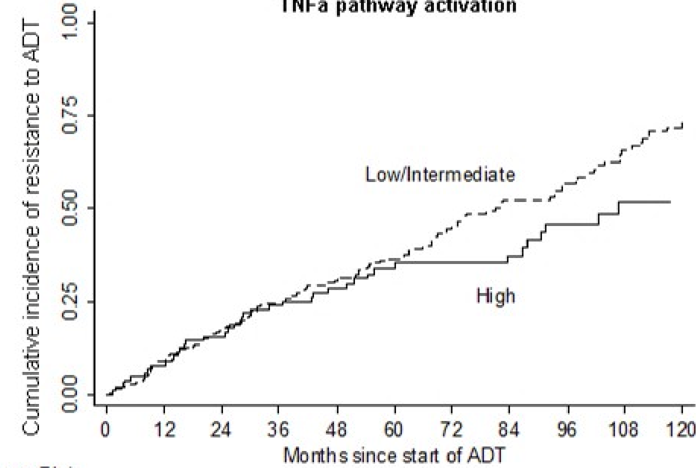
No. at Risk											
T carriers	223	195	166	144	112	78	61	45	28	20	7
GG	241	220	194	170	140	107	78	54	43	31	27



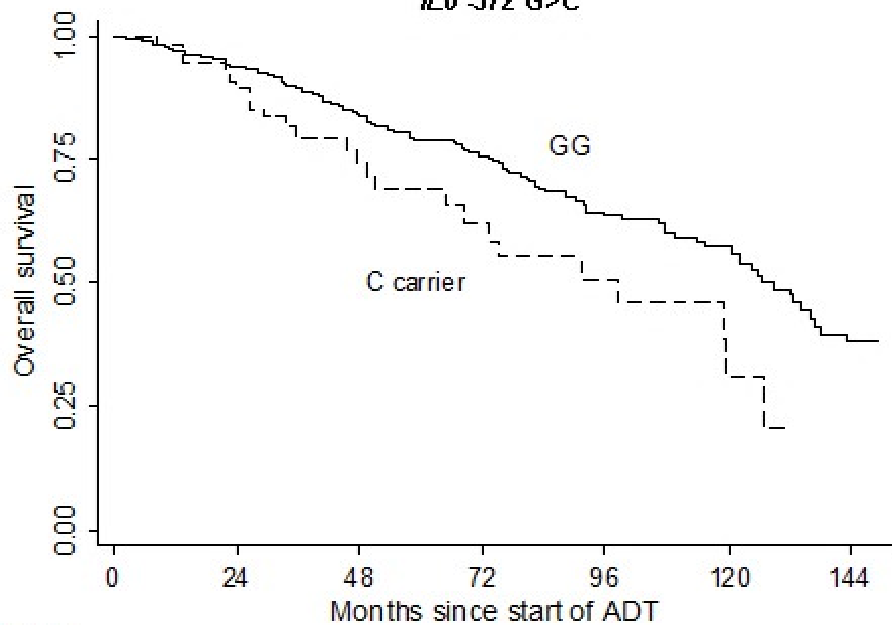
No. at Risk											
CC	66	52	45	38	31	20	16	9	5	4	2
A carriers	395	360	312	273	218	162	120	87	64	46	31

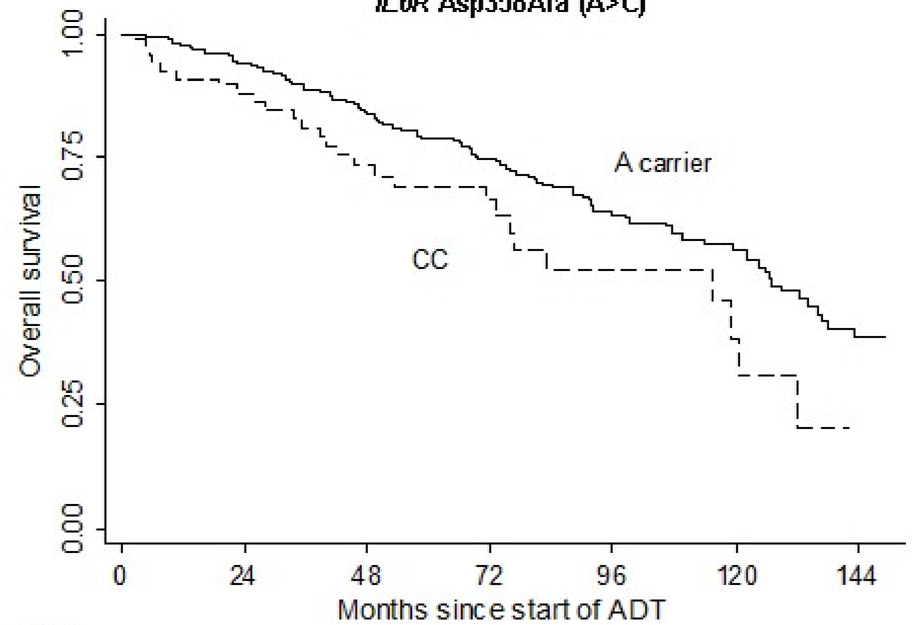


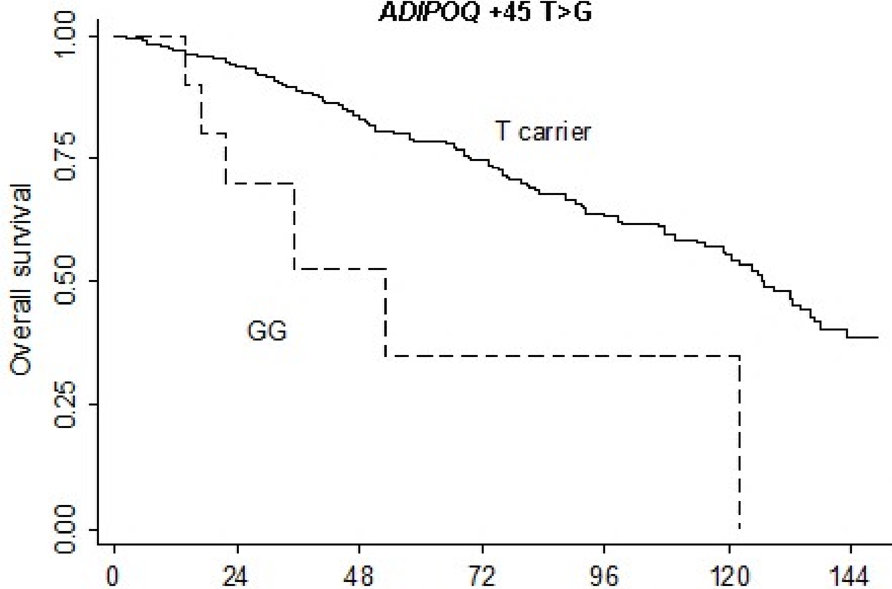
No. at Risk											
Low	191	168	143	123	105	78	49	33	24	19	11
High/Interm	266	240	211	186	143	104	87	63	45	31	22

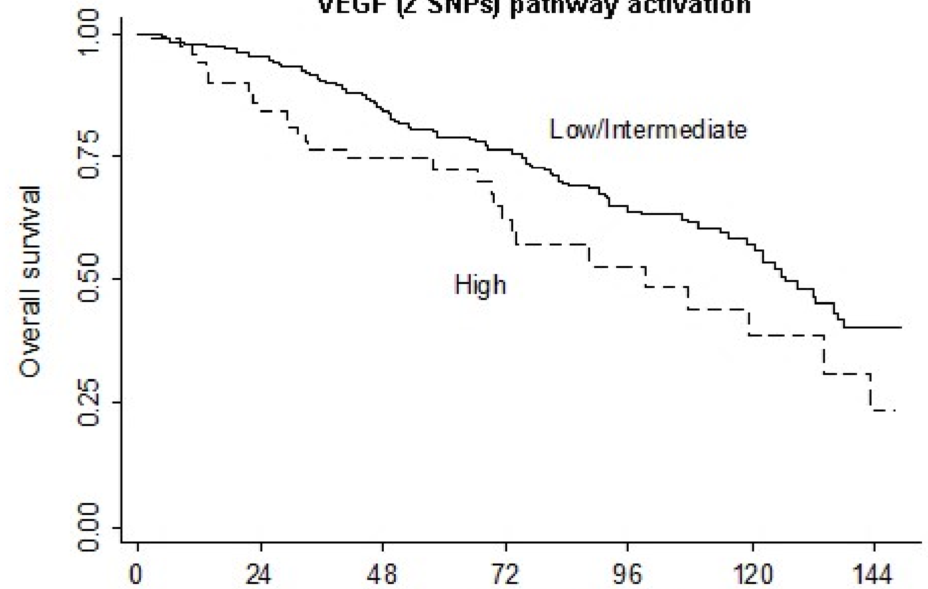


No. at Risk											
Low/Interm	349	311	270	237	189	136	94	65	47	34	19
High	107	96	83	71	58	46	42	31	22	16	14


IL6 -572 G>C
Overall survival
1.00
0.75
0.50
0.25
0.00
GG
C carrier
0
24
48
72
96
120
144
Months since start of ADT
No. at Risk
GG 399 348 257 161 85 50 20
C carriers 57 48 29 18 11 4 1


IL6R Asp358Ala (A>C)
Overall survival
1.00
0.75
0.50
0.25
0.00
A carrier
CC
0
24
48
72
96
120
144
Months since start of ADT
No. at Risk
CC 68 53 35 22 11 5 0
A carriers 398 352 258 159 87 50 22


ADIPOQ +45 T>G
Overall survival
1.00
0.75
0.50
0.25
0.00
T carrier
GG
0
24
48
72
96
120
144
Months since start of ADT
No. at Risk
T carriers 460 403 294 184 100 57 22
GG 10 6 3 1 1 1 0


VEGF (2 SNPs) pathway activation
Overall survival
1.00
0.75
0.50
0.25
0.00
Low/Intermediate
High
0
24
48
72
96
120
144
Months since start of ADT
No. at Risk
Low/Interm 391 347 251 155 84 47 19
High 71 56 40 25 13 7 3

Supplementary table 1. Studied single nucleotide polymorphisms (SNPs) in genes coding for molecules involved on adipokine pathways

Pathway	Gene	Reference SNP ID	Nucleotide substitution	Genomic location	MGF (%)
Adiponectin	*ADIPOQ*	rs1501299	G>T	+ 276 intron 2	TT (11.7)
	ADIPOQ	rs2241766	T>G	+45 intron 2	GG (2.3)
	ADIPOQ	rs16861194	A>G	-11426 promoter	GG (1.1)
Interleukin - 6	*IL6*	rs1800795	G>C	-174 promoter	CC (13.1)
	IL6	rs1800796	G>C	-572 promoter	CC (0.4)
	IL6	rs1800797	G>A	-597 promoter	AA (13.9)
	IL6	rs10499563	T>C	-6331 promoter	CC (4.8)
	IL6R	rs2228145	A>C	Asp358Ala	CC (14.7)
	IL6ST	rs3729960	G>C	Gly148Arg	CC (1.5)
Vascular Endothelial Growth Factor	*KDR*	rs2071559	T>C	-604 promoter	TT (22.2)
	VEGF	rs2010963	G>C	+405 5'-UTR	CC (15.3)
	VEGF	rs833061	C>T	-460 promoter	CC (20.8)
	VEGF	rs3025039	C>T	+936 3'-UTR	TT (3.1)
Leptin	*LEP*	rs7799039	G>A	-2548 promoter	AA (16.8)
	LEPR	rs1137100	A>G	Lys109Arg	GG (5.7)
	LEPR	rs1137101	A>G	Gln223Arg	GG (16.4)
	LEPR	rs8179183	G>C	Lys656Asn	CC (2.9)
Peroxisome proliferator-activated receptor	*PPARGC1A*	rs8192678	A>G	Gly482Ser	GG (13.7)
	PPARD	rs2016520	T>C	-87 5'-UTR	CC (5.9)
	PPARG	rs1801282	C>G	Pro12Ala	GG (0.8)
Osteopontin	*SPP1*	rs28357094	T>G	-66 promoter	GG (5.9)

Insulin growth factor 1	*IRS1*	rs1801278	C>T	Gly972Arg	TT (1.9)
	IGF1R	rs2229765	G>A	+3174 exon 16	AA (21.1)
Fibroblast growth factor 2	*FGFR2*	rs2981582	C>T	Intron 2	TT (3.8)
Tumor necrosis factor alpha	*TNFA*	rs1800629	G>A	-308 promoter	AA (5.1)
	TNFA	rs1800630	C>A	-863 promoter	AA (3.8)
	TNFRSF1A	rs4149570	G>T	-329 promoter	TT (15.7)

MGF, minor genotype frequency in present study; *ADIPOQ*, adiponectin gene; *IL6*, interleukin-6 gene; *IL6R*, interleukin-6 receptor gene; *IL6ST*, 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; *SPP1*, 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.

Supplementary table 2. Rationale for functional combination of Single Nucleotide Polymorphisms (SNPs), according to adipokine pathways

Pathway	SNP	Genotypes	Functional outcomes	SNP functional combinations
Adiponectin	*ADIPOQ* +45 T>G	G carrier [1,2]	↑ expression	**haplotype** (combined according to reference [5]).
	ADIPOQ +276 G>T	T carrier [2,3]	↑ expression	
	ADIPOQ -11426	AA [4]	↑ expression	
Interleukin - 6	*IL6* -174	C carrier [6 7]	↑ expression, ↑ activation	**Signaling:** *high*, IL6R Ccarrier/IL6ST GG; *intermediate*, IL6R Ccarrier/IL6ST Ccarrier and IL6R AA/IL6ST GG; *low*, IL6R AA/IL6ST Ccarrier.
	IL6 -572	C carrier [7]	↑ expression, ↑ activation	**Expression:** *high*, ≥ 3/4 risk genotypes; *low*, 0-2/4 risk genotypes.
	IL6 -597	A carrier [7]	↑ expression, ↑ activation	**Activation:** *high*, high expression/high or intermediate signaling; *intermediate*, high expression/low signaling and low expression/high signaling; *low*, low expression/low or intermediate signaling.
	IL6 -6331	TT [8]	↑ expression, ↑ activation	
	IL6R 358	C carrier [9,10]	↑ signaling, ↑ activation	
	IL6ST 148	GG [11]	↑ signaling, ↑ activation	
Vascular Endothelial Growth Factor	*KDR* -604	TT [12]	↑ signaling, ↑ activation	**Expression 2 SNPs** (-460/+405, according to ref [13]): *low* vs. *high*.
	VEGF -460	C carrier [13,14]	↑ expression, ↑ activation	**Expression 3 SNPs:** *high*, -460/+405 high/936 CC; *intermediate*, -460/+405 high/936 T carrier and -460/+405 low/936 CC; *low*, -460/+405 low/936 T carrier.
	VEGF +405	GG [13,14]	↑ expression, ↑ activation	**Activation 2SNPs:** *high*, -460/+405 high/KDR TT; *intermediate*, -460/+405 high/KDR Ccarrier and -460/+405 low/KDR TT; *low*, -460/+405 low/KDR Ccarrier.
	VEGF +936	CC [15]	↑ expression, ↑ activation	**Activation 3SNPs:** *high*, high or intermediate expression/ KDR TT; *intermediate*, high expression/ KDR Ccarrier and low expression/ KDR TT; *low*, low or intermediate expression/ KDR Ccarrier.
Leptin	*LEP* -2548	AA [16]	↑ expression	**Signaling:** *high*, 3/3 risk alleles; *intermediate*, 2/3 risk alleles; *low*, 0-1/3 risk alleles
	LEPR 109	GG [17,18]	↑ signaling	**Activation:** *high*, high signaling/LEP AA or G carrier and intermediate signaling/LEP AA; *low*, low signaling/LEP AA or G carrier and intermediate signaling/LEP G carrier.
	LEPR 223	GG [17,19]	↑ signaling	
	LEPR 656	C carrier [20]	↑ signaling	
Peroxisome proliferator-activated receptor	*PPARGC1A* 482	AA [21]	↑ expression	**Number of risk alleles:** 0-3/3
	PPARD -87	TT [22]	↑ expression	

	PPARG 12	G carrier [23]	↓ activation	
Osteopontin	*SPP1* -66	TT [24]	↑ expression	---
Insulin growth factor	*IRS1* 972 *IGF1R* +3174	CC [25] GG [26]	↑ signaling ↑ signaling	**Signaling:** *high*, IRS1 CC/IGF1R GG; *intermediate*, IRS1 CC/IGF1R A carrier and IRS1 T carrier/IGF1R GG; *low*, IRS1 T carrier/IGF1R A carrier.
Fibroblast growth factor 2	*FGFR2*, rs2981582	T carrier [27]	↑ signaling	---
Tumor necrosis factor alpha	*TNFA* -308 *TNFA* -863 *TNFRSF1A* -329	A carrier [28] A carrier [29,30] T carrier [31]	↑ expression, ↑ activation ↑ expression, ↑ activation ↑ signaling, ↑ activation	**Expression:** *high*, -308 Acarrier/-863 Acarrier; *intermediate*, -308 Acarrier/-863 CC and -308 GG/-863 Acarrier; *low*, -308 GG/-863 CC. **Activation:** *high*, high or intermediate expression/TNFRSF1A GG; *intermediate*, high expression/TNFRSF1A Tcarrier and low expression/TNFRSF1A GG; *low*, low or intermediate expression/TNFRSF1A Tcarrier.

MGF, minor genotype frequency in present study; *ADIPOQ*, adiponectin gene; *IL6*, interleukin-6 gene; *IL6R*, interleukin-6 receptor gene; *IL6ST*, 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; *SPP1*, 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.

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Supplementary table 3. Association of SNPs in genes of adipokine pathways with resistance to ADT and all-cause mortality

	MGF	Resistance to ADT				All-cause mortality			
SNP IDs and combined SNPs [1]	(%)	Model	No.	LR	aHR (95%CI)	Model	No.	LR	aHR (95%CI)
KDR-604, *rs2071559*	48	Dominant	463	2.07	1.13(0.84-1.52)	Recessive	468	16.29	1.16(0.80-1.68)
VEGF+405, *rs2010963*	38	Dominant	463	3.24	1.20(0.92-1.58)	Recessive	468	16.87	1.28(0.83-1.96)
VEGF-460, *rs833061*	44	Recessive	**457**	**3.90**	**1.30(0.95-1.78)**	Recessive	462	13.85	1.14(0.77-1.68)
VEGF+936, *rs3025039*	13	Recessive	440	2.68	1.80(0.58-5.65)	Dominant	445	18.24	1.23(0.83-1.83)
VEGF expression 2 SNP [1]	---	---	457	2.35	1.16(0.86-1.55)	---	462	13.43	1.02(0.72-1.45)
VEGF expression 3 SNP LI_H [1]	---	---	436	2.51	1.18(0.89-1.55)	---	441	15.93	1.07(0.77-1.49)
VEGF activation 2 SNP LI_H [1]	---	---	**457**	**3.77**	**1.33(0.93-1.90)**	**---**	**462**	**18.51**	**1.62(1.09-2.41)**
VEGF activation 3 SNP LI_H [1]	---	---	436	2.69	1.19(0.89-1.59)	---	441	17.24	1.28(0.87-1.88)
IL6-174, *rs1800795*	38	Recessive	460	1.76	0.86(0.58-1.29)	Recessive	465	18.00	0.76(0.48-1.21)
IL6-572, *rs1800796*	6	Dominant	451	3.11	1.32(0.89-1.96)	Dominant	**456**	**22.58**	**1.71(1.10-2.66)**
IL6-597, *rs1800797*	37	Recessive	449	1.46	1.03(0.68-1.55)	Recessive	455	15.90	1.12(0.80-1.56)
IL6-6331, *rs10499563*	25	Dominant	462	1.61	1.16(0.59-2.28)	Recessive	467	15.94	0.89(0.64-1.24)
IL6R, *rs2228145*	39	Dominant	**461**	**5.96**	**0.66(0.46-0.96)**	Dominant	**466**	**19.20**	**0.62(0.40-0.94)**
IL6ST, *rs3729960*	16	Dominant	**461**	**4.26**	**1.27(0.94-1.72)**	Recessive	466	16.72	2.90(0.40-21.02)
IL6 activation LI_H [1]	---	---	**453**	**4.85**	**1.30(0.97-1.72)**	---	458	16.52	1.19(0.84-1.67)
IL6 expression risk [1]	---	---	456	2.60	1.18(0.89-1.56)	---	461	17.21	1.07(0.92-1.25)
IL6R signaling L_IH [1]	---	---	460	3.27	1.31(0.84-2.04)	---	465	14.67	1.13(0.68-1.88)
ADIPOQ+45, *rs2241766*	16	Recessive	**465**	**6.46**	**2.69(1.26-5.73)**	Recessive	**470**	**23.47**	**4.22(1.85-9.64)**
ADIPOQ +276, *rs1501299*	30	Recessive	**464**	**5.35**	**0.76(0.58-0.99)**	Recessive	**469**	**19.29**	**0.74(0.54-1.02)**

ADIPOQ -11426, *rs16861194*	9	Dominant	447	2.91	0.54(0.20-1.45)	Recessive	452	14.78	0.78(0.49-1.24)
ADIPOQ haplotype LI_H [1]	---	---	**461**	**1.51**	**1.51(1.10-2.06)**	---	466	17.53	1.29(0.87-1.90)
LEP-2548, *rs7799039*	39	Dominant	463	2.60	0.86(0.65-1.13)	Dominant	468	15.84	1.08(0.71-1.65)
*LEPR*109, *rs1137100*	21	Dominant	463	1.97	1.23(0.71-2.12)	Recessive	468	16.42	1.16(0.82-1.63)
*LEPR*223, *rs1137101*	42	Dominant	**462**	**4.13**	**1.27(0.95-1.71)**	Dominant	**467**	**19.31**	**1.42(0.98-2.05)**
*LEPR*656, *rs8179183*	19	Dominant	461	1.44	1.10(0.49-2.47)	Recessive	466	15.17	0.81(0.57-1.16)
LEP activation [1]	---	**---**	**460**	**4.42**	**0.50(0.20-1.21)**	---	465	13.93	0.84(0.37-1.90)
LEPR signaling LI_H [1]	---	---	460	2.45	1.18(0.86-1.60)	---	465	13.99	1.10(0.76-1.59)
PPARG, *rs1801282*	8	Recessive	461	1.70	0.68(0.25-1.85)	Recessive	466	16.23	0.58(0.18-1.86)
PGC1A, *rs8192678*	36	Recessive	460	1.68	1.02(0.77-1.34)	Recessive	465	15.21	1.22(0.75-2.00)
PPARD, *rs2016520*	23	Recessive	459	2.80	0.85(0.64-1.13)	Recessive	464	16.78	0.60(0.24-1.46)
PPARs risk alleles 0_123 [1]	---	---	457	1.25	1.02(0.79-1.31)	---	462	16.14	1.17(0.87-1.57)
OPN, *rs28357094*	26	Recessive	463	2.81	1.19(0.90-1.56)	Dominant	468	16.31	0.78(0.36-1.67)
IRS1, *rs1801278*	10	Recessive	452	2.56	0.68(0.28-1.65)	Recessive	456	10.84	0.62(0.23-1.69)
IGF1R, *rs2229765*	45	Recessive	459	1.94	0.90(0.64-1.25)	Recessive	464	15.62	0.93(0.63-1.39)
IGF signaling L_IH [1]	---	---	447	3.62	0.76(0.51-1.15)	---	451	11.48	0.71(0.44-1.14)
FGFR2, *rs2981582*	37	Recessive	459	2.93	1.20(0.84-1.71)	Recessive	464	15.70	0.77(0.48-1.23)
TNFA-308, *rs1800629*	19	Recessive	458	3.09	0.85(0.63-1.14)	Dominant	463	14.34	0.91(0.46-1.80)
TNFA-863, *rs1800630*	19	Dominant	**459**	**4.42**	**0.79(0.59-1.06)**	Dominant	464	15.92	0.78(0.55-1.10)
TNFRSF1A-329, *rs4149570*	39	Recessive	457	2.43	0.86(0.58-1.27)	Recessive	462	14.20	0.84(0.52-1.37)
TNFA activation LI_H [1]	---	**---**	**456**	**6.51**	**0.70(0.50-0.98)**	---	461	15.10	0.80(0.58-1.12)
TNFA expression L_IH [1]	---	**---**	**457**	**6.94**	**0.73(0.56-0.96)**	---	459	13.90	0.91(0.66-1.27)

Results are presented only for the best-fitting genetic model (based on the minor allele as dominant: aa + Aa genotype versus AA genotype, or recessive: aa genotype versus Aa + AA genotype) for each SNP or functional combination of SNPs by pathway (the model with the highest likelihood ratio was selected).[1] Functional combinations of SNPs in pathways are detailed in supplementary table 2. ADT, androgen deprivation therapy; No., number of subjects; MGF, minor genotype frequency in the cohort; LR, likelihood ratio; aHR (95%CI), age-adjusted hazard ratio and respective 95% confidence interval; SNP, single nucleotide polymorphism. *ADIPOQ*, adiponectin; *FGFR2*, fibroblast growth factor receptor 2; *IL6*, interleukin 6; *IL6R*, interleukin 6 receptor; *IL6ST*, interleukin 6 signal transducer; *IRS1*, insulin receptor substrate 1; *IGF1R*, insulin growth factor receptor 1; *KDR*, kinase insert domain receptor; *LEP*, leptin; *LEPR*, leptin receptor; *OPN*, osteopontin; *PPARD*, peroxisome proliferator-activated delta; *PPARG*, peroxisome proliferator-activated receptor gamma; *PGC1A*, peroxisome proliferator-activated receptor gamma coactivator 1; *TNFA*, tumoral necrosis factor alpha; *TNFRSF1A*, tumor necrosis factor receptor superfamily, member 1A; *VEGF*, vascular endothelial growth factor. Significant associations are in boldface.

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ORIGINAL ARTICLE

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

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-β_1) 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-β_1 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-β_1 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 *TGFB1* gene (*TGFB1+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-β_1 (in homozygous C).[20,21]

Genetic variants, which influence *EGF* and *TGFB1* expressions and protein serum levels, may impact PC development and prognosis. Our purpose was to investigate the combination of *EGF+61G>A* and *TGFB1+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 *TGFB1+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 *TGFB1* polymorphism in control group (*EGF+61G>A*: PC group, $P=0.082$, control group, $P=0.073$; *TGFB1+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 *TGFB1+869T>C* combined proliferation functional genetic profile

	Control group	*PC group*	*OR*	*95% CI*	P-*value*
Combined genetic profile					
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.

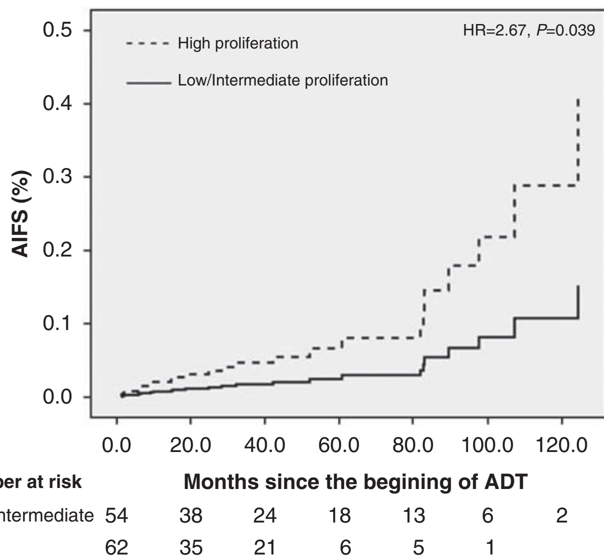

Figure 1 Androgen independence-free survival (AIFS) according to *EGF-TGFB1* combined proliferation genetic profile in patients submitted to ADT.

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 paradoxal 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 *TGFB1+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-allele[21] 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 *TGFB1* 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 inhibition[45] 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 Cooffey[48] 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 *TGFB1* 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 (T_1–T_{2b}), 37.9% with locally advanced disease (T_3–T_4) 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 TGFB1 +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-μl reaction mixture containing *EGF+61G>A* specific primers, PCR buffer 1×, Taq Polymerase 1 U, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM, DNA 100 ng. PCR products (242 bp) were incubated overnight with *Alu*I 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-β_1 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 ($\geqslant$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.

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