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Molecular Markers of
Epithelial to Mesenchymal Transition in Prostate Cancer

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Dissertation to a Master's Degree in Oncology

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Dissertation for applying to a Master's Degree in Oncology – Specialization in Molecular Oncology submitted to the Institute of Biomedical Sciences Abel Salazar-University of Porto

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Summary

Prostate cancer (PCa) is a leading cause of cancer-related morbidity and mortality worldwide and especially in developed countries. Clinically, PCa behavior varies from indolent, not requiring therapeutic intervention, to highly aggressive, entailing radical treatment. Current methods for stratifying PCa aggressiveness are mostly based on the Gleason score, serum PSA level and TNM stage, and these are unable to accurately predict tumor outcome in an individual basis. Thus, one of the current challenges in PCa management is the ability to discriminate indolent from aggressive tumors before treatment, avoiding overtreatment and the consequent harms.

Epithelial to mesenchymal transition (EMT) is a process that allows an epithelial cell to acquire a mesenchymal phenotype, and it has been considered a fundamental process for tumor invasion and metastization. Taking into account the important role of EMT in tumor progression, markers of this process might be used as indicators of tumor progression, allowing for a better assessment of tumor aggressiveness.

Hence, the main goal of this study was to evaluate the expression of EMT-related genes in PCa tissue samples and correlate these findings with standard clinical and pathological parameters, to unveil new prognostic markers that may improve characterization of PCa aggressiveness.

Using a RealTime ready Custom Panel 384 assay, 93 EMT-related genes were assessed in a series of normal prostatic tissues (n=5), stage pT2a+b-PCa (n=5) and stage pT3b-PCa (n=5), from which five candidate genes for EMT markers in PCa emerged: *CAMK2N1*, *CD44*, *KRT14*, *TGFβ3* and *WNT5A*.

Expression levels of the five genes were then validated in a larger independent series of tissue samples using quantitative RT-PCR. Globally, *CAMK2N1*, *CD44* and *WNT5A* displayed higher expression levels in higher stage and less differentiated PCa. These results suggest that *CAMK2N1*, *CD44* and *WNT5A* expression is associated with more aggressive forms of PCa, reflecting a more active transition to an EMT state by tumor cells. Further studies, including the evaluation of protein expression by immunohistochemistry, are, however, required to validate these preliminary findings in larger series of PCa patients and to correlate molecular findings with patient outcome.

Resumo

O cancro da próstata (CaP) é uma das principais causas de mortalidade e morbilidade relacionada com o cancro em todo o mundo e especialmente nos países desenvolvidos. Clinicamente, o comportamento do CaP pode variar de indolente, onde nenhum tratamento é necessário, a altamente agressivo, que implica tratamentos radicais. Os actuais métodos de estratificação destes tumores são baseados no score de Gleason, nível de PSA e estadio TNM, e não conseguem prever completamente a progressão da doença nem a agressividade do tumor. Assim, um dos atuais desafios na área do CaP é a discriminação entre tumores agressivos e indolentes antes dos pacientes iniciarem qualquer tipo de tratamento, para evitar que estes sejam expostos a tratamentos desnecessários e aos efeitos secundários associados.

A transição epitélio-mesenquimal (TEM) é um processo que permite à célula epitelial assumir um fenótipo mesenquimal e tem sido descrita como um processo fundamental para a invasão tumoral e metastização. Tendo em conta o importante papel da TEM na progressão tumoral, os seus marcadores poderão ser utilizados como marcadores de progressão tumoral, permitindo uma melhor avaliação a agressividade dos tumores.

O principal objectivo deste trabalho foi a avaliação da expressão de genes relacionados com a TEM em amostras de tecido de CaP e correlacionar os resultados com dados clínicos e patológicos, com a finalidade de encontrar novos marcadores de prognóstico que possam melhorar a caracterização da agressividade do CaP.

Utilizando *RealTime ready Custom Panel 384 assay* foi avaliada a expressão de 93 genes relacionados com a TEM em amostras de tecido prostático normal (n = 5), CaP estadio pT2a+b (n = 5) e CaP estadio pT3b (n = 5), a qual conduziu à selecção de cinco genes candidatos para marcadores de TEM em CaP: *CAMK2N1*, *CD44*, *KRT14*, *TGFβ3* e *WNT5A*.

Os níveis de expressão dos cinco genes foram de seguida avaliados numa série independente a alargada de amostras através de RT-PCR quantitativo. Globalmente, os genes *CAMK2N1*, *CD44* e *WNT5A* apresentaram níveis de expressão mais elevados nos estadios mais avançados e menos diferenciados de CaP. Estes resultados sugerem que a expressão de *CAMK2N1*, *CD44* e *WNT5A* está associada a formas mais agressivas de CaP, reflectindo uma maior actividade da TEM nas células tumorais. No entanto, são necessários mais estudos, como a avaliação da expressão proteica por imunohistoquimica, para validar estes resultados preliminares em séries mais alargadas de pacientes com CaP e correlacionar os resultados com a evolução da doença.

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Introduction

Prostate Cancer

Prostate Cancer Epidemiology

Prostate cancer (PCa) is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males, accounting for 14% of the total new cancer cases and 6% of the total cancer deaths in males in 2008 [1].

In Europe, the estimated incidence in 2008 was 370,733 new cases accounting for 11.6% of all diagnosed cancers (Figure 1) [2]. The same trend was reported for Portugal, in which the estimated incidence rate in the same year was of 5,140 new cases accounting for 11.9% of all cancers (Figure 1) [2].

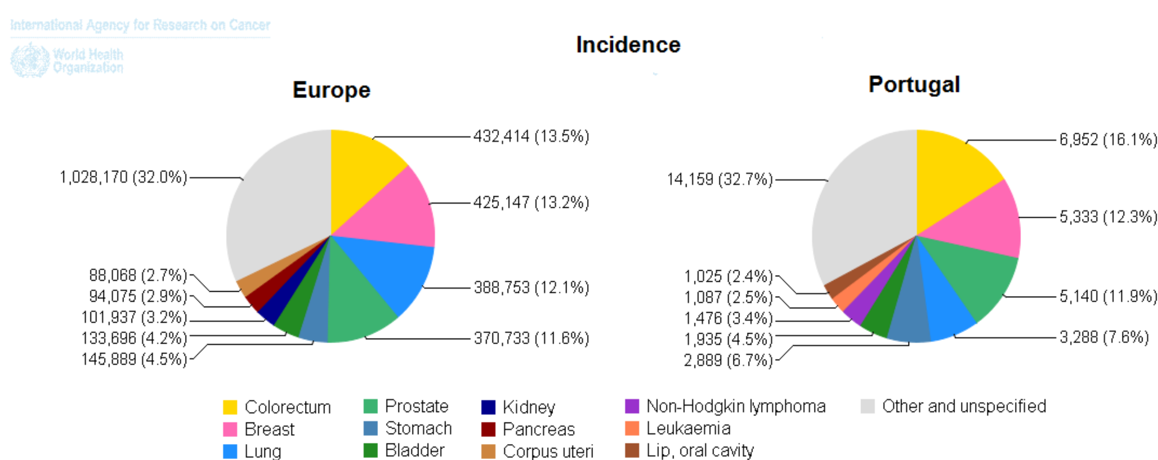

Figure 1- Estimated incidence of different types of cancer in Europe and Portugal in 2008, number of newly diagnosed cases and proportion of each cancer comparing to all types of cancer (in both genders and excluding non melanoma skin cancers). Prostate cancer is represented in lighter green. (Adapted from [2])

This cancer is one of the most age-dependent cancer, is rare before the age of 50 increasing exponentially thereafter [3].

Incidence rates of PCa vary more than 25-fold worldwide, with the highest rates recorded primarily in developed countries of Oceania, Europe, and North America, where PCa is the most frequent cancer and the third cause of cancer death in males (Figure 2) [1]. This variation is largely due to the wide utilization of prostate-specific antigen (PSA) testing in developed countries, that detects clinically important tumors as well as other slow-growing cancers that might otherwise escape diagnosis [1].

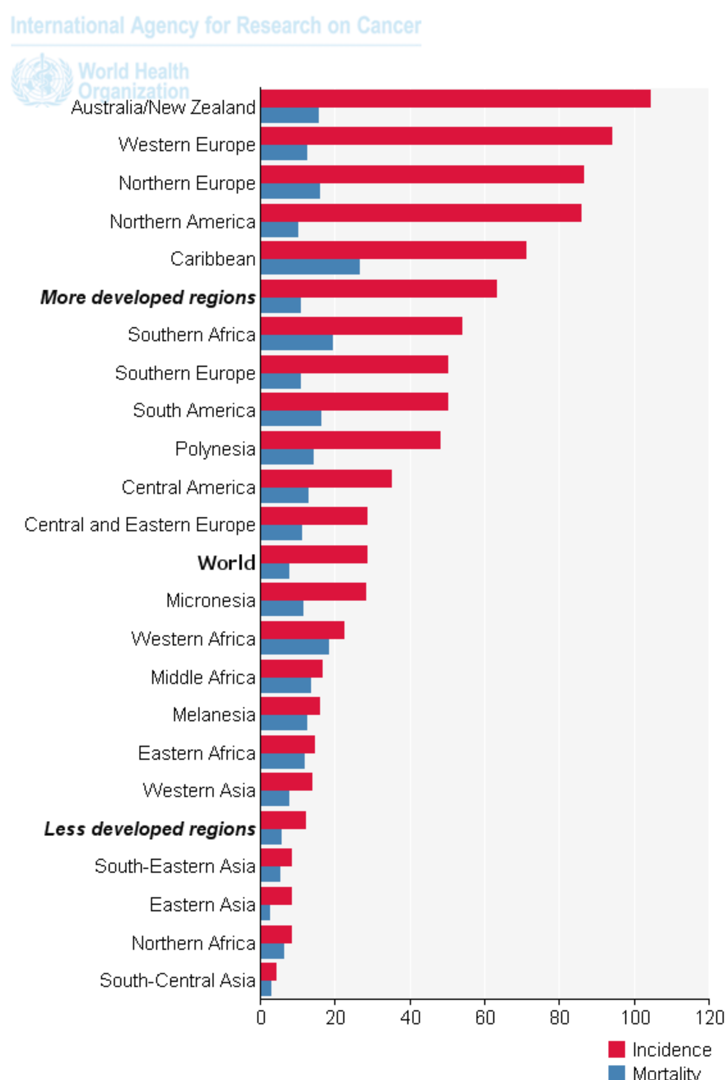

Figure 2- Estimated age-standardized rates of incidence and mortality for prostate cancer (World) per 100,000 [2].

Mortality rates differ much less, with a 10-fold variation between the countries [2]. PSA testing has a much greater effect on incidence than in mortality, leading to a less accentuated difference in mortality rates between developed and developing countries [2]. In contrast, African descent males in the Caribbean region have the highest mortality rates in the world, which is thought to reflect a genetic susceptibility of this group [4,5].

Incidence and mortality rates suffered some variations across time, mainly due to PSA testing. Before PSA testing implementation, PCa incidence was characterized by a high rates in North America, especially for Afro-America men, intermediate rates in Europe and low rates in Africa and Asia [3]. After the advent of PSA, the incidence of PCa in developed countries remarkably increased, and then fell, but to higher levels than previously [3]. Mortality rates have decreased but that changes can be described as modest [3].

Prostate Cancer Precursor Lesions

Several lesions have been proposed as precursors of PCa. Adenosis, also known as atypical adenomatous hyperplasia (AAH), has been reported, by some authors, as a precursor to low-grade transition of PCa, do to some morphological similarities between the two lesions [6]. Although, a long-term prospective study showed that men with adenosis are not at increased risk for developing PCa [7]. Another lesion that has been associated with the development of PCa is proliferative inflammatory atrophy (PIA). In fact, there is some molecular and epidemiological as well as some morphological, evidence that long-standing PIA may predispose to PCa [8]. However, PIA is a lesion frequently identified on biopsy material in the absence of carcinoma and on follow-up has not been associated with increased risk of cancer [9]. Therefore, adenosis and PIA should not be considered, at present, as direct precursors lesions of PCa.

Currently, the most well characterized and well established precursor lesion of PCa is prostatic intraepithelial neoplasia (PIN). PIN consists of architecturally benign prostatic acini lined by cytologically atypical cells, and it is a pathological entity that ranges from low- to high-grade (LGPIN and HGPIN) [6]. There is epidemiological, morphological, and molecular evidence that HGPIN is a precursor lesion to PCa [6]. A fact that strongly supports the association between HGPIN and PCa, is that the prevalence of both HGPIN and PCa increases with patients' age and HGPIN precedes the onset of PCa by a decade, approximately [10]. An increased incidence, size and number of HGPIN foci have been observed in prostates with carcinoma when compared to prostates without carcinoma [10]. Furthermore, several studies have reported that HGPIN, like prostate cancer, occurs most frequently in the peripheral zone of the prostate [11]. Genetic similarities between these two lesions have also been found, including the loss of chromosome 8p and gains of 8q, two of the most frequent genetic alterations occurring both in PCa and HGPIN [6]. However, there is evidence that not all PCa arise from HGPIN. The majority of prostates with early carcinomas lack any HGPIN [6]. Moreover, low-grade carcinomas, especially those present within the transition zone are not closely related to HGPIN [6]. Therefore, it appears that HGPIN might be a precursor lesion to many, but not all, prostate adenocarcinomas.

Prostate Cancer Diagnosis

Screening programs based on PSA testing combined with digital rectal examination (DRE) have been introduced in some parts of the world, especially in developed countries, with main goal of detect early PCa, because only organ confined disease can be cured by treatment [12]. However, diagnosis can only be defined based on examination of prostate samples [12].

The current standard method for detection of prostate cancer is by transrectal ultrasound (TRUS) guided core biopsies [13]. The need for prostate biopsies should be determined based on PSA level and/or suspicious DRE, concerning patient's age, potential co-morbidities and therapeutic consequences [13]. A positive PSA test, indicative for biopsy, is usually defined as a value greater than 3 to 4 ng/mL [14,15].

The collected samples should be as far as posterior and lateral in the peripheral gland as possible [13]. For a glandular volume of 30-40 ml, 8 to 12 cores are recommended and additional cores from suspect areas by DRE/TRUS should be obtained [13].

The PCa diagnosis is based on histological examination of the collected samples [16]. From this analysis, proportion of tumor involvement of the biopsies, number of positive biopsies, Gleason score as well as extraprostatic extension, if present, should be reported [13,16]. Ancillary techniques, like histochemistry and immunohistochemistry , as well as additional sections of the samples should be considered if a suspected glandular lesion is identified [16].

After diagnosis, based on biopsies evaluation, further diagnostic or staging procedures should be used if they might affect the treatment decision [13].

Histopathological Evaluation of Prostate Cancer

Gleason Score

Gleason grading system was firstly described in 1966 by Donald F. Gleason and at the present, with some changes, it is the most commonly grading system used for PCa, being recommended by Word Health Organization (WHO) [17,18]. This grading system is based on microscopic assessment of glandular architecture, without consideration of nuclear morphology [19]. It recognizes five basic patterns with decreasing of glandular differentiation (Figure 3).

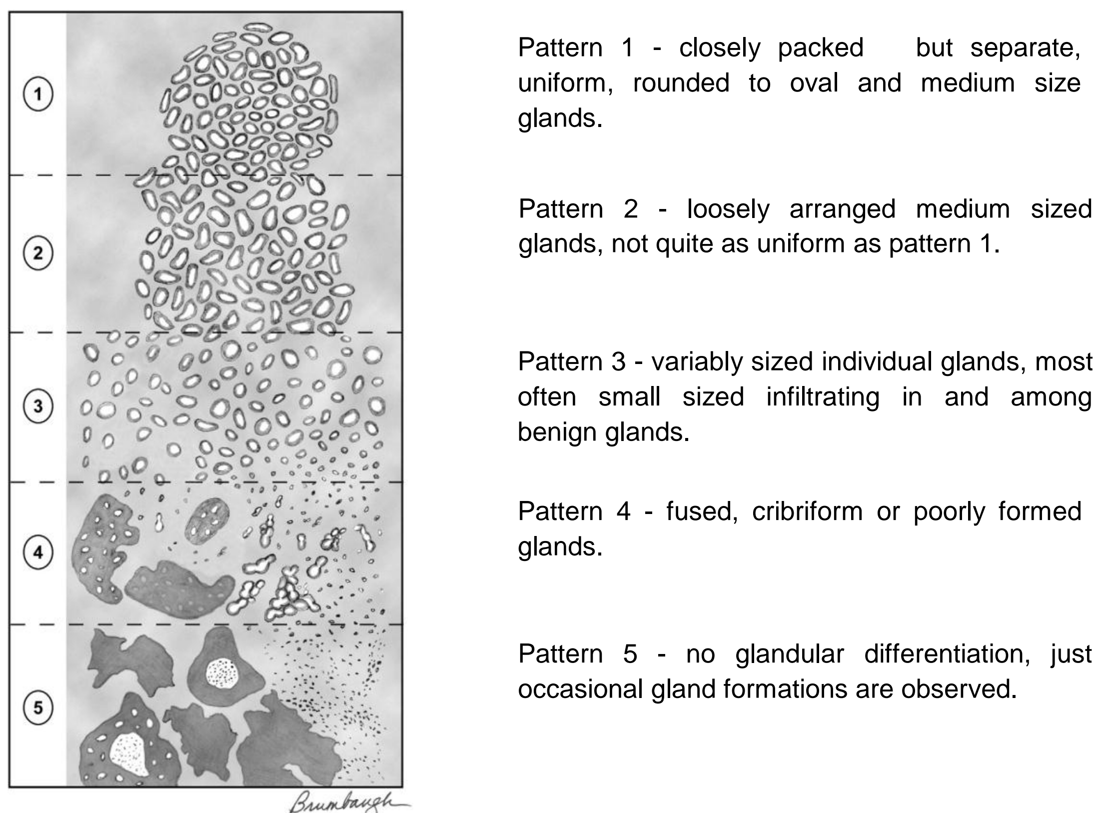

Figure 3- Updated Gleason grading system for histological grading of prostate cancer. (Adapted from [17])

Taking into account the heterogeneity of prostate cancer, more than one Gleason pattern might be present in the same tumor [19]. Due to this heterogeneity, the Gleason grading is made by a score resulting from the sum of the two predominant patterns in the tumor [19]. If the tumor is composed only for one pattern, the Gleason score is obtained by doubling the numerical value of this pattern [19].

The heterogeneity of prostate cancer also affects the biopsy evaluation, because all patterns present in the tumor might not be represented in biopsy cores. A Gleason score between 2 and 4 should not be assigned in biopsy samples, due to the risk of under-grading the tumor [17]. To decrease the frequency of under-grading, the present rules to assess Gleason score depends on the nature of the evaluated sample [17].

In clinical practice, Gleason score, along with pathological staging (pTNM staging), is one of the most powerful prognostic factors [19].

Clinical and Pathological Staging

The TNM system is the most important global classification in oncology and is used for staging tumors. In TNM system, the extent of the primary tumor (T-category), regional lymph nodes involvement (N-category) and distant metastasis (M-category) are

evaluated [20]. Clinical TNM (cTNM) staging is based on information that is available without surgery, such as clinical examination, imaging modalities and laboratory tests [20]. Pathological TNM staging (pTNM) is based on histological examination of the cancer resection specimen [20].

This staging system has been adopted by International Union Against Cancer (UICC)[20]. The current TNM staging system for PCa is represented in Table 1.

Concerning that disease anatomic extent is probably the most important prognostic factor in oncology, TNM staging is a powerful prognostic factor [20]. As already mentioned, Gleason score is also a powerful prognostic factor in PCa, and these two factors together have a huge impact in treatment and prognosis. Indeed, there is already evidence that TNM staging and Gleason score have independent prognostic value [20].

Table 1- TNM staging system for prostate cancer. (Adapted from [21])

T- Primary tumor	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent tumor, not palpable or visible by imaging
T1a	Tumor incidental histological finding in 5% of tissue resected during TUR
T1b	Tumor incidental histological finding in more 5% of tissue resected during TUR
T1c	Tumor identified by needle biopsy
T2	Confined within the prostate
T2a	Tumor involves half of the lobe or less
T2b	Tumor involves more than one half of one lobe, but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostate capsule but has not spread to other organs
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles
T4a	Tumor invades bladder neck and/or external sphincter and/or rectum
T4b	Tumor invades levator muscles and/or is fixed to pelvic wall
N- Regional lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph nodes metastasis
N1	Regional lymph node metastasis
M- Distante metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Metastasis at other site(s)

Abbreviations: TUR- transurethral resection

Treatment

Therapeutic management of PCa has become far more complex because of the availability of various stage-specific therapeutic options namely active surveillance, surgery (radical prostatectomy), radiotherapy and hormonal therapy [22]. Despite all therapeutic options available, metastatic disease remains without curative treatment [12].

Active surveillance consists in a periodic monitoring with PSA tests, physical examinations and repeated prostate biopsy, which is converted to a potentially curative treatment at the sign of disease progression, and should be applied in patients with low-risk PCa (PSA<10ng/mL, Gleason score <7 and stage cT1c to cT2a) [13,23]. This therapeutic option was conceived with the aim to reduce the overtreatment of patients with low-risk PCa because this tumors usually have a very indolent course even when left without treatment [24]. A recent study demonstrated that men with low-risk PCa and with a life expectancy over 10 years are good candidates for active surveillance, and only about 30% of these men will require delayed radical intervention [25].

The surgery intervention, radical prostatectomy, is the standard curative treatment for PCa, and is the only treatment for localized PCa that proved to reduce mortality and metastasis risk when compared to active surveillance [26]. However, for a follow-up over 10 years, a little or no further increase of benefit has been shown for surgery over active surveillance [26,27]. Although, for the subgroup of patients with high-risk PCa (PSA>20 or Gleason score 8 to 10 or stage≥cT3a) has been suggested that surgery might reduce mortality even for a follow-up above 10 years [13,27].

External beam-radiotherapy and brachytherapy are considered alternative therapeutical options to radical prostatectomy in patients with disease-free survival rates similar to those of surgical procedure [28]. External beam-radiotherapy is also often applied for treatment of patients with locally advanced disease and with local failure after prostatectomy [12]. Excellent outcomes have been reported for treatment of localized PCa with brachytherapy and it has been also suggested as a viable primary treatment option for patients with low, intermediate and high-risk localized prostate cancer [29].

Being prostate a hormone-responsive organ, hormonal therapy (i.e. androgen deprivation) is another therapeutic option available, and it has been the mainstay for management of patients with metastatic disease [12]. Although hormonal therapy is applied with palliative intent, it might have an important role in mortality impact by delaying death from PCa [12]. Due to the hormonal therapy most tumors acquire a hormone-refractory phenotype, and for these patients the only available option is conventional quimiotherapy, which effectiveness still limited [12]. Hormonal therapy may also be

applied combined with radiotherapy for treatment of localized or locally advanced disease, in order to improve the outcome [12].

Harms and Benefits of PSA Testing

Currently PCa screening is based on measurement of serum PSA levels, but other methods of detection, such as digital rectal examination and ultrasunography, are often included in screening programs [23].

The goal of PCa screening is to reduce deaths due to PCa, increasing length of life, and additionally reduce the development of symptomatic metastatic disease [23].

There is convincing evidence that PSA-based screening programs result in a detection of many cases of asymptomatic PCa [23]. However, the majority of cancer detected by screening will never cause symptoms or morbidity and most cancer deaths from PCa will not be avoided by screening [30] For these reasons, PSA screening has been associated with an “over-diagnosis” [23]. The magnitude of over-diagnosis caused by PSA screening is difficult to determine, but two largest trials suggest an over-diagnosis rate of 17% to 50% [31].

Men with screen-detected cancer can potentially fall into one of the three categories [30]:

- Those whose cancer will result in death despite early diagnosis and treatment;
- Those who will have good outcomes in absence of treatment;
- Those whom early diagnosis and treatment improves survival.

The reduction of PCa specific death after screening implementation is, rather small. Two of the largest trials of PSA screening, U.S. PLCO (Prostate, Lung, Colorectal and Ovarian) and ERSPC (European Randomized Study of Screening for Prostate Cancer), aimed to evaluate PSA screening impact in PCa mortality. The USA trial did not demonstrate any PCa mortality reduction [32]. The European trial found a reduction in prostate cancer deaths of approximately 1 death per 1000 men screened [33].

PSA test has been also associated with false-positive results, indeed approximately 80% of positive PSA test results are false-positive when cutoffs between 2.5 and 4.0ng/mL are used [15]. False-positive results have been associated with negative psychological effects, including persistent worry about PCa, and with frequent exposure to additional testing, including one or more biopsies in the following years [34]. About one third of the men submitted to prostate biopsy, have experienced pain, fever, bleeding, infection and transient urinary difficulties, considering these effects as a “moderate or major problem” [35].

The vast majority (about 90%) of men with PSA-detected PCa are early treated by surgery, radiation or androgen deprivation therapy [36]. Prostatectomy and radiotherapy often cause long-term adverse effects, including urinary incontinence and erectile dysfunction, in at least 200 to 300 of 1000 men treated [37]. Prostatectomy have been also associated with perioperative mortality (up to 5 in 1000 men will die within 1 month after surgery) and radiotherapy have also been associated with bowel dysfunction [37]. Conversely, androgen deprivation therapy is associated with erectile dysfunction as well as gynecomastia and hot flashes [37].

Concerning over-diagnosis associated with PSA screening, man with cancer that would remain asymptomatic cannot benefit from screening or treatment. An active surveillance, as an alternative of curative treatment is an available option that reduces the effects associated with others treatments.However, both physicians and patients tend to elect treatments with curative intent, given to the current inability to distinguish tumors that will remain indolent from those that will be lethal [38]. Thus, a huge number of men harboring PCa that will never become symptomatic are being subjected to the harms of treatment.

Discrimination between Indolent and Aggressive Disease - A major challenge in prostate cancer management

PCa behavior can vary from indolent, who do not require treatment, to aggressive, where radical treatment is required [23]. The current challenge of managing localized PCa is to distinguish patients with aggressive tumors from those which tumor do not need any intervention. The absence of prognostic factors that could fully predict PCa behavior has led to a significant over-treatment of patients who would otherwise require only conservative management, exposing then to treatments associated morbidity. Indolent PCa refers to a cancer that would never exhibit clinical manifestation according to its pathologic features, regardless lifespan of the patient [39]. Currently, from indolent tumors, only a proportion of these can be classified as been insignificant PCa, taking into account patients' age and possible comorbidities [39]. The concept of insignificant PCa is well established as low-grade, small volume and organ confined PCa that is unlikely to progress to clinical and biologic significance [39]. In clinical practice, insignificant PCa is a cancer diagnosed in the absence of related symptoms that would not cause mortality or morbidity during patients life if left without treatment [39]. To date, the most widely used preoperative criteria for predicting insignificant PCa after positive biopsies and before surgery are the Epstein criteria. These criteria have emerged in 1994 and in 2004 an

update was reported [40]. The updated Epstein criteria to identify insignificant PCa consist:

- PSA density ≤0,15 ng/mL per gram;
- Gleason score ≤6;
- Fewer than three positive cores;
- <50% of cancer involvement in any core.

Although Epstein criteria area useful in insignificant PCa predicting, they might lead to a misclassification of about 30% of the patients, who would have unfavorable pathological features in prostatectomy specimen [41,42]. Nevertheless, Epstein criteria seem to be highly predictive for favorable disease that is cured by surgery, because just a small percentage of patients considered to harbor an insignificant PCa demonstrated extraprostatic extension in prostatectomy specimens [41,42]. However, predicting favorable disease at prostatectomy should not be the main end point of interest, since the major goal is to avoid surgery or other treatments, protecting patients from associated morbidity. Several preoperative predictive models have emerged in an attempt to better identify insignificant PCa. However, the main nomograms reported and validated are just slightly superior to Epstein criteria in their ability to predict insignificant PCa, remaining a significant number of patients under-staged [39].

Hence, to date the current classification of insignificant PCa does not perfectly predict the biologic behavior of cancer, and indolent tumors out of this subgroup do not have any predictive criteria. Furthermore, there is a need to find more precise and replicated tools to better predict the indolent behave of PCa. This challenge in PCa management might be addressed by better understanding of the molecular basis of cancer progression.

Epithelial to mesenchymal transition (EMT) is a process that has been associated with tumor aggressiveness, playing a central role in tumor invasion and metastasis [43]. Thus, molecular markers of EMT can be used as markers of tumor progression in PCa, allowing to a better characterization of PCa aggressiveness.

Epithelial to Mesenchymal Transition

What is Epithelial to Mesenchymal Transition?

Epithelial to mesenchymal transition (EMT) is a biologic process that allows an epithelial cell to undergo multiple biochemical changes enabling it to acquire a mesenchymal phenotype, enhancing migratory capacity, invasiveness, resistance to apoptosis, and augmenting the production of extracellular matrix (ECM) components (Figure 4) [44].

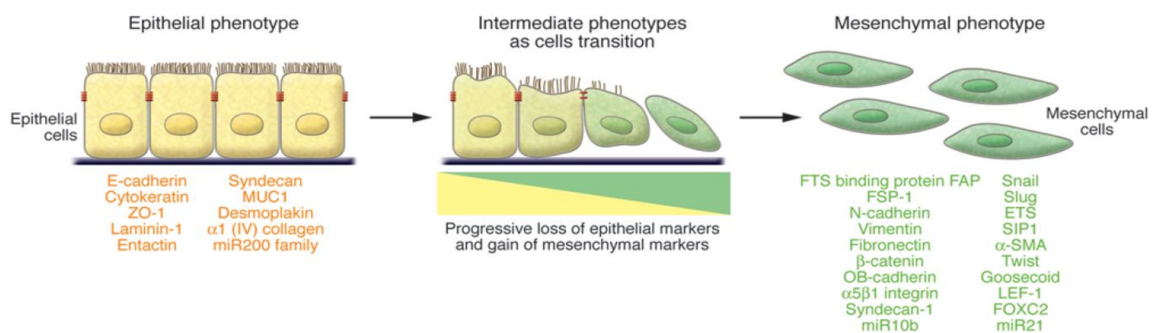

Figure 4- Molecular and phenotypic changes in epithelial cells during EMT. EMT involves a functional transition of a polarized epithelial cell into a mobile and ECM component secreting mesenchymal cell. During this process epithelial cells progressively loose expression of epithelial markers and gain expression of mesenchymal markers, while alterations in cell shape are also observed [43].

During this transition, epithelial cells progressively lose expression of typical epithelial markers, such as E-cadherin and keratins, and gain expression of mesenchymal markers, including vimentin, α-smooth muscle actin (SMA), and N-cadherin [43,44]. EMT is completed when the underlying basement membrane is degraded, allowing for mesenchymal cells to migrate away from the original epithelial layer [43]. Importantly, the mesenchymal phenotype acquired by EMT is not an irreversible stage, and a mesenchymal to epithelial transition (MET) is likely to occur, reconstituting the original epithelial phenotype of the cell [43].

Distinct molecular processes are associated with EMT and these include activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, production of ECM degrading enzymes, and altered expression of specific microRNAs [43].

Although a common set of genetic and biochemical elements underlie EMT, this process may occur in three distinct biological contexts being classified accordingly [43]:

- Type 1: occurring during implantation, embryogenesis and organ development;
- Type 2: associated with tissue regeneration and organ fibrosis;
- Type 3: associated with cancer progression and metastasis.

Briefly, EMT type 1 can generate mesenchymal cells that have potential to undergo MET and form secondary epithelia, and it is not associated with an invasive phenotype which would result in systemic spread via the circulation [43]. On the other hand, EMT type 2 acts as a part of the repair system and normally generates fibroblasts and other related cells deemed to reconstruct tissues following trauma and inflammatory injury [43]. This type of EMT is associated with inflammation and ceases when inflammation is attenuated [43]. In a situation of persistent inflammation, EMT type 2 continues to respond to ongoing inflammatory stimuli, generating fibrosis and eventually leads to organ destruction [43]. Finally, EMT type 3 occurs in cancer cells and endows the capacity to invade and metastasize [43].

EMT and Cancer Progression

The majority of human solid tumors are of epithelial origin, *i.e.*, carcinomas [45]. In these tumors, invasion of the basement membrane is thought to be one of the last stages of tumor progression, allowing for subsequent metastatic dissemination with life-threatening consequences [43]. The mechanisms underlying the acquisition of the invasive phenotype and the ensuing systemic spread of the cancer cells have been object of intensive research. Thus, EMT has been reported as a critical mechanism for invasion and metastasis formation in carcinomas [46]. Studies with animal models and cell cultures have demonstrated that mesenchymal cancer cells are typically seen at the invasive front of primary tumors and these are the cells that eventually enter into subsequent steps of invasion and metastization [45]. According to this model, EMT provides mobility to cancer cells and the capacity to invade the tissues and organs surrounding the tumor, and also to enter the circulation (Figure 5) [43]. After intravasation, these cancer cells are transported through the circulation and can leave the blood stream at a remote site where they may form metastasis [43].

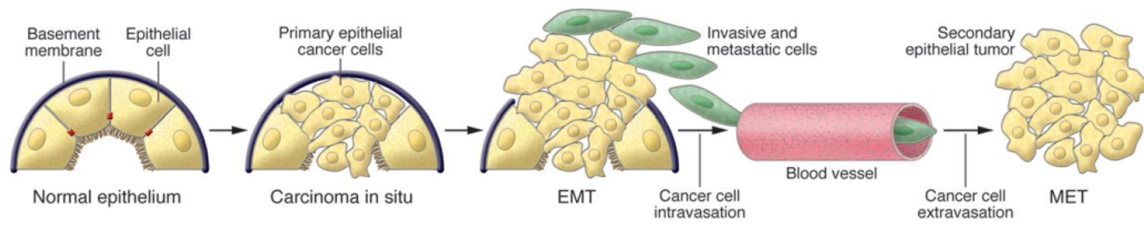

Figure 5- Contribution of EMT to cancer progression: evolution from carcinoma *in situ* to invasive carcinoma and then to metastatic disease, through EMT and MET. The invasive carcinoma stage involves acquisition of mobility by epithelial cancer cells, which may be provided by EMT. EMT endows cancer cells with mobility and capacity to invade the tissues and organs surrounding the tumor. The EMT transformed cancer cells also have the capacity to enter the circulation and exit the blood stream at a remote site, where they may form metastases. Metastasis formation may involve the reversion to the epithelial phenotype, giving rise to a secondary epithelial tumor similar to the primary tumor, through the process of MET [43].

Histologically, metastasis usually resemble the primary tumor from which cells arose, thus metastatic cells no longer exhibit the mesenchymal phenotype [43]. This fact indicates that metastasizing cancer cells lose their mesenchymal phenotype during metastasis formation, probably via MET [45]. The induction of MET in disseminated cancer cells likely reflects the microenvironment which they encounter after extravasation [46]. Therefore, EMT seems to be an important mechanism for carcinoma progression to a metastatic stage and the opposite process, MET, is required for subsequent metastasis formation.

Inducing Signals of EMT

The regulation of EMT in cancer cells remains unclear. Genetic and epigenetic alterations undergone by cancer cells during tumorigenesis seem to render them especially responsive to EMT-inducing heterotypic signals originating in the tumor-associated stroma [43]. These EMT-inducing signals activate EMT-signaling pathways, including transforming growth factor beta (TGFβ), nuclear factor kappa B (NFkB), wingless type (Wnt), and Notch which target transcription factors, notably Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), zinc finger E-box binding homeobox 2 (ZEB2), Twist and others (Figure 6) [43,46,47]. Once expressed and activated, each of these transcriptional factors can control the complex EMT program, often coupled with other transcriptional factors [43].

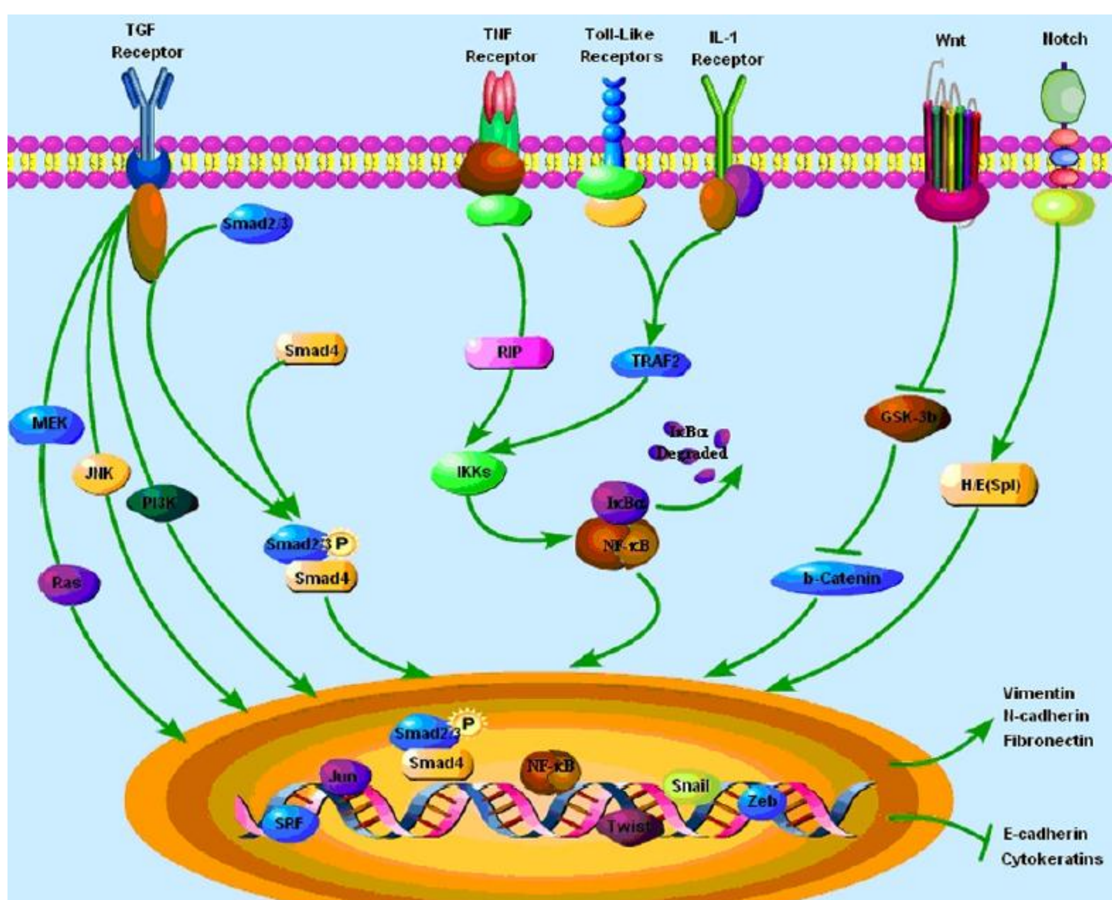

Figure 6 - Signaling pathways involved in regulation of EMT during tumor progression. EMT induction in tumor microenvironment is a complex phenomenon that remains unclear. Therefore, the proposed EMT-related signaling pathways are diverse, including TGFβ (through Smad-dependent and Smad-independent transcriptional pathways), activation of NF-kB signaling, Wnt/β-catenin and Notch signaling pathway, among others. These signaling pathways target transcription factors, such as Snail, Slug, ZEB1, ZEB2 and Twist, that once expressed and activated can control the complex EMT program, often coupled with other transcriptional factors [48].

Currently, the best studied EMT induction pathway involves TGFβ signaling, which is a suppressor of epithelial cell proliferation and has an important role in tumorigenesis [49]. However, it is now clear that TGFβ can also act as a positive regulator of tumor progression and metastasis [49,50]. Genetic and epigenetic mechanisms work in concert to convert TGF-β from a tumor suppressor to a promoter of growth, survival, and metastization [50].

TGFβ induces EMT in cancer cells through Smad-dependent and Smad-independent transcriptional pathways [43]. In Smad-dependent pathway, the binding of TGFβ results in the formation of TGF-β receptor type I and II tight complexes, which lead

to phosphorylation of Smad2 and Smad3 [51]. Phosphorilated Smads form heterodimeric complexes with Smad4 and translocate into the nucleus, where they control the transcription of target genes related with EMT, through interaction with specific binding motifs in their gene regulatory regions, such as Snail, ZEB, Slug and Twist [52]. In Smad-independent pathway, TGFβ directly activate various types of non-Smad signaling pathways that control the transcription of EMT target genes, such as MEK/Ras/Erk, c-Jun N-terminal kinase (JNK) and phosphatidylinsitol-3 kinase (PI3K) [53].

Another signaling pathway that induces EMT involves NFkB. NF-kB proteins are usually sequestered in the cytoplasm in a complex with an inhibitor protein, named IkB [54]. The activation of NF-kB is, in most cases, proceeded by the activation of an IkB kinase (IKK) complex, wich phosphorylates IkB leading to its degradation and consequently to activation of NF-kB [54]. The increased expression of inflammatory cytokines (TNF-α, ILs, LPS) in the tumor microenvironment, activates IKKs and consequently NFkB pathway [48]. This pathway directly activates the expression of EMT inducers, including Snail, ZEB1 and ZEB2 factors [54]. Since NFkB signaling can induce EMT in cells unresponsive to TGFβ, the cooperation between TGFβ and NFkB signaling pathway may be critical for EMT induction in some cases [55].

Wnt/β-catenin and Notch signaling pathways are also important for EMT induction. When the Wnt signaling pathway is activated, β-catenin is moved to the nucleus, and such accumulation is often associated with loss of E-cadherin expression and with susceptibility to enter in EMT [46]. Concerning the Notch signaling pathway, it has been reported that Slug is a direct target of this pathway, which is one of the EMT transcription factors [56]. These two signaling pathways contribute to EMT induction and have also been reported as important for the maintenance of the stemness of cancer stem cells [57,58]. TGF-β and Wnt signaling pathways may collaborate to induce EMT and thereafter function in an autocrine fashion to sustain the resulting phenotype [59].

Epigenetic mechanisms seem also to be also involved in EMT regulation. These mechanisms, unlike mutations, can be reversed, which is consistent with the reversible nature of EMT. Indeed, an epigenetic regulation of E-cadherin during EMT and MET has been already suggested [60]. Noncoding microRNAs have been reported as components of the cellular signaling pathway that regulates EMT. MicroRNA-200 (miR-200) inhibits the expression of ZEB1 and ZEB2, resulting in an increase of E-cadherin expression, thereby contributing to the maintenance of the epithelial phenotype [61,62]. In breast carcinoma, loss of miR-200 correlates both with increased vimentin and decreased E-cadherin expression [62]. MicroRNA-21 has the opposite role and is up regulated in many cancers, facilitating TGF-β-induced EMT [63].

EMT and Cancer Stem Cells

Cancer stem cells (CSCs) are often compared with normal stem cells, which are multipotent or pluripotent progenitor cells that can self-renew and endure asymmetric cell division, to give rise to differentiated or committed progenitors [64]. CSCs are cancer cells that exhibit a stem cell–like phenotype and which are capable to reconstruct the heterogeneity of the originating tumor [65].

The first evidence of CSCs came in 1997 through studies on acute myeloid leukemia, where a subpopulation of CD34+/CD38- cells with the ability to initiate tumor growth was identified [66]. More recent studies have also identified CSCs in brain tumors (CD113+)[67], breast cancer (CD44highCD24lowESA+)[68], oral squamous cell carcinoma (CD44high)[69], pancreatic cancer (CD44+CD24+ESA+)[70], prostate cancer (CD44+)[71] and in many other carcinomas.

Due to those characteristics and also to the fact that metastases often recapitulate the organization of the primary tumor, it is believed that CSCs in association to EMT play a major role in tumor dissemination [72].

As previously mentioned, a decisive step in tumor progression is the induction of EMT in tumor cells, including CSCs. It has been suggested that CSCs undergo EMT, acquiring the capacity to migrate out of primary tumor and colonize distant sites, where they may undergo MET to establish metastases with the same features of primary tumor (

Figure 7) [72]. Therefore, according to this model, CSCs play a central role in this process, being the main responsible for tumor metastization, which may explain the similarity observed between primary tumor and respective metastases.

Supporting this model, recent studies have suggested that CSCs are capable to undergo EMT and the reverse process MET, switching between a mesenchymal and an epithelial phenotype [73]. However, not all cancer cells exhibiting EMT features are cancer stem cells, as some cells that undergo EMT do not have the ability to reverse the process through MET and become fixed in a mesenchymal phenotype, representing a more differentiated EMT stage [73]. The role of this population of cells in tumor progression is unknown, but it is possible that they may make an important contribution to the tumor stroma [74]. Studies have also demonstrated that metastatic cancer cells, that presumably underwent EMT, exhibit a CSC phenotype [68,75].

Some authors provide a different interpretation of the CSCs / EMT connection, associating EMT with induction of a CSCs phenotype in cancer cells [65]. This association is supported by the fact that Wnt and Noch pathways, which are involved in EMT regulation, also drive both normal and CSC renewal and maintenance [57,58].

As well as being implicated in metastasis formation, CSCs that have undergone

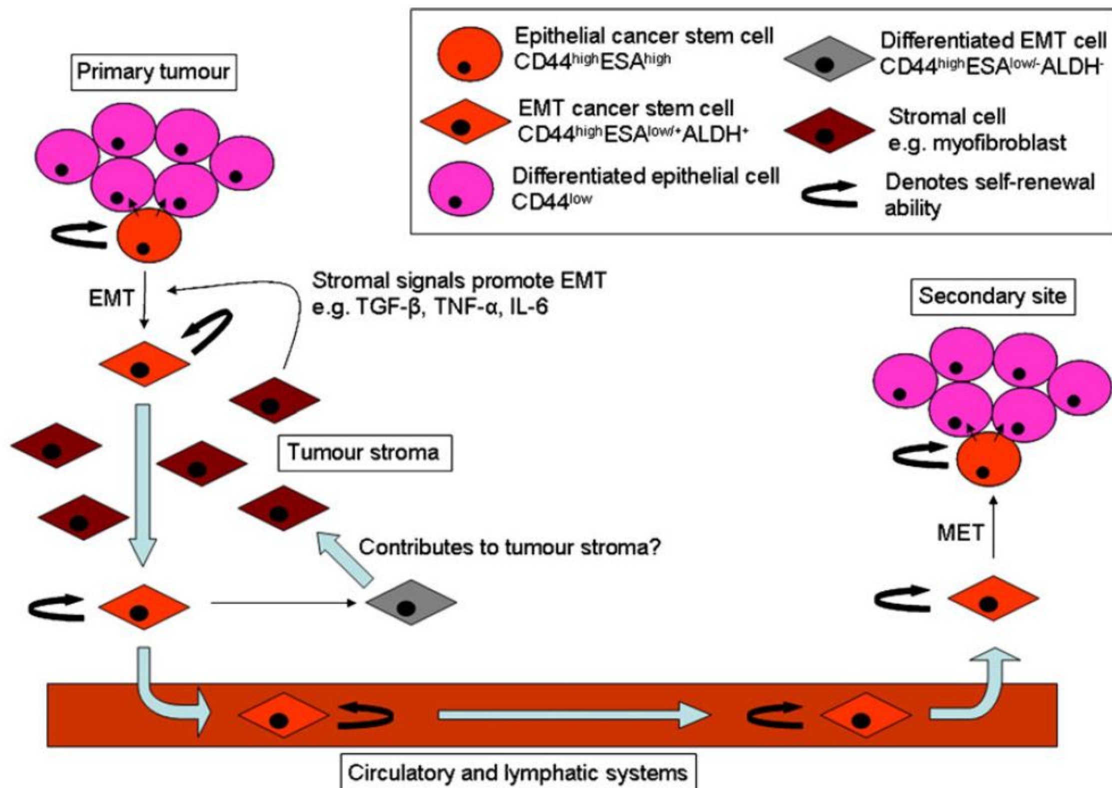

Figure 7- Metastasis formation involving CSCs: a model of metastatic dissemination with a central role for CSCs is depicted. CSCs can undergo EMT, acquiring the capacity to migrate out of the primary tumor and reach the circulatory and lymphatic systems, through which they can travel to distant sites. Once arriving at the distant sites, these cells can undergo MET to resume their proliferative phenotype and produce a metastasis. A sub-population of EMT-CSCs do not have the ability to undergo MET and become fixed in a mesenchymal phenotype. The role of these cells is unknown, but it is possible that they may contribute to tumor stroma [74].

EMT are also associated with therapeutic resistance. Cancer therapies are usually capable to kill bulk tumor cells but often spare CSCs, which are then responsible for tumor recurrence after an apparently successful therapy [74]. Clinically, recurrence often results in a more aggressive tumor, possibly due to a higher content of CSCs [74]. The mechanism underlying the therapeutic resistance of CSCs is not fully understood. However, drug efflux transporters of the ABC family have been implicated in therapeutic resistance of CSCs [74]. Therefore, CSCs that have undergone EMT are now an important focus of research aimed at preventing tumor metastasis and recurrence.

Evidence of EMT in Prostate Cancer Progression

Over the last years, EMT has been an area of intensive research in PCa, and one of the most controversial aspects is the extent to which one can find evidence of EMT in human pathological specimens [76]. In PCa, the Gleason score might be viewed as morphological evidence of EMT. An increasing Gleason score is associated with a progressive loss of epithelial glandular architecture, including loss of defined basement membrane and cell polarity, and with augmented invasive patterns, like cell cords, sheets or individual cells invading tumor stroma [77]. Further evidence of EMT is delivered from analysis of molecular markers of this process. Concerning the metastasis model previously mentioned, changes in the expression of EMT-associated genes should be evident in primary tumors and the regression of these alterations must be evident in metastatic lesions.

Numerous studies have been performed to evaluate the expression levels of E-cadherin in PCa specimens. A decreased of E-cadherin expression correlates with higher Gleason score and more advanced pathologic stage [78,79]. Regarding metastasis, some studies report a decrease of E-cadherin expression in bone and lymph node metastasis and others clearly show unchanged expression [78,79]. Concerning an epigenetic regulation for E-cadherin expression, a study using PCa specimens reported that methylation of the E-cadherin gene is common in prostate cancer and that the density of E-cadherin promoter methylation correlates with tumor progression [60]. In PCa cell lines, derived from bone metastasis, an unmethylated state of E-cadherin was found, corresponding to a re-expression of the protein [80]. Despite these results, definitive molecular evidence of MET in PCa metastases has not emerged yet.

In contrast to E-cadherin, markers of mesenchymal phenotype, like N-cadherin, are up-regulated in more aggressive tumors. A recent study showed that N-cadherin is up-regulated in castration-resistant PCa, both in animal models and in human specimens, and it is also associated with metastatic disease and higher Gleason score [81]. The up-regulation of this cadherin has been also reported as a major cause of PCa metastasis formation and castration resistance [82].

Transcriptional regulators of EMT have drawn most of the attention because they are arguably the best diagnostic markers of EMT to date [83]. Much of what has been reported about EMT in prostate cancer, as well in other cancers, is based on studies using *in vitro* and *in vivo* models. TGFβ is an inducer of EMT and has been one of the most studied EMT pathways. The capacity to induce EMT-like states in PCa cells lines has been shown by a number of research teams. TGFβ induced EMT in PCA cells lines is mediated by NFκB, which accumulates in the nucleus, inducing vimentin expression [84].

In tissue samples, the expression of TGFβ, NFκB and vimentin has been correlated with tumor recurrence following surgery [84].

Recent studies suggest an interesting role for DOC-2/DAB2 interacting protein (DAB2IP) in PCa EMT. This gene is frequently down-regulated by epigenetic mechanisms in PCa [85] and this inhibition has been associated, in PCa cells an xenograft models, with induction of EMT, with DAB2IP involved in Wnt signaling modulation, and development of metastases [86]. The involvement of the Wnt signaling pathway in PCa EMT has been also reported. Over-expression of Wnt antagonists, secreted Frizzled related proteins (sFRPs) or Wnt inhibitory factor 1 (WIF1), in PCa cells results in increased expression of epithelial markers, decreased invasiveness and down-regulation of SNAIL2 and TWIST [87,88]. A recent study has also unveiled a connection between TMPRSS2-ERG gene fusions and Wnt pathway activation, as ERG may drive the expression of the Wnt receptor Frizzled 4 (FZD4), which is required for expression of a number of EMT markers [89].

Taking into account the role of androgen receptor (AR) in PCa progression, it is not surprising that AR signaling has also been implicated in EMT induction. In PCa cell lines, androgens may induce an EMT pattern through Snail activation, leading to significant changes in cell migration and invasion potential [90]. Expression levels of AR are inversely correlated with androgen-mediated EMT, indicating that a low AR content is required for the EMT phenotype, whereas a decrease of androgen-mediated EMT with over-expression of AR has been observed [90].

Transcriptional factors including members of the Snail, Twist and ZEB families have also been reported to be involved in EMT in PCa. SNAIL1 represses E-cadherin gene expression as well as that of Raf kinase inhibitor protein (RKIP), which has been identified as a metastasis suppressor in PCa [91]. Moreover, TWIST1 induces EMT-like stages in PCa cells, including up-regulation of N-cadherin [92] and ZEB1 is a direct suppressor of E-cadherin in PCa cell lines, facilitating transendothelial migration [93].

Currently, there is wide evidence that EMT-like states do exist and are important for PCa progression and metastasis formation. However, there is no compelling evidence of EMT as a reversible transdifferentiation mechanism in response to local inductive stimuli, which might account for the routine pathological findings.

Aims

Taking in consideration the important role of played by EMT in tumor progression and metastasis formation, the markers of this process might be used as indicators of tumor aggressiveness, allowing for improved PCa patients' management.

Hence, the aims of this study are:

- Identify EMT-related genes that might be relevant for PCa progression.
- Evaluate expression levels of the EMT-related genes in PCa tissue samples.
- Correlate the expression levels of EMT-related genes with standard clinical and pathological parameters routinely used to assess tumor aggressiveness.

Experimental Methodology

Clinical Samples

All samples used for the purpose of this Thesis were collected at Portuguese Oncology Institute-Porto, Portugal between 2001 and 2012, and included 97 prostate adenocarcinoma (PCa) samples prospectively collected from patients with clinically localized disease consecutively diagnosed and treated with radical prostatectomy. Simultaneously, in 49 radical prostatectomy specimens, prostatic intraepithelial neoplasia (PIN) lesions distant to the tumor were also identified and collected.

Additionally, five samples of prostate cancer metastasis (MET) from brain, bone, lymph node, glans and omentum were also included in our study. As controls, 16 samples of normal prostatic tissue (NPT) were collected from peripheral zone of prostates obtained from cystoprostatectomy specimens of bladder cancer patients that did not harbor PCa. In an attempted to increase the series of non-neoplastic samples were also evaluated 14 samples of benign prostatic hyperplasia (BPH) collected by transurethral resection (TUR).

All tissue samples, excepted metastasis, were promptly frozen immediately after the surgery and stored at -80ºC for further analysis. After histological identification of PCa, PIN, NPT and BPH by an experienced pathologist (Rui Henrique, M.D., PhD), fresh-frozen tissue fragments were trimmed to maximize the yield of target cells (>70% of target cells). Subsequently, an overage of fifty 12µm thick sections were cut and at the end a section was stained to ensure a uniform percentage of target cells and to exclude contamination from neoplastic cells in normal tissues. Metastatic samples were obtained from formol fixed and paraffin embedded tissues.

Gleason score and TNM stage of all cases of PCa were previously assessed by histological slides from formalin-fixed, paraffin embedded tissues fragments from the same surgical specimens. Relevant clinical data was collected from clinical charts.

This study, as well as the use of samples and the access to clinical data, was approved by the institutional review board (Comissão de Ética) of Portuguese Oncology Institute – Porto, Portugal.

Evaluation of Expression of EMT-Related Genes in Prostate Cancer

Based on an intensive literature review, 93 potential EMT-related genes were selected for gene expression evaluation in prostate cancer samples (Annex 1). Concerning those genes, we started by accessing the mRNA expression using a specific

platform and the best genes were selected for validation on a large number of independent samples.

RNA Extraction

Total RNA from all clinical samples, except from metastasis samples, was extracted using a trizol method. Briefly, 1500µL of Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) was added to each 2.0mL tube and tissues were homogenized using rotor-shaker. Tubes were incubated for 5 to 10min at room temperature and then 300µL of Chloroform (Merck, Germany) were added. The tubes were vigorously hand shacked for 15 sec and incubated for 3min at room temperature followed by a 15min 12000g centrifugation at 4ºC. Then, 600µL of the upper phase of each tube, phase containing RNA, was transferred to a new tube. RNA was purified using the PureLinkTM RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Since MET samples were formol fixed and paraffin embedded (FFPE), their RNA extraction was performed using High Pure FFPE RNA Micro KIT v.7 (Roche Applied Science, Mannhein, Germany), according to the manufacturer's instructions.

RNA concentration and purity ratios were then evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Aditionally, RNA quality was checked by ectrophoresis in a 2% agarose gel.

cDNA Synthesis

To performed the screening of the 93 EMT-related genes, cDNA was synthesized from 1000ng of total RNA by reverse transcription using Transcriptor High Fidelity cDNA Synthesis Kit v.6 (Roche Applied Science, Mannhein, Germany), according to manufacturer's instructions. For the validation of the firstly identified differentiated expressed genes by QRT-PCR, 300ng of total RNA were used to synthesize and amplify cDNA using TransPlex® Whole Transcriptome Amplification (WTA) Kit (Sigma-Aldrich®, Germany) according to manufacturer’s protocol. WTA reaction products were purified using QIAquick PCR Purification Kit (QIAGEN, Germany), according to manufacturer’s protocol, and stored ate -20ºC.

Screening of 93 EMT-Related Genes Expression

Expression levels of 93 EMT-related genes previous selected were evaluated in NPT (n=5), pT2a+b-PCa (n=5), pT3b-PCa (n=5) and MET (n=5) samples using a RealTime ready Custom Panel 384 assay (Roche Applied Science, Mannhein, Germany). This experiment was performed in triplicates for PCa samples and without replicates for NPT and MET.

Additionally to the 93 selected genes RealTime ready Assays pre-plated and dried-down, a custom panel of three reference genes, *GUSβ, TFRC* and *RN18S1*, were also included. *GUSβ* and *TFRC* have been reported as two of the most suitable genes to be used as reference genes in QRT-PCR assays in prostate tissue samples [94]. Since the RealTime ready Custom Panel 384 assay had three wells available for reference genes, we also add *RN18S1*, which is one of the most commonly used reference genes in this type of analysis in a variety of tissue samples [95,96].

The RealTime ready Assays are based on Universal ProbeLibrary (UPL) technology, which are short hydrolysis probes, labeled at the 5' end with a reporter dye (FAM) and at the 3' with a dark quencher dye. These probes, in order to maintain the specificity and melting temperature, have a Loked Nucleic Acids (LNA) incorporated into sequence.

In each well 0.1µL of cDNA (corresponding to 5ng), 5 µL of LightCycler® 480 Probes Master v.9 (Roche Applied Science, Mannhein, Germany) and 4.9µL LightCycler® 480 Probes Master H2O (included in LightCycler® 480 Probes Master v.9) were added. The QRT-PCR reaction was performed in a RealTime termocycler LightCycler® 480 II (Roche Applied Science, Mannhein, Germany), following manufacturer's instructions.

Expression levels of the 93 genes were analyzed using the comparative Ct method (ΔΔCt), which is suitable when a high number of genes are analyzed [97]. The ΔΔCt method was applied according to the following expressions:

Difference of expression between two groups = $2^{-\Delta\Delta Ct}$
ΔΔCt = MedianΔCt (group A) – Median ΔCt (group B)
ΔCt = Ct (gene of interest) – Ct (reference gene)

In order to calculate ΔCt, the most constant endogenous control and simultaneously the control that amplified in all samples was selected. It was considered a significant reduction of expression values of $2^{-\Delta\Delta Ct}$ bellow 0.5 and a significant increase of expression values of $2^{-\Delta\Delta Ct}$ above 2.0.

From the 93 EMT-related genes analyzed, five genes were selected as potential markers of EMT in PCa based on the following criteria:

- Higher differences of expression between (in order of importance):
  - pT3b-PCa and NPT
  - pT3b-PCa and pT2a+b-PCa
- Correlation between gene behavior in the analysis and described literature.

Evaluation of Selected Genes Expression Levels in a Largest Independent Series

In order to validate previous results, expression levels of genes selected were evaluated in a largest independent series of NPT (n=16), HBP (n=14), PIN (n=49), pT2a+b-PCa (n=50) and pT3b-PCa (n=47) samples. For that were used TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA, USA) for the selected genes and for the endogenous controls *GUSβ* and *TFRC*.

This assay comprise a unlabeled PCR primer pair and a TaqMan® probe with a reporter FAMTM dye label and a minor groove binder (MGB) linked to the 5' end and a nonfluorescent quencher (NFQ) on the 3' end. The quantitative QRT-PCR reaction performed using TaqMan® assays is based on the 5' nuclease activity of the Taq DNA polymerase. During QRT-PCR, TaqMan® probes hybridize to the target DNA between the two unlabeled PCR primers. Signal from the fluorescent dye on the 5' of TaqMan® probes is quenched by NFQ on its 3' end through fluorescence resonance energy transfer (FRET). A Taq polymerase extends the unlabeled primers using the template strand as a guide. When the polymerase reaches the TaqMan® probe, it cleaves the molecule, separating the dye from the quencher, and allowing it to fluoresce. The QRT-PCR instrument detects fluorescence from the unquenched FAMTM dye. With each cycle of PCR, more dye molecules are releases, resulting in an increase in fluorescence intensity proportional to the amount of amplicon synthesized.

The QRT-PCR assay was performed in 96-well plates on an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA), according to the recommended protocol. Briefly, in each well was added 2µL of WTA-cDNA diluted sample (dilution factor of 5x), 1µL of TaqMan® Gene Expression assay, 12.5µL of TaqMan® Universal PCR Master Mix and 9.5µL (Applied Biosystems, Foster City, CA, USA) of bidestilated water (B.BRAUN, Melsungen, Germany). The QRT-PCR conditions were the recommended by the manufacturer: 50ºC for 2 min, 95ºC for 10 min, 40 cycles at 95ºC for 15 sec and 60ºC for 1 min.

All samples were run in triplicate and two water blanks were added to each plate as negative controls. WTA-cDNA synthesized from prostate total RNA was used to prepare five consecutive cDNA dilutions (dilution factor of 10x) that were analyzed as standards, allowing the construction of a standard curve for relative quantification and PCR efficiency assessment.

The expression levels were analyzed using the relative standard curve method, which provides more accurate quantitative results in comparison with the $\Delta\Delta$Ct method [97]. The results from the QRT-PCR were analyzed using the 7500 Software version 2.0.5 (Applied Biosystems, Foster City, CA, USA). In each sample, the mean quantity of each gene was normalized with mean quantity of endogenous controls *GUSβ* and *TFR*, according to the expression:

$$\textbf{Gene expression} = \frac{\textbf{Gene Mean Quantity}}{\dfrac{\textbf{\textit{GUS}}\boldsymbol{\beta}\ \textbf{Mean Quantity} + \textbf{\textit{TFR}}\ \textbf{Mean Quantity}}{\textbf{2}}}$$

Statistical Analysis

Differences in expression levels of the EMT-related genes between the different groups of samples (NPT, BPH, PIN, pT2a+b-PCa and pT3b-PCa) were firstly analyzed using Kruskal-Wallis nonparametric test, followed by nonparametric Mann-Whitney U-test, when appropriate.

The association between expression levels of EMT-related genes and Gleason score was also evaluated using the Kruskal-Wallis and Mann-Whitney-U tests. A nonparametric Spearman test was performed to assess a correlation between expression levels and PSA and age.

All tests were two-sided and p-values were considered significant when inferior to 0.05. For multiple comparisons, Bonferroni’s correction was used to adjust p-values.

Statistical analysis was performed using the software IBM® SPSS® Statistics 20.

Results

Clinical Samples

For the purpose of this study, tissue samples of normal prostate tissue (NPT, n=16), benign prostatic hyperplasia (BPH, n=14), high-grade prostatic intraepithelial neoplasia (PIN, n=49), prostate adenocarcinoma (PCa, n=97), and prostate cancer metastasis (Met, n=5) were used to evaluate the transcript levels of EMT-related genes. Relevant clinical and pathological data of the patients included on this study are summarized in Table 2.

Table 2- Clinical and pathological features of patients included in this study

Clinicopathological Features	**NPT**	**BPH**	**PIN**	**PCa**	**Met**
Patients, *n*	16	14	49	97	5
Median Age, *yrs* (range)	63 (45-80)	67 (61-74)	65 (51-75)	63 (50-75)	73 (65-89)
PSA (ng/mL), *median* (range)	*n.a.*	4 (1.0-22.0)	*n.a.*	9.1 (3.0-24.7)	*n.a.*
Pathological Stage, *n* (%)					
pT2a+b	*n.a.*	*n.a.*	*n.a.*	50 (52)	*n.a*
pT3b	*n.a.*	*n.a.*	*n.a.*	47 (48)	*n.a*
Gleason Score, *n* (%)					
<7	*n.a.*	*n.a.*	*n.a.*	27 (28)	*n.a*
=7	*n.a.*	*n.a.*	*n.a.*	56 (57)	*n.a*
>7	*n.a.*	*n.a.*	*n.a.*	14 (15)	*n.a*

Abbreviations: Normal prostatic tissue (NPT), benign prostatic hyperplasia (BPH), high-grade prostatic intraepithelial neoplasia (PIN), prostate cancer (PCa) and not available/applicable (*n.a.*).

Screening of 93 EMT-Related Genes' Expression

After analyzing expression levels of the 93 EMT-related genes according to criteria previously described, *CAMK2N1, CD44, KRT14, TGFβ3* and *WNT5A* were identified as differentially expressed in PCa (Table 3 and Table 4). Results for the 93 EMT-related genes analyzed are discriminated in Annex 2.

Table 3- Values of $2^{-\Delta\Delta Ct}$ between pT3b-PCa and NPT, and between pT2a+b-PCa and pT3b-PCa for the selected genes.

	$2^{-\Delta\Delta Ct}$	
Target Name	**pT3b-PCa Vs. NPT**	**pT3b-PCa Vs. pT2a+b-PCa**
CD44	**0.16**	**0.31**
KRT14	**0.19**	**0.28**
TGFB3	**0.21**	**0.26**
WNT5A	**2.68**	**9.16**
CAMK2N1	**4.76**	**4.04**

Table 4- Variation of expression of the five selected genes between the three groups of samples.

Variation of Expression				
CD44	**KRT14**	**TGFB3**	**WNT5A**	**CAMK2N1**
NPT ↘ pT2a+b-PCa ↘ pT3b-PCa	NPT ↘ pT2a+b-PCa ↘ pT3b-PCa	NPT ↘ pT2a+b-PCa ↘ pT3b-PCa	pT3b-PCa NPT ↘ ↗ pT2a+b-PCa	pT3b-PCa ↗ pT2a+b-PCa ↗ NPT

Legend: ↘- decrease of expression; ↗- increase of expression

CD44 is involved in a variety of process including cell adhesion and has been recently proposed as a CSC marker, namely for PCa stem cells [71,98]. Both tumor suppressive and oncogenic functions have been suggested for *CD44* [99,100]. Thus, both an increase or a decrease of expression with tumor progression could be expected. In this first analysis was observed a decrease in expression with tumor progression, supporting a tumor suppressive role for *CD44*.

CAMK2N1 encodes for a protein that inhibits CAMK2 activity, and this inhibition has been associated with a reduction of proliferation and invasion capacities [101]. Indeed, a recent study in PCa tissue samples has reported a higher expression of *CAMK2N1* in more aggressive tumors [102]. Our preliminary results suggested a decreased expression of *CAMK2N1* with tumor progression, corroborating the previously reported results.

WNT5A encodes a ligand that can activate the Wnt pathway, which has been associated with EMT induction in tumor cells [43]. Thus, an increased expression with tumor progression would be expected. At this point, our results revealed an inconsistent variation of expression (Table 4), but a similar behavior has been already reported [103].

TGFβ3 encodes for a ligand that can activate the TGF-β signaling pathway, which has been associated with EMT induction. This signaling pathway, however, has been also

reported as tumor suppressive [43,49]. Thus, either an increase or a decrease of *TGFβ3* expression with tumor progression could be anticipated. In this first analysis a decrease of expression with tumor progression was observed, supporting a tumor suppressive role for *TGFβ3*.

KRT14 encodes for a keratin protein, which is a major component of the epithelial cells' cytoskeleton [104]. During EMT, epithelial cells progressively loose expression of typical epithelial markers, such as keratins, and, thus, a decrease of *KRT14* expression with tumor progression would be expected [43], and this corresponded to to our observations

Other genes have demonstrated significant differences in expression (e.g., *VCAN*, *COL5A2* and *PROM1*) but the variations of expression observed were not consistent and, thus, further analyses were not carried out.

The results of metastasis samples were not further analyzed because a significant number of genes did not adequately amplify. In line with these findings, the evaluation of metastasis' RNA quality by electrophoresis demonstrated poor quality (data not shown).

Evaluation of Selected Genes Expression Levels in a Largest Independent Series

Expression levels of the five EMT selected genes were analyzed in a larger and independent series of NPT (n=16), BPH (n=14), PIN (n=49), pT2a+b-PCa (n=50) and pT3b-PCa (n=47). BPH samples were used in an attempt to increase the series of non-neoplastic samples, but statistically significant differences were observed for expression levels of the majority of the analyzed genes between BPH and NPT. Thus, BPH samples were excluded from further analysis.

No correlations were found between expression levels of any of the EMT-related genes and serum PSA levels or age of PCa patients. Regarding Gleason score, analysis of variance identified significant differences in the expression levels of *CAMK2N1*, *CD44* and *WNT5A* among Gleason score groups (<7, =7 and >7), and increased expression levels were depicted for tumors with higher Gleason score (Figure 8, Figure 9 and Figure 10). Comparing expression levels using Mann-Whitney U-test (MW), only *CAMK2N1* and *WNT5A* displayed significant differences among all three groups (Figure 8 and Figure 10).

Analysis of variance also identified significant differences in *CAMK2N1*, *CD44* and *WNT5A* expression levels among NPT, pT2a+b-PCa and pT3b-PCa. In locally advanced tumors (pT3b-PCa), *CAMK2N1* expression levels were significantly higher than those of

organ-confined tumors (pT2a+b-PCa) or in NPT (Figure 11). However, no statistically significant differences were observed between the latter two groups.

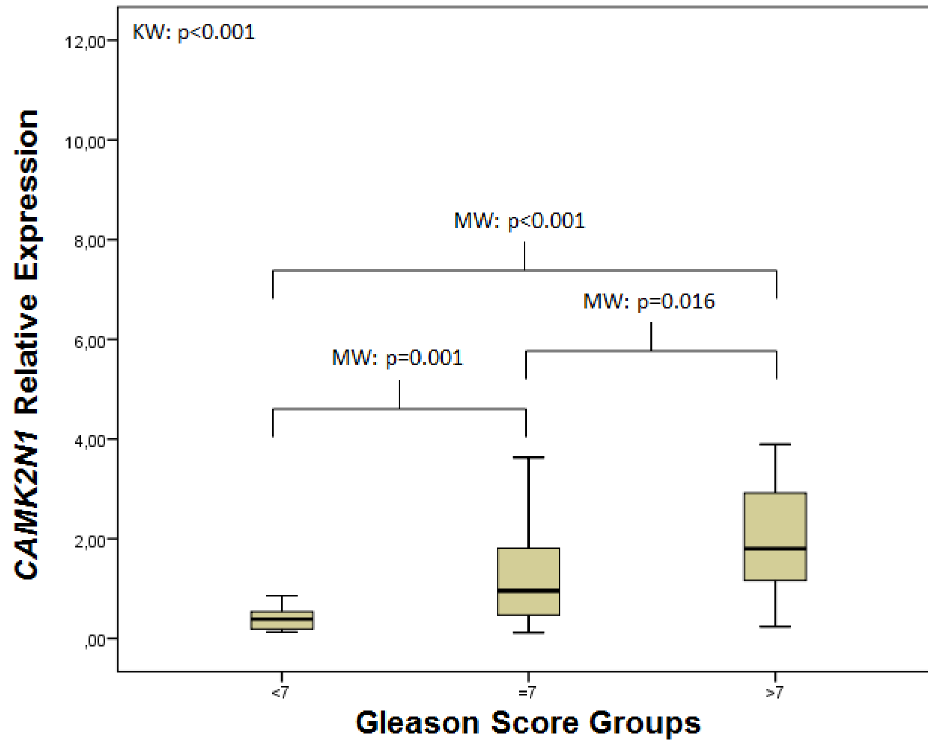

Figure 8 - Relative expression levels of *CAMK2N1* with tumors Gleason score.

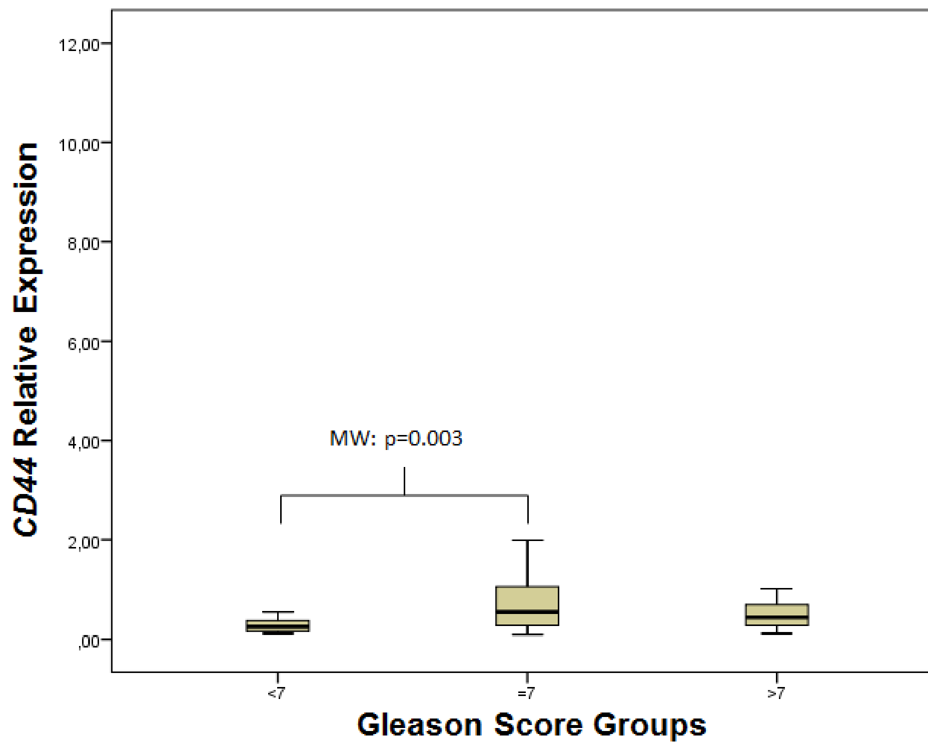

Figure 9 - Relative expression levels of *CD44* with tumors Gleason score.

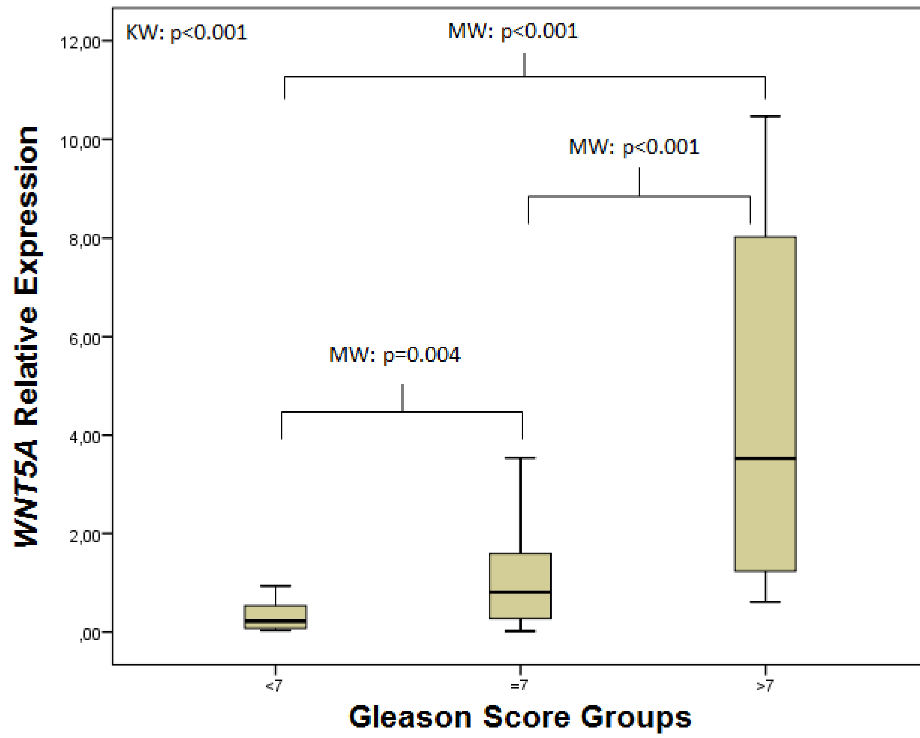

Figure 10 - Relative expression levels of *WNT5A* with tumors Gleason score.

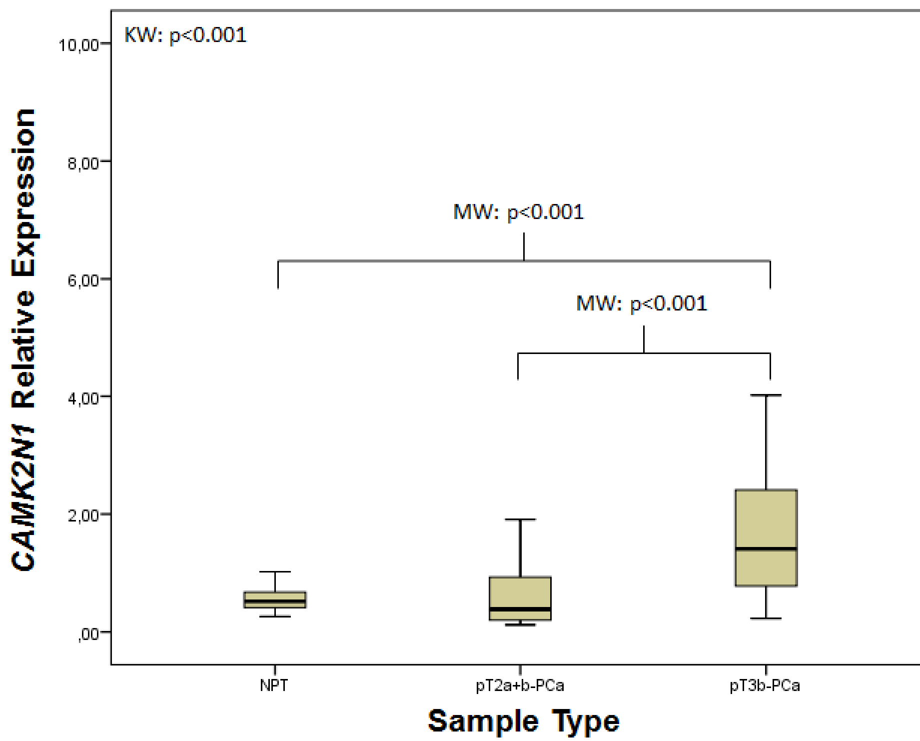

Figure 11- Relative expression levels of *CAMK2N1* in NPT, pT2a+b-PCa and pT3b-PCa.

Likewise, *CD44* expression levels were significantly higher in advanced stage PCa cases in comparison with organ-confined tumors, but no significant differences were observed between NPT and pT2a+b-PCa or pT3b-PCa (Figure 12). Surprisingly, both NPT and pT3b-PCa displayed significantly higher *WNT5A* expression levels compared to those of pT2a+b-PCa, and no significant differences were observed between NPT and pT3b-PCa (Figure 13).

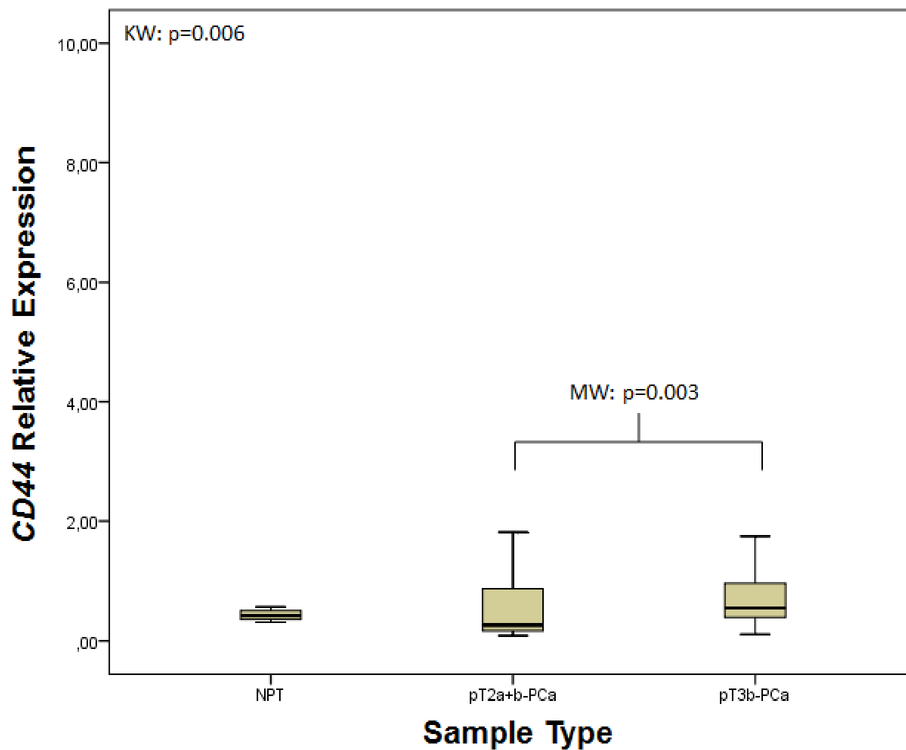

Figure 12- Relative expression levels of *CD44* in NPT, pT2a+b-PCa and pT3b-PCa.

Contrarily, for *KRT14* and *TGFB3,* no significant differences in expression levels were observed among the different groups of tissue samples (NPT, pT2a+b-PCa and pT3b-PCa) (Figure 14 and Figure 15).

Additionally, the expression levels of the same genes were also assessed in the pre-malignant lesions of the prostate (PIN). A considerable heterogeneity was apparent for the expression levels of almost all genes in PIN lesions (data not shown). For *CAMK2N1*, expression levels observed in PIN lesions were significantly higher than those observed in NPT and pT2a+b-PCa. Nonetheless PIN lesions displayed significantly lower levels than locally advanced tumors (Figure 16). CD44 expression levels in PIN samples were significantly higher than those observed in any other group of samples (NTP, pT2a+b-PCa and pT3b-PCa) (Figure 17). Contrarily, PIN samples showed significantly lower *WNT5A* expression levels compared to NPT and pT3b-PCa, but the same trend

was not found for organ-confined tumors. Indeed, no significant differences were observed between PIN and pT2a+b-PCa (Figure 18).

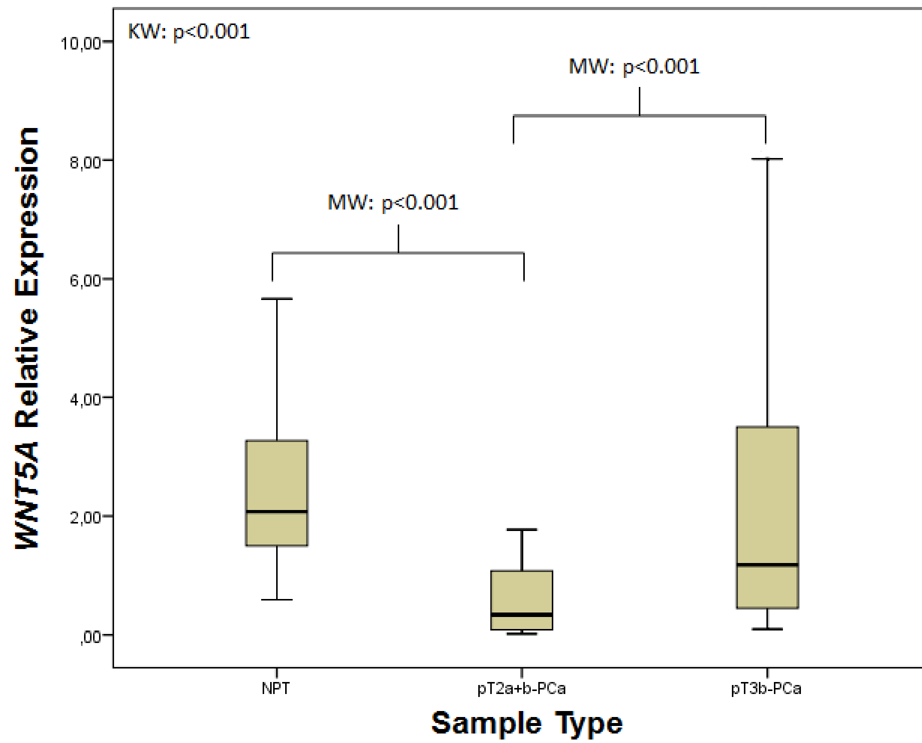

Figure 13- Relative expression levels of *WNT5A* in NPT, pT2a+b-PCa and pT3b-PCa.

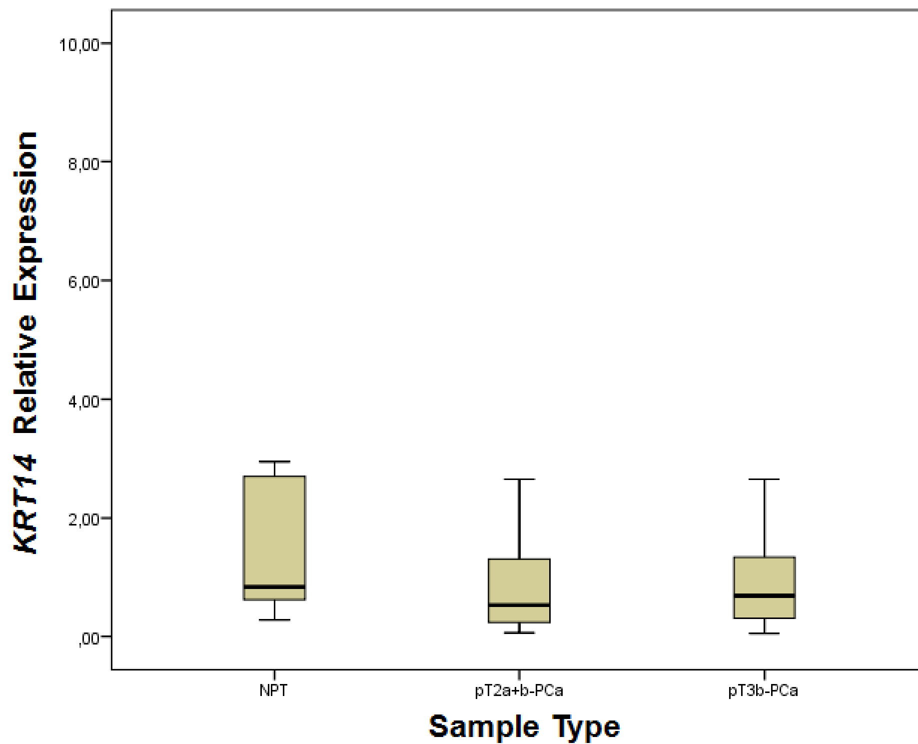

Figure 14- Relative expression levels of *KRT14* in NPT, pT2a+b-PCa and pT3b-PCa.

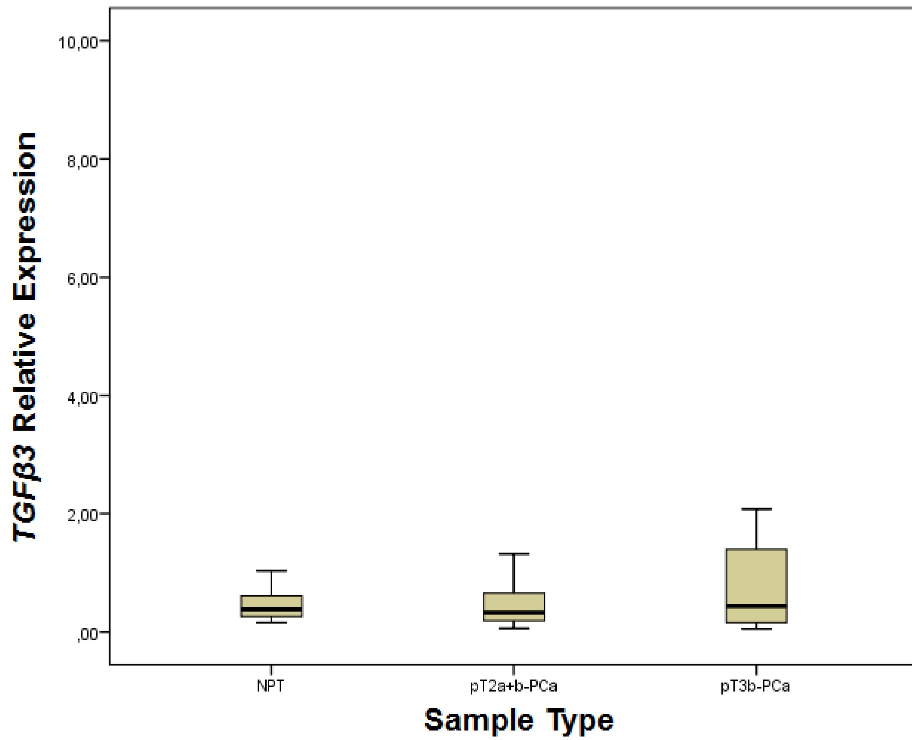

Figure 15- Relative expression levels of *TGFβ3* in NPT, pT2a+b-PCa and pT3b-PCa.

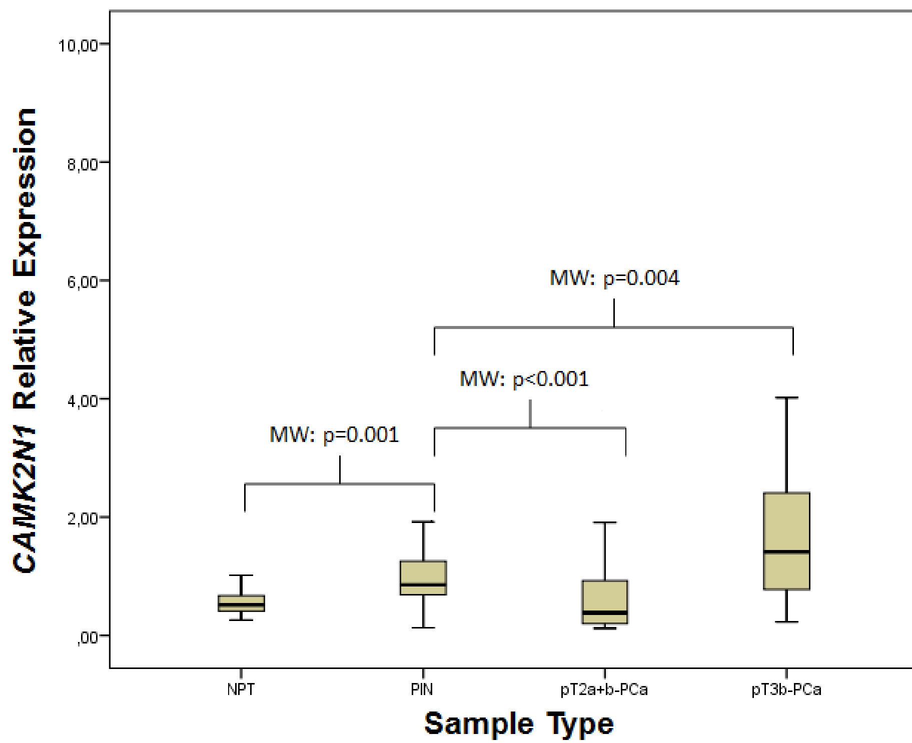

Figure 16- Relative expression levels of *CAMK2N1* in NPT, PIN, pT2a+b-PCa and pT3b-PCa.

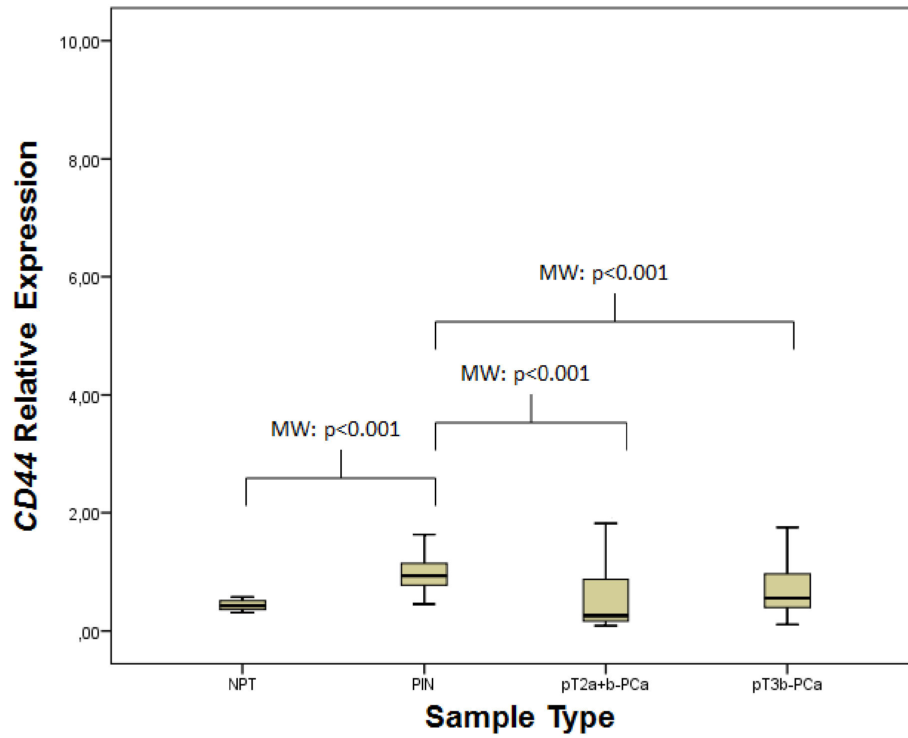

Figure 17- Relative expression levels of CD44 in NPT, PIN, pT2a+b-PCa and pT3b-PCa.

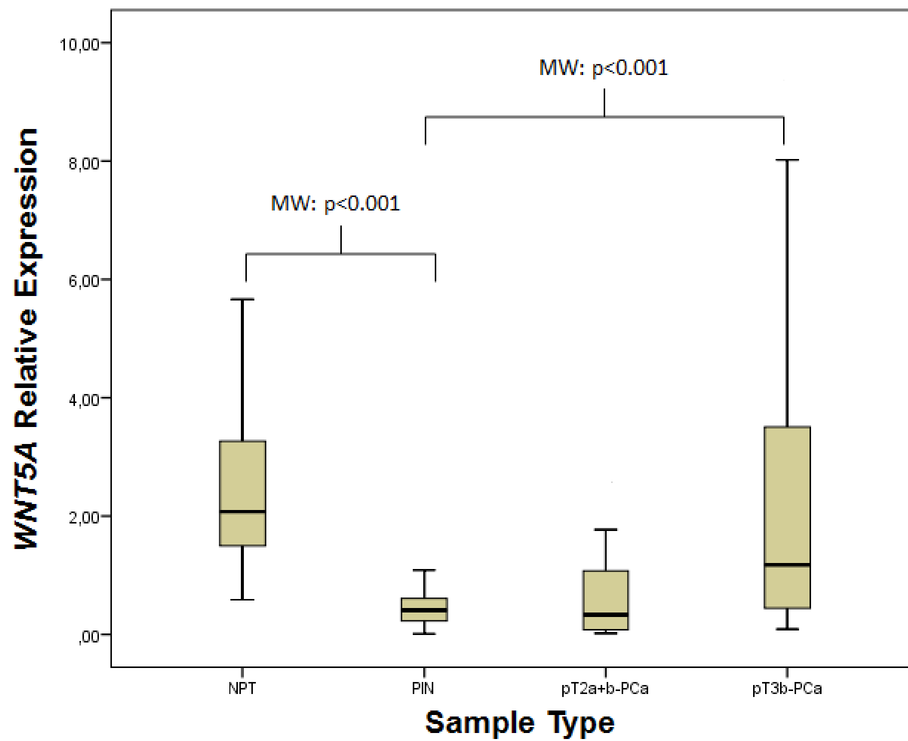

Figure 18- Relative expression levels of *WNT5A* in NPT, PIN, pT2a+b-PCa and pT3b-PCa

Discussion

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death among males worldwide, representing the most common form of cancer and the third cause of cancer-related death in men in developed countries [1]. Currently, PCa management and treatment is decided based on serum PSA levels, histopathological tumor characteristics and patient's life expectancy, among other factors [12]. However, PCa behavior is frequently unpredictable, ranging from indolent, in which active surveillance might be the best choice, to highly aggressive, in which case radical treatment is required [23]. On the other hand, organ-confined PCa is mostly curable, in contrast to locally or systemically advanced disease, for which no effective curative treatments are available [12]. Due to the current inability to distinguish tumors that will remain indolent from those that will be lethal, physicians and patients tend to elect treatments with curative intent, but which lead to undesirable side-effects in a large proportion of patients [38]. The current methods for stratifying PCa tumors before patients' treatment are based on PSA level, clinical stage (cTNM) and evaluation of Gleason score in biopsies samples as well as the number of cores / percentage of tissue that contain tumor cells [19,40]. Although all these clinical data are helpful, they cannot fully predict outcome or tumor aggressiveness [19,39]. Thus, a better characterization of tumor aggressiveness is critical for reducing both over-treatment and mortality due to this malignancy and it should be ideally based on the biological properties of PCa cells.

EMT promotes cancer cell motility and the capacity to invade tissues and organs surrounding the tumor, as well as facilitates intravasation, allowing for metastasis formation at remote sites [43]. Hence, taking into account the important role of EMT in tumor progression, EMT-related genes might be used as markers of tumor progression, allowing for an improved assessment of tumor aggressiveness. Thus, we evaluated the transcript levels of EMT-related genes at different stages of prostate cancer progression, in an attempt to disclose new prognostic markers which might allow a better stratification of PCa patients according to the likelihood of progression to lethal forms of the disease.

Firstly, we assessed the expression levels of 93 EMT-related genes using a RealTime ready Custom Panel 384 assay, using a limited series of prostatic tissues [NPT (n=5), pT2a+b-PCa (n=5), pT3b-PCa (n=5) and MET (n=5)], representing well defined stages of the disease. This assay allowed for the identification of five candidate genes: *CAMK2N1, CD44, KRT14, TGFβ3* and *WNT5A*. Subsequently, the expression levels of these genes were further analyzed in a large independent series of samples. From these analyses, statistically significant differences in the expression levels of *CAMK2N1*, *CD44* and *WNT5A* among NPT, pT2a+b-PCa and pT3b-PCa were depicted, and these were

highest in the more advanced stage PCa cases. Thus, these gene panel constitutes a departing point for further exploitation of their potential as markers of clinically aggressive disease.

CAMK2N1 encodes for a protein that inhibits CAMK2 activity, which has been implicated in cell cycle progression through activation of the MEK/ERK and Notch-1 pathway [105,106]. As previously mentioned, Notch-1 and MEK/ERK pathways are both involved in EMT induction and, thus, CAMK2 proteins may play an important in EMT. In PCA cell lines, over-expression of *CAMK2* was found to induce a decrease in apoptosis, whereas its inhibition reduces proliferation and invasion capacity [101]. Indeed, a recent study in PCa tissue samples has reported a higher expression of *CAMK2N1* in PCa patients that recurred compared to patients in which no disease recurrence was detected after a five-year follow-up period [102]. Likewise, in our study, a significant increase in *CAMK2N1* expression was observed in more advanced tumors (pT3b-PCa), supporting a correlation between *CAMK2N1* expression and tumor progression, as well as, eventually, with EMT. However, because CAMK2 promotes proliferation and invasion, its inhibition in more advanced stages would not be expected. A possible explanation for this finding is that tumor cells actively engaged in EMT are usually less proliferative [73]. Thus, the inhibitory activity of *CAMK2N1* on CAMK2 in PCa would provide conditions for cancer cells to endure EMT through slowing cell proliferative activity. However, the pathway through which CAMK2N1 expression promotes tumor aggressiveness remains unclear and there is a need to clarify which is the specific role of this gene in tumor progression.

CD44 is a transmembrane glycoprotein involved in cell adhesion, migration, differentiation, signal transduction and apoptosis [98,107]. The extracellular domain of CD44 binds to numerous components of ECM, especially to hyaluronic acid (HA), whereas the intracytoplasmatic domain interacts with cytoskeletal proteins and intracellular signaling proteins [108]. Several studies have been undertaken to clarify the role of CD44 in PCa progression, and most have suggested that decreased, albeit variable, *CD44* expression is associated with PCa progression. Indeed, a strong correlation between Gleason score and the loss of CD44 expression has been reported [109,110], but that is not a consensual finding [111]. Although, some studies suggested a tumor suppressive function for *CD44* [99,110], others have implicated *CD44* in PCa cell migration and invasion *in vitro* as well as in metastatic dissemination *in vivo* and chemoresistance [71,100,112]. Thus, both a tumor suppressive or an oncogenic function have been proposed for CD44, and the actual role of *CD44* in PCa progression is still unclear. Our data favors an oncogenic function, since higher expression levels were found in more advanced and less differentiated tumors. Furthermore, CD44 has been recently

proposed as a CSC marker, namely for PCa stem cells [71]. These cells, as previously referred, play a central role in tumor progression, particularly for metastasis formation. Thus increased CD44 expression in more advanced tumors, as observed in our study, may be correlated with an increase of CSCs in these tumors, compared to early stage disease. Indeed, the increase of CSCs populations in these tumors may be one of the explanations for there more aggressive behavior. Nevertheless, this increase of *CD44* expression may also be seen as the results of an increase of CSCs induced by EMT.

Taking into account the role of CD44 in cell adhesion and migration, the alteration of expression found in this study might reflect alterations in cell mobility. Although CD44 was originally identified as a receptor for HA, there is evidence indicating that the binding to HA does not promote CD44-mediated cell migration and metastasis [113,114]. Later studies revealed thatt CD44 had affinity for other ligands, such as matrix metalloproteinase 9 (MMP9) [115]. Matrix metaloproteinases are essential for migration through the EMC and have a crucial role in ECM degradation, allowing cells to detach and migrate [116]. Previous studies have demonstrated that CD44 functions as a docking molecule for MMP9 on the cell surface, suggesting a mechanism for CD44-mediated cell migration and metastasis [100,115]. In this context, if the increased expression of CD44 in more aggressive tumor that we observed might be associated with an increased expression of MMP9, which could foster cell migration and subsequent invasion and metastasis formation in prostate cancer.

Surprisingly, no differences were observed in CD44 expression levels between NPT and pT2a+b-PCa or pT3b-PCa, although our results suggest a decrease in CD44 expression in pT2a+b-PCa and an increase in pT3b-PCa samples, when compared to NPT. It is likely that these results are due to the relatively small number of cases analyzed (especially NPT), which jeopardizes statistical significance.

WNT5A is a member of Wnt family, implicated in tumor progression and osteomimicky (a process in which prostate cancer cells acquire an osteoblast-like phenotype) [117]. Thus, *WNT5A* may play an important role in PCa metastization since this cancer has a strong propensity to metastasize to bone. Indeed, a recent study showed a significant increase in *WNT5A* expression in advanced metastatic disease compared to benign prostatic lesions or early stage PCa [103]. However, significant differences between earlier disease stages were not reported [103]. Herein, WNT5A was stage-dependently expressed, showing a significant increase of expression along tumor stage. Surprisingly, *WNT5A* expression was significantly decreased in organ confined disease when compared to NPT, and no significant differences were observed between pT3b-PCa and NPT. These observations may indicate that suppression of *WNT5A* in

tumors renders them less aggressive and that when expression is kept they may acquire a more aggressive behavior.

WNT5A is a non-canonical ligand which can activate the non-canonical Wnt pathway (Wnt/Ca^{2+}), although it has been suggested that WNT5A may also interact with the canonical pathway (Wnt/β-catenin), which has been associated with tumor progression and stemness features [118].*CD44* is one of the target genes of the Wnt/β-catenin pathway and, interestingly, we found that the variation of expression observed for *WNT5A* was similar to that of *CD44* [119]. This fact suggests a link between *WNT5A* expression and Wnt/β-catenin pathway activation in PCa.

Concerning correlations between gene expression levels and standard clinicopathologic parameters, increased expression of *CAMK2N1*, *CD44* and *WNT5A,* was associated with increased Gleason score, which is indicative of more aggressive disease. The Gleason score may also be considered a morphological marker of EMT, since the increase in the score is associated with the loss of epithelial glandular features and an increase of invasiveness. From this point of view, because *WNT5A* and *CAMK2N1* displayed the strongest correlations with the Gleason score, it is suggested that they might play an important role in EMT and, consequently, in the metastasis formation process of PCa.

On the other hand, no correlation was found between expression levels and pathological stage or Gleason score for *KRT14* and *TGFβ3*.

TGFβ signaling has been reported as a signaling pathway involved in the EMT activation [43,46]. *TGFβ3* encodes one of the ligands that, through the binding to the TGFβ receptor, can activate this signaling pathway [49]. Thus, in the context of TGFβ signaling as an inducer of cell migration an invasion, and increased expression of activators, like TGFβ3, would be expected. The TGF-β proteins are released in an inactive form to the ECM, where they are sequestered [120,121]. Thus, ECM acts as a reservoir from which inactive TGF-β proteins can readily be activated without the need for new synthesis [49]. This fact may be one of the explanations for ours results, because the induction of EMT through TGFβ signaling may not require an increased expression of TGFB proteins, but only the activation of already synthesized proteins.

Keratins are a major component of epithelial cells cytoskeleton and play an important functional role in the integrity and mechanical stability of these cells [104]. During EMT, epithelial cells progressively loose expression of typical epithelial markers, such as keratins, and, thus, decreased expression with with tumor stage and Gleason score would be expected [43]. However, it has been reported that epithelial tumors largely

maintain the features of specific keratin expression associated with the respective cell type origin and, moreover, the expression spectrum of KRT14 in tumors is similar to that of the native normal epithelium, thus supporting our results [104].

Concerning the preliminary results of the screening of expression of the 93 EMT-related genes and the results of the posterior validation, some inconsistency between the results of the two assays was apparent. For CD44, an increase of expression with tumor stage was observed in the second analysis, contrarily to the results of the first assay, whereas for TGFB3 and KRT14, in the second analysis, no significant differences in expression between the different groups of samples were observed. These discrepancies are probably due to de number of samples analyzed: in the first analysis the number of samples analyzed was rather small, which may jeopardize the results. However, a screening of expression of 93 genes in a larger series of samples would be unaffordable.

Although the expression analysis performed provided information about the amount of mRNA encoded by the selected genes, there is a need to verify the functional impact in terms of protein synthesis. The importance of this evaluation is mainly due to the fact that some epigenetic post-transcriptional regulation may occur and affect mRNA, eventually having no impact in protein expression. In addition, the identification of the mechanisms involved in deregulated gene expression regulation is also critical.

Because PIN represents a precursor, non-invasive, lesion of PCa, we also evaluated the expression levels of the five EMT-related genes in PIN samples. Our expectations were that expression levels would be lower than those o PCa and, eventually, higher than those of NPT. However, the results of these analyses were mostly inconsistent, probably reflecting a substantial heterogeneity in the malignant potential of these precursor lesions. Indeed, it became clear over the last decade that albeit morphologically similar, PIN lesions do not harbor equal potential for progression to invasive carcinoma [122]. Thus, EMT-related gene expression heterogeneity might simply reflect different potential for developing an invasive phenotype. Expression levels of *WNT5A* in PIN samples were those that more closely followed the initial expectations, displaying a significant lower expression compared with pT3b-PCa, and thus supporting an association with tumor progression and invasion.

In an attempt to increase the series of non-neoplastic samples, the evaluation of expression of the five selected genes was also performed in BPH samples. However, statistically significant differences in expression levels for the majority of analyzed genes

between the BPH and NPT samples were observed. We, thus, decided not to combine these two non-neoplastic groups of samples in the same series, excluding BPH samples from further analysis. A possible explanation for these results, is based on the fact that BPH and NPT samples, used in the present work, were collected from different regions of prostate: BPH samples were collected from the transition zone and NPT samples were collected from peripheral zone of prostate. Thus, the observed differences in gene expression might be due to the different anatomical origin of the samples. Interestingly, the diverse biological characteristics of anatomic prostate regions have been proposed as the main reason for the ,dissimilarities in gene expression between tumors located in the transition and peripheral zone of prostate previously reported [123]. On the other hand, it has been suggested that EMT may be involved in genesis of BPH, and some studies have already reported alterations in EMT-related genes in those lesions, which may further explain our results [124,125].

A major limitation of this preliminary study is that we assessed the expression of EMT-related genes in bulk tumor samples. EMT is a process that, when activated in carcinomas, does not occur in all cancer cells, but only in a variable, probably small proportion of them, which lead the invasion and metastization processes. Thus, the expression levels which represent an average of the tumor might not be representative of the cancer cells that undergo EMT. This may impair the detection of subtle differences in gene expression patterns which might be restricted to the invasive front of the tumor. To overcome this limitation, analysis of microdissected tumor cells is required.. However, if it holds true that more aggressive and advanced tumors are enriched in cells enduring EMT, then the significant differences in expression found for the selected genes between tumors with different grades of aggressiveness, are likely to reflect such differences in tumor cell subpopulations. Another limitation of this study was the inability to use data from metastasis of PCa. Because the available samples were obtained from formalin-fixed, paraffin-embedded tissues, mRNA was of poor quality and it jeopardized the molecular assays. These are difficult tissue samples to obtain and the relevant information enclosed in metastatic tumor cells might be better revealed through immunohistochemical assays targeting the proteins encoded by the EMR-related genes. Evaluation of the expression of EMT-related genes in samples of metastases is likely to provide relevant data about the occurrence of MET during metastasis formation.

Conclusion and Future Perspectives

There is a current need to identify new biomarkers of PCa progression in an attempt to better discriminate indolent from aggressive tumors and thus improve patient management. Because EMT is associated with the acquisition of invasive and metastasizing behavior by tumor cells, which are features that denote tumor aggressiveness, we determined the expression of EMT-related genes in a series of PCa in an attempt to identify novel molecular markers of aggressive disease.

Our results showed that three EMT-related genes - *CAMK2N1*, *CD44* and *WNT5A* - were mostly overexpressed in more advanced and less differentiated PCa cases. Thus, they may constitute candidate biomarkers for the assessment of PCa clinical aggressiveness.

The validation of these preliminary results will require the analysis of protein expression in routine tissue samples of PCa (e.g., biopsies) and a correlation with patient outcome. Immunohistochemistry is the obvious choice for that purpose as it constitutes, nowadays, a routine technique is most departments of Pathology.

From a biological standpoint, to clarify the role of *CAMK2N1* in PCa progression will require functional studies to determine the effectiveness of CAMK2 inhibition and the consequent effects in tumor cell phenotype.

Finally, there is a need to better characterize the EMT-related genes concerning the mechanisms involved in gene expression regulation. Because EMT is a reversible process, and indeed this reversion seems to occur during metastasis formation, it is likely that epigenetic mechanisms are involved in both EMT and MET. The fact that some of these genes display CpG islands at their promoter region make then susceptible for regulation by methylation and this constitutes a starting point for further investigations of the plasticity and dynamics of EMT in PCa.

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Annex 1

Table 5- Characteristics of the of 93 EMT-related genes studied.

Related Gene Function	Gene Symbol	Gene Full Name	Other Functions
Differentiation and Development	*AHNAK*	AHNAK nucleoprotein	
	CD44	CD44 molecule (Indian blood group)	Cell adhesion
	ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	cell proliferation and cell adhesion
	MITF	microphthalmia-associated transcription factor	Transcription factor
	NODAL	nodal growth differentiation factor	cell proliferation and cell migration
	POU5F1	POU class 5 homeobox 1	Regulator of Wnt pathway
	PROM1	prominin 1	
	RARB	retinoic acid receptor beta	cell proliferation
	RGS2	regulator of G-protein signaling 2	cell proliferation
	SOX10	SRY (sex determining region Y)-box 10	cell proliferation, cell morphogenesis and regulator of Wnt pathway
	TCF3	transcription factor 3	transcription factor
	TCF4	transcription factor 4	transcription factor
	TMEFF1	transmembrane protein with EGF-like and two follistatin-like domains 1	
	TMEM132A	transmembrane protein 132A	
	TWIST1	twist basic helix-loop-helix transcription factor 1	Transcription factor
Morphogenesis	*SNAI1*	snail homolog 1 (Drosophila)	Cell differentiation and cell migration
	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	Extracellular matrix organization and cell migration
Cell Growth and Proliferation	*AKT1*	v-akt murine thymoma viral oncogene homolog 1	Cell differentiation
	CAV2	caveolin 2	Cell migration
	EGFR	epidermal growth factor receptor	Cell migration and cell adhesion
	FGFBP1	fibroblast growth factor binding protein 1	Cell differentiation and cell migration
	IGFBP4	insulin-like growth factor binding protein 4	
	ILK	integrin-linked kinase	Cell adhesion, cell migration and regulation of Wnt pathway
	NKX3-1	NK3 homeobox 1	
	PDGFRB	platelet-derived growth factor receptor, beta polypeptide	Cell migration and differentiation
	PTP4A1	protein tyrosine phosphatase type IVA, member 1	Cell differentiation and cell migration
	RASSF1	Ras association (RalGDS/AF-6) domain family member 1	
	ZEB1	zinc finger E-box binding homeobox 1	Cell differentiation and transcription factor
Migration and Motility	*CALD1*	caldesmon 1	

Related Gene Function	Gene Symbol	Gene Full Name	Other Functions
Migration and Motility	*MSN*	moesin	
	MST1R	macrophage stimulating 1 receptor	Cell differentiation, cell proliferation
	STAT3	signal transducer and activator of transcription 3	Cell proliferation and regulation of Notch pathway
	TSPAN13	tetraspanin 13	Cell growth
	VIM	vimentin	cytoskeleton organization
Cytoskeleton organization	*KRT7*	keratin 7	Cell differentiation
	KRT14	keratin 14	Cell differentiation, cell adhesion
	KRT19	keratin 19	Cell differentiation
	MAP1B	microtubule-associated protein 1B	
	PLEK2	pleckstrin 2	
Extracellular Matrix and Cell Adhesion	*CDH1*	cadherin 1, type 1, E-cadherin (apithelial)	
	CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)	
	CDH2	cadherin 2, type 1, N-cadherin (neuronal)	
	CDH3	cadherin 3, type 1, P-cadherin (placental)	
	COL1A2	collagen, type I, alpha 2	
	COL5A2	collagen, type V, alpha 2	
	DSC2	desmocollin 2	
	DSP	desmoplakin	cytoskeleton organization
	FN1	fibronectin 1	Cell migration and cell morphogenesis
	ITGA5	integrin, alpha 5	
	ITGAV	integrin, alpha V	Cell proliferation and cell migration
	ITGB1	integrin, beta 1	cell migration
	MMP2	matrix metallopeptidase 2	
	MMP3	matrix metallopeptidase 3	
	MMP9	matrix metallopeptidase 9	cell proliferation
	OCLN	occludin	
	PTK2	protein tyrosine kinase 2	Cell proliferation and cell migration
	SERPINE1	serpin peptidase inhibitor, clade E	Cell migration
	SPP1	secreted phosphoprotein 1	
	STEAP1	six transmembrane epithelial antigen of the prostate 1	Cell adhesion
	TFPI2	tissue factor pathway inhibitor 2	
	TIMP1	TIMP metallopeptidase inhibitor 1	Cell proliferation and cell migration
	VCAN	versican	Cell proliferation and migration
Signaling Pathways Notch	*FOXC2*	forkhead box C2	Cell differentiation, cell proliferation, cell adhesion

Related Gene Function	Gene Symbol	Gene Full Name	Other Functions
Signaling Pathways Notch	*JAG1*	jagged 1	Cell differentiation, cell morphogenesis
	NOTCH1	notch 1	Cell differentiation, cell morphogenesis
Signaling Pathways TGFβ	*BMP1*	bone morphogenetic protein 1	Cell differentiation, extracellular matrix organization
	BMP7	bone morphogenetic protein 7	Cell differentiation, cell proliferation
	COL3A1	collagen, type III, alpha 1	Extracellular matrix organization
	F11R	F11 receptor	Cell differentiation, cell adhesion
	SMAD2	SMAD family member 2	Cell differentiation, cell proliferation, cell morphogenesis
	SMAD4	SMAD family member 4	Cell differentiation, cell morphogenesis
	TGFB1	transforming growth factor, beta 1	Cell proliferation, cell migration, cell adhesion
	TGFB2	transforming growth factor, beta 2	Cell differentiation, cell proliferation, cell morphogenesis, cell migration, cell adhesion
	TGFB3	transforming growth factor, beta 3	Cell differentiation, cell proliferation, cell adhesion
Signaling Pathways Wnt	*CTNNB1*	catenin (cadherin-associated protein), beta 1	Cell morphogenesis, cell proliferation, cell adhesion, cell-extracellular matrix adhesion and WNT pathway
	DAB2IP	DAB2 interacting protein	Cell proliferation, cell migrations
	FZD7	frizzled family receptor 7	Cell differentiation, stem cell division, stem cell maintenance
	GSC	goosecoid homeobox	Cell differentiation and Wnt pathway
	GSK3B	glycogen synthase kinase 3 beta	Cell proliferation, cell migration
	RAC1	ras-related C3 botulinum toxin substrate 1	Cell morphogenesis, cell adhesion, cell-extracellular matrix adhesion, cytoskeleton organization and Wnt pathway
	SFRP1	secreted frizzled-related protein 1	
	SNAI2	snail homolog 2 (Drosophila)	Cell differentiation, stem cell proliferation, Notch pathway
	WNT11	wingless-type MMTV integration site family, member 11	Cell differentiation, cell proliferation, cell migration
	WNT5A	wingless-type MMTV integration site family, member 5A	Cell differentiation
	WNT5B	wingless-type MMTV integration site family, member 5B	Cell differentiation, cell migration
Other functions	*CAMK2N1*	calcium/calmodulin-dependent protein kinase II inhibitor 1	Calcium-dependent protein kinase inhibition
	GNG11	guanine nucleotide binding protein (G protein), gamma 11	Modulation/transduction of transmembrane signaling
	IL1RN	interleukin 1 receptor antagonist	Modulation of immune and inflammatory responses
	NUDT13	nudix (nucleoside diphosphate linked moiety X)-type motif 13	mitochondrial NADH diphosphatase

Related Gene Function	Gene Symbol	Gene Full Name	Other Functions
Other functions	*SIP1*	survival motor neuron interacting protein 1	Transcription factor
	SNAI3	snail homolog 3 (Drosophila)	Transcription factor
	VPS13A	vacuolar protein sorting 13 homolog A (S. cerevisiae)	Proteins transport
	ZEB2	zinc finger E-box binding homeobox 2	Transcription factor

Based on information available in *http://www.ncbi.nlm.nih.gov/*

Annex 2

Table 6- Results from Screening of 93 EMT related genes expression, $2^{-\Delta\Delta Ct}$.

elated Gene Function	Gene Symbol	$2^{-\Delta\Delta Ct}$	
		pT3b-PCa vs. NPT	pT3b-PCa vs pT2a+b-PCa
Differentiation and Development	*AHNAK*	0,82	0,6
	CD44	0,16	0,31
	ERBB3	2,11	0,76
	MITF	0,8	0,78
	NODAL	n.a.	n.a.
	POU5F1	0,39	1,13
	PROM1	0,14	0,38
	RARB	0,62	0,64
	RGS2	0,54	0,58
	SOX10	n.a.	n.a.
	TCF3	1,78	1,72
	TCF4	0,68	0,45
	TMEFF1	1,23	1,57
	TMEM132A	2,58	1,32
	TWIST1	6,8	0,86
Morphogenesis	*SNAI1*	0,28	1,19
	SPARC	1,77	2,91
Cell Growth and Proliferation	*AKT1*	1,73	1,01
	CAV2	0,6	0,81
	EGFR	0,35	0,76
	FGFBP1	n.a.	n.a.
	IGFBP4	0,82	1,29
	ILK	0,82	0,9
	NKX3-1	4,01	1
	PDGFRB	0,53	1,92
	PTP4A1	1,31	1,25

elated Gene Function	Gene Symbol	$2^{-\Delta\Delta Ct}$	
		pT3b-PCa vs. NPT	pT3b-PCa vs pT2a+b-PCa
Cell Growth and Proliferation	*RASSF1*	0,94	1,08
	ZEB1	0,77	0,66
Migration and Motility	*CALD1*	0,4	0,59
	MSN	0,74	1,35
	MST1R	n.a.	n.a.
	STAT3	1,02	0,88
	TSPAN13	5,39	1,24
	VIM	0,73	1,11
Cytoskeleton organization	*KRT7*	0,17	0,71
	KRT14	0,19	0,28
	KRT19	0,83	1,13
	MAP1B	0,58	1,81
	PLEK2	0,97	0,66
Extracellular Matrix and Cell Adhesion	*CDH1*	1,33	0,7
	CDH11	1,41	1,52
	CDH2	0,66	1,39
	CDH3	0,37	0,95
	COL1A2	1,12	5,26
	COL5A2	2,66	3,11
	DSC2	6,08	0,86
	DSP	1	0,84
	FN1	0,58	1,38
	ITGA5	0,21	0,65
	ITGAV	1,66	0,9
	ITGB1	0,96	0,79
	MMP2	0,5	1,56
	MMP3	n.a.	n.a.

elated Gene Function	Gene Symbol	$2^{-\Delta\Delta Ct}$	
		pT3b-PCa vs. NPT	pT3b-PCa vs pT2a+b-PCa
Extracellular Matrix and Cell Adhesion	*MMP9*	0,65	0,23
	OCLN	8,63	1,55
	PTK2	1,37	0,93
	SERPINE1	0,85	1,63
	SPP1	2,53	0,43
	STEAP1	3,58	0,72
	TFPI2	0,7	0,78
	TIMP1	1,31	0,99
	VCAN	2,69	5,94
Signaling Pathways Notch	*FOXC2*	0,55	1,02
	JAG1	1,29	1,53
	NOTCH1	0,4	1,38
Signaling Pathways TGFβ	*BMP1*	0,23	0,93
	BMP7	0,42	0,6
	COL3A1	1,54	2,31
	F11R	1,04	0,93
	SMAD2	1,81	0,92
	SMAD4	0,99	0,63
	TGFB1	0,79	1,87
	TGFB2	0,46	1,02
	TGFB3	0,21	0,26
Signaling Pathways Wnt	*CTNNB1*	1,25	0,73
	DAB2IP	0,3	0,78
	FZD7	0,49	1,27
	GSC	0,69	1,68
	GSK3B	2,31	0,91
	RAC1	1,83	1,16

elated Gene Function	Gene Symbol	$2^{-\Delta\Delta Ct}$	
		pT3b-PCa vs. NPT	pT3b-PCa vs pT2a+b-PCa
Signaling Pathways Wnt	*SFRP1*	1,59	1,33
	SNAI2	0,17	0,54
	WNT11	0,49	2,91
	WNT5A	2,68	9,16
	WNT5B	0,39	0,58
Other functions	*CAMK2N1*	4,76	4,04
	GNG11	1,03	0,54
	IL1RN	0,82	2,13
	NUDT13	n.a.	n.a.
	SIP1	1,63	1,01
	SNAI3	0,13	0,94
	VPS13A	1,99	1,52
	ZEB2	0,46	0,71

Abbreviations: not available (*n.a.*)