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MICRORNA PROMOTER METHYLATION:

POTENTIAL BIOMARKERS IN PROSTATE CANCER

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MicroRNA promoter methylation: potential biomarkers in Prostate Cancer

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"Success consists of going from failure to failure without loss of enthusiasm"

Winston Churchill

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Resumo

Introdução: O cancro da próstata (CaP) é uma doença heterogénea, sendo, atualmente, a neoplasia maligna não-cutânea mais comum em homens nos países desenvolvidos e a sexta principal causa de morte por cancro a nível mundial. O conhecimento sobre o processo de iniciação e progressão da doença é ainda limitado, sendo reconhecida a necessidade de desenvolver e aperfeiçoar métodos inovadores para apoio à deteção precoce, diagnóstico e decisão terapêutica. A descoberta da metilação do DNA como mecanismo de regulação génica essencial à homeostasia celular e cuja desregulação constitui um evento comum na carcinogénese, tem possibilitado uma maior compreensão da biologia do CaP. De facto, diversos estudos revelaram que a alteração dos padrões de metilação nos promotores dos genes ocorre em etapas precoces do desenvolvimento do CaP. Os microRNAs constituem um outro mecanismo de regulação da expressão génica e cuja desregulação está também relacionada com a carcinogénese. A expressão desta classe de RNAs não codificantes de cadeia curta pode ser, igualmente, regulada por meio da metilação do DNA. Contudo, o potencial desta alteração como biomarcador de CaP não foi, até à data, cabalmente explorado.

Objetivos: Determinar, em doentes de CaP, o potencial como biomarcador diagnóstico e prognóstico da quantificação da metilação de regiões promotoras de microRNAs recentemente identificados.

Materiais e métodos: A análise prévia através de HumanMethylation450 BeadChip permitiu a identificação dos dinucleótidos CpGs mais diferencialmente metilados localizados em regiões promotoras de microRNAs. A partir desta análise, os níveis de metilação das regiões promotoras de miR-34b/c, miR-129-2, miR-152, miR-193b, miR-663a e miR-1258 foram selecionados para validação através de qMSP, numa série alargada de tecidos prostáticos. Os níveis de metilação das regiões promotoras destes microRNAs foram igualmente analisados em amostras tumorais e não tumorais de bexiga e rim a fim de avaliar a sua especificidade para CaP. Posteriormente, os níveis de metilação das regiões promotoras de miR-34b/c, miR-193b e miR-1258 foram avaliados em sedimentos urinários, e os de miR-34b/c e miR-129-2 em biópsias prostáticas, para determinação do seu valor diagnóstico e prognóstico, respetivamente.

Resultados: Foi confirmada a elevação significativa dos níveis de metilação dos promotores dos microRNAs candidatos (selecionados a partir do *array*) em CaP comparativamente com tecido prostático normal. Excetuando miR-152, todos os candidatos apresentaram valores de AUC superiores a 0.90 nesta série de amostras. O miR-193b e miR-1258 demonstraram os melhores resultados, com valores de AUC=0.96 e AUC=0.99, respetivamente. Globalmente, níveis de metilação mais elevados associaram-se a estadio patológico mais avançado, tendo os níveis de metilação de miR-129-2 associado com grau combinado de Gleason mais elevado. Quando testados em amostras de tecido vesicais e renais, quer normais, quer neoplásicos, o miR-129-2 e miR-663a apresentaram níveis de metilação mais elevados em tumores vesicais comparativamente a CaP. Dos candidatos testados em sedimentos urinários, o miR-193b demonstrou melhor desempenho, com AUC=0.96, 91.6% de sensibilidade e 95.7% de especificidade. Numa série de amostras de biópsias prostáticas, níveis de metilação mais elevados do miR-129-2 demonstraram ser preditores independentes de menor sobrevivência específica e livre de doença.

Discussão: A validação dos resultados do *array* confirmou a quantificação da metilação dos promotores de miR-34b/c, miR-193b e miR-1258 como biomarcadores específicos de CaP em amostras de tecido. Para efeitos de deteção precoce, sendo urina a amostra ideal, a quantificação dos níveis de metilação do promotor de miR-193b demonstrou um desempenho comparável ao dos melhores biomarcadores epigenéticos para CaP até à data descritos. No que respeita à avaliação do valor prognóstico, elevados níveis de metilação do promotor de miR-129-2 demonstraram ser preditores independentes de um menor intervalo de tempo até recorrência bioquímica e mortalidade devida à neoplasia, sendo apenas superado pelo estadio clínico.

Conclusão: Neste estudo, foi demonstrado que a quantificação dos níveis de metilação dos promotores dos miR-193b e miR-129-2 tem potencial utilidade como biomarcadores de diagnóstico e prognóstico, respetivamente. Assim, poderão constituir ferramentas úteis no apoio à avaliação clínica e decisão terapêutica de pacientes com CaP. Contudo, a confirmação deste valor clínico requer validação em séries independentes.

Summary

Background: Prostate cancer (PCa) is a heterogeneous disease that constitutes the most common cancer in men from western countries and the sixth leading cause of death by cancer among men worldwide. Current knowledge about its onset and progression is still limited and better tools for improved diagnosis and therapeutic decision-making are needed. Insight into DNA methylation has shed new light on PCa biology. This gene regulation mechanism is essential for cellular homeostasis and its alteration is common in cancer. Indeed, altered patterns of DNA methylation occur at early steps of PCa development, including those occurring at gene promoter regions. MicroRNAs are a class of small non-coding RNAs involved in gene expression regulation and its deregulation has been implicated in tumorigenesis. MicroRNA expression regulation by DNA methylation has been previously reported but its potential use as PCa biomarker has not been systematically explored.

Aims: To assess the diagnostic and prognostic biomarker potential of quantitative promoter methylation of newly identified microRNAs in PCa patients.

Materials and methods: HumanMethylation450 BeadChip-based analysis previously enabled the identification of the most differentially methylated CpGs in known microRNA promoter regions. From this analysis, methylation levels in miR-34b/c, miR-129-2, miR-152, miR-193b, miR-663a e miR-1258 promoter regions were selected to be validated by qMSP in a large set of tissue samples. Promoter methylation levels of those microRNAs were also assessed in bladder and kidney non-tumorous and tumorous samples, to determine its PCa-specificity. Then, promoter methylation levels of miR-34b/c, miR-193b and miR-1258 were evaluated in urine sediments and miR-34b/c and miR-129-2 in biopsy samples to test for diagnostic and prognostic value, respectively.

Results: Higher promoter methylation levels in PCa compared to normal prostate were confirmed for all six candidate microRNAs selected from the array. Except for miR-152, all candidates displayed AUC values higher than 0.90 in this sample set. MiR-1258 and miR-193b disclosed the best performance with AUC=0.99 and AUC=0.96, respectively. Higher methylation levels of all candidates,

excepting miR-152, correlated with higher pathological stage. Moreover, higher miR-129-2 methylation levels also correlated with a higher Gleason grade. When tested in bladder and kidney tissues (normal and neoplastic), miR-129-2 and miR-663a showed higher methylation levels in bladder cancer compared to PCa. Of the candidates tested in urine samples, miR-193b showed the best performance, with AUC=0.96, 91.6% sensitivity and 95.7% specificity. In a prostate biopsy sample set, higher miR-129-2 methylation levels independently predicted for shorter DSS and DFS. Although a similar trend was apparent for miR-34b/c, it did not achieve statistical significance.

Discussion: In the initial validation of the array results, miR-34b/c, miR-193b and miR-1258 promoter methylation levels were shown to be PCa-specific biomarkers in tissues, emerging as promising candidates for subsequent testing. For early diagnosis purposes, urine is an ideal sample and miR-193b quantitative promoter methylation demonstrated biomarker performance similar to the best epigenetic biomarkers thus far reported for PCa. Concerning assessment of prognosis, high miR-129-2 promoter methylation levels independently predict for shorter time to biochemical recurrence and decreased disease-specific survival. Its performance as prognostic biomarker was only surpassed by clinical stage.

Conclusion: Herein, we demonstrate that quantitative miR-193b and miR-129-2 promoter methylation have potential clinical usefulness as early diagnostic and prognostic biomarkers, respectively. Nevertheless, these findings require validation in larger and independent sample sets.

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List of abbreviations

ACTβ – Actin β

ADT – Androgen deprivation therapy

AFMS - Anterior fibromuscular stroma

APC – Adenomatous poliposis coli

AR – Androgen receptor

AUC – Area under the curve

BlCa – Bladder cancer

BPH – Benign prostatic hyperplasia

CGI – CpG islands

DNA – Deoxyribonucleic acid

DNMT - DNA (cytosine-5)-methyltransferase

DFS – Disease-free survival

DRE – Digital rectal examination

DSS – Disease-specific survival

ER – Estrogen receptor

FDA – Food and Drugs administration

FFPE – formalin-fixed paraffin embedded

GSTP1 – Glutathione S-transferase pi 1

HCC – Hepatocellular carcinoma

hK2 - kallikrein-related peptide 2

LH - luteinizing hormone

LHRH - luteinizing hormone-releasing hormone

MSP – Methylation-specific PCR

NPV - Negative predictive value

NSCLC – Non-small cell lung carcinoma

PAP – Prostatic acid phosphatase

PCa – Prostate cancer

PCA3 – prostate cancer antigen 3

PC8 - Phenol-chlorophorm at pH8

PCR – Polymerase chain reaction

PIN - Prostatic intra-epithelial neoplasia

PPV - Positive predictive value

PSA – Prostate-specific antigen

qMSP – quantitative methylation-specific PCR

RARβ2 - Retinoic Acid Receptor B

RASSF1A - RAS association domain family protein 1 isoform A

RCC – Renal cell carcinoma

RCT – Renal cell tumor

RISC - RNA-induced silencing complex

RNA - Ribonucleic acid

ROC – Receiver operating characteristic

RP – radical prostatectomy

SDS – Sodium dodecyl sulfate

TRBP - transactivating response RNA-binding protein

TRUS – Transrectal ultrasonography

Introduction

1 The prostatic gland

The prostate is a retroperitoneal organ that surrounds the bladder neck and the urethra, being posteriorly separated from the rectum by the Denonvillier's fascia [5, 6]. It has a pear-like shape, with its base at the bladder neck and the apex at the urogenital diaphragm, weighting about 20g in the adult male with a volume around 25cm^3 [5-7].

The prostate, as an exocrine organ, consists of glands secreting a fluid that comprises the bulk of the seminal emissions and is emptied into the urethra. Due to its location and function, the development of prostatic cancer as well as its treatment options brings risks to the sexual, urinary and bowel functions [7].

Anatomically, the prostate gland is a heterogeneous organ. Throughout the twentieth century, the regional anatomy of the prostate did not gather consensus between investigators. In 1981 McNeal proposed the currently accepted model of zonal organization [5, 8]. According to this model, four basic anatomic regions can be identified. The peripheral zone constitutes over 70% of the glandular component and is located at the lateral and posterior sides of the organ. The vast majority of carcinomas, as well as the other pathologies affecting this organ, arise from this region [8]. The central zone represents 25% of the glandular prostate and is located posteriorly to the urethra surrounding the ejaculatory ducts. There are marked architectural differences between the central and peripheral zones, suggesting different biological roles [8]. The transition zone is centrally located, surrounding the prostatic urethra. Finally, the anterior fibromuscular stroma (AFMS) which is a band of fibromuscular tissue, contiguous with the bladder's smooth muscle and the external sphincter, forming the anterior surface of the gland as a thick, non-glandular layer [7, 8]. A partial capsule encloses the posterior and lateral sides of the prostate while the anterior and apical surfaces are covered by the AFMS [7].

Concerning the gland's histology, its architecture is that of a branched duct gland embedded in a fibromuscular stroma [5]. Prostate epithelium is composed of two layers of cells: secretory luminal cells (PSA producing and androgen receptor positive) and basal cells, with rare neuroendocrine cells being present as well [7, 9].

2 Prostate cancer

Prostate cancer (PCa) is the most common male cancer in western countries [10]. PCa is an age-related and a very heterogeneous disease, both genetically as well as clinically, ranging from relatively indolent to highly aggressive tumors. This disease is typically asymptomatic, thus leading to commonly late diagnosis which, in turn, impairs not only prognosis but also therapeutical strategies [11].

2.1 Epidemiology

Currently, PCa is a major health concern in western countries standing as the second most common cause of cancer and the sixth leading cause of death by cancer among men, worldwide [12]. The burden brought by this malignancy is increasing simply due to the global population's growth and aging [13]. In 2012, the number of PCa cases diagnosed worldwide accounted to approximately 15% of all new cases of cancer diagnosed in men [14]. The American Cancer Society yearly predictions for 2015 suggest that prostate cancer alone will account for 26% (about 220,800) of newly diagnosed cancer cases and 9% (27,540) deaths in men in the USA alone [15].

Geographically, PCa incidence and mortality rates have a highly heterogeneous distribution. Specifically, incidence rates vary more than 25-fold worldwide with the highest rates being observed in North-America, Europe and Oceania mainly due to wide use of PSA based screening [12]. Temporal trends are influenced by usage of PSA testing as a diagnostic tool. Soon after the introduction of PSA testing, incidence rates rapidly arose followed by a sharp decline. This pattern was particularly evident in the USA, Canada, Australia and Nordic countries. On the other hand, in countries such as the UK and Japan, with a low and gradual increase in the prevalence of PSA-testing, rates continue to increase slightly [12]. Death rates have been declining in high-income countries, in part due to improvements in treatment with curative intent. The role of PSA-testing in this trend remains elusive with studies in Europe and the USA reporting little to no gains. On the other hand, mortality rates are increasing rapidly in Asian, central and eastern European countries [12, 13].

2.2 Risk factors

Currently, there are only three risk factors well-established for PCa, namely: age, race and family history. However, smoking and increased body-mass have also been found to be associated with PCa, particularly with more aggressive tumors. Other factors, including exposition to ionizing or ultraviolet radiation, endogenous hormones and urinary tract infections were suggested to be linked to PCa, but still require additional studies for confirmation [16].

2.2.1 Aging

Age is the most important factor in PCa. In unscreened populations, PCa has the steepest age-dependent incidence curve, with an increased slope in the seventh decade of life [16]. Similarly to other epithelial malignancies, PCa is a disease mainly related to aging, commonly afflicting men above 65 years of age with diagnosis earlier than this age being rare and incidence peaking between 70 and 74 years old [16, 17]. Cases in men with less than 50 years of age are extremely uncommon, accounting for 0.1% of all cases [17].

2.2.2. Ethnic origins

Incidence and mortality rates vary significantly between races. Compared to their Caucasian counterparts, African-american individuals have a younger median age of diagnosis, 58% higher incidence and 144% greater mortality. On the other hand, individuals with Hispanic ancestry show 14% lower incidence and 17% lower mortality. Individuals of Asian origin appear to have a lower risk to develop the disease. For instance, Asian, Indians and Pakistanis living in the USA have a standardized incidence ratio of 0.54 when compared to American Caucasian populations [16].

2.2.3. Familial and genetic factors

Men with strong family history of PCa have two to four times higher incidence, when compared with control groups. This risk is particularly higher in patients with a first degree relative who was afflicted with the disease and also with relatives that were diagnosed at younger ages [18]. Association with X-chromosome has also been suggested. It has been found that individuals with a brother that harbored the disease have higher risk of developing this malignancy comparing to individuals in which the father was a PCa patient [18]. Although rare, *BRCA2* mutations increase by 8.6 times the risk of developing the disease at an age below 65 years and are correlated with a more aggressive behavior [16, 18]. Other

mutations that confer higher risk, albeit rare, have been reported in *BRCA1*, *HOXB13*, *NSB1* and *CHEK2* genes [16].

2.3 Detection and diagnosis

The adoption and effectiveness of a screening program for PCa is still a matter of debate [12, 19]. In many countries, screening programs based on PSA testing in combination with digital rectal examination have been introduced [19]. A suspicion of PCa usually arises on the basis of DRE and PSA levels although a definitive diagnosis is always dependent in cytological or histological analysis from core biopsies or surgical specimens [20].

2.3.1. DRE

Since most prostate cancers are located in the peripheral zone, their detection is possible by DRE once their volume is 0.2mL or higher. In about 18% of the patients, PCa detection is made by DRE, irrespective of the patients PSA values. Abnormal DRE is associated with a higher Gleason score and, thus, is an indication for prostate biopsy [20].

2.3.2. PSA

Usage of PSA as a serum marker revolutionized PCa detection. This enzyme is produced by prostatic epithelial cells and is organ, but not cancer, specific. Therefore, PSA serum levels may be increased in benign conditions such as BPH and prostatitis [20, 21]. The PSA value is a continuous parameter and a higher PSA value represents a higher likelihood of PCa existence. Currently, a positive PSA test with a value greater than 3 to 4 ng/mL is indicative for prostate biopsy [19, 20].

2.3.3. Histopathological examination: the Gleason Score

The standard way to obtain material for histopathological examination is ultrasound-guided transrectal or transperineal 18G core biopsy [20]. On the histological analysis of the cores obtained, the glands are typically smaller and are lined by a single layer of cuboidal or low columnar cells. They have a more crowded aspect and are devoid of the basal cell layer that is present on the normal architecture and also in the benign afflictions of the gland. Nuclear size is increased and one or two nucleoli may be present [6].

Prostatic adenocarcinomas are often multifocal and heterogeneous neoplasms [20]. In this context, the Gleason Score was developed. Gleason's grading evaluates the architectural features of the cancer glands, categorizing them into five distinct patterns from well to poorly differentiated. The Gleason Score is then obtained as the sum of the two most common growth patterns observed in the specimen, incorporating both a primary (the most prevalent) grade and a secondary (next most prevalent) grade, and ranging from 2 (1+1) to 10 (5+5) [5, 7].

Both in radical prostatectomy specimens and needle biopsy samples, the Gleason Score is currently the strongest prognostic factor [20].

Albeit being globally accepted and used, the Gleason system displays several limitations. In particular, the Gleason grading is observer dependent, varying with the experience of the pathologist. Moreover, nowadays, most of the diagnosed patients fall in the Gleason 6-7 category, an intermediate prognostic range that limits the usefulness of the 10-point scale [11]. On the other hand, in neoplasms that have a minor high grade component, a tertiary Gleason Score may be reported, since it carries significant prognostic information [7]. Although the Gleason Score is the most powerful prognostic factor, other features must be taken into account during histopathological evaluation such as the presence of extracapsular invasion, perineural invasion, surgical margin status, lymph node status and seminal vesicle invasion [11].
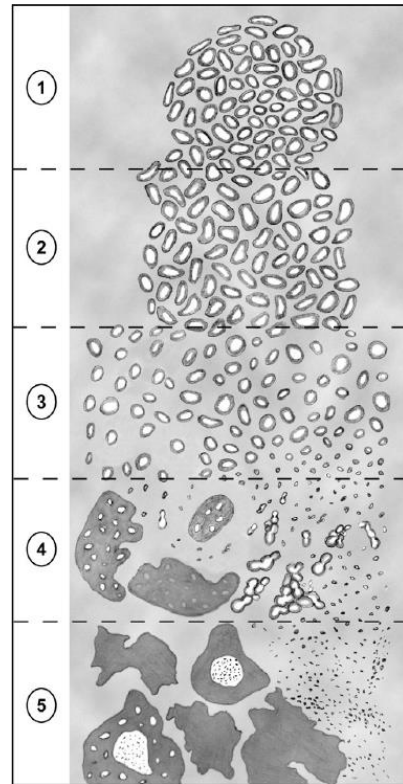
Figure 1 - The Gleason grades range from closely packed and uniform glands (grade 1) to almost complete loss of glandular differentiation (grade 5). Adapted from [1].

2.4 Clinical and pathological staging

Cancer staging is a key factor to ascertain prognosis and determining treatment. Several cancer staging systems are used worldwide and the most clinically useful is the tumor node metastasis or TNM system [22]. This system classifies cancers by the size and extent of the primary tumors (T), the presence of

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involvement of regional lymph nodes (N) and presence or absence of distant metastases (M) [22].

There are two types of staging: the clinical and pathological. In prostate, clinical staging is determined mainly from data collected by DRE, TRUS and magnetic resonance imaging, although PSA level is also a factor taken into account. Clinical staging remains unchanged apart from the pathological stage which is, in turn, ascertained after radical prostatectomy and its data is provided by macroscopic and microscopic observation of the surgical specimen and dissected lymph nodes [23].

In PCa, T-staging is the first and most important since the distinction between organ confined (T1-T2) and extraprostatic disease (T3-T4) is the most impactful aspect on treatment decisions [20]. On the other hand, N-staging should only be pursued in cases where the potential findings will impact treatment decisions, particularly in patients where curative treatments are planned. The gold standard for N-staging is surgical lymphadenectomy although computer tomography and magnetic resonance imaging may be used [20].

Table 1 - Pathological staging of prostate cancer. Adapted from [19].

PRIMARY TUMOR (T)	
Clinical	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent tumor neither palpable nor visible by Imaging
T1a	Tumor incidental histologic finding in 5% or less of tumor resected
T1b	Tumor incidental histologic finding in more than 5% of tumor resected
T1c	Tumor identified by needle biopsy
T2	Tumor confined within prostate gland
T2a	Tumor involves one half of one side 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 prostate capsule
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as: external sphincter, rectum, bladder, levator muscles, and/or pelvic wall

Pathological (pT)*	
pT2	Organ confined
pT2a	Unilateral, involving one-half of one side or less
pT2b	Unilateral, involving more than one-half of one side but not both sides
pT2c	Bilateral disease
pT3	Extraprostatic extension
pT3a	Extraprostatic extension or microscopic invasion of bladder neck
pT3b	Seminal vesicle invasion
pT4	Invasion of rectum, elevator muscles and/or pelvic wall
REGIONAL LYMPH NODES (N)	
Clinical	
Nx	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
Pathological	
pNx	Cannot be assessed
pN0	No positive regional nodes
pN1	Metastasis in regional node(s)
DISTANT METASTASIS (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

***There is no pathologic pT1 classification**

2.5 Treatment

Currently, the difference between PCa incidence and death rates is quite considerable. Many autopsy studies showed that 60-70% of aged men who die from different causes, harbor histological PCa, with an estimated 10-15% of these having a Gleason score of 7 or higher. Indeed, it is estimated that about 15-20% of men are diagnosed with PCa during their lifetime, but only 3% of those men die from it. At the same time, the incidence of small localized tumors is increasing, essentially as a result of early screening procedures. Therefore, there is a number of men who do not need aggressive and definitive forms of treatment. On the other hand, in patients with limited co-morbidities and limited life-expectancy, the need of a

definitive treatment is questionable. Thus, the risk of overtreatment, ie, treatment of a disease that will not pose a threat to the patient's well-being during his lifetime, is one of the major issues of PCa screening [24, 25]. As a result, two different approaches have been proposed: active surveillance and watchful waiting. Active surveillance was developed aiming to reduce overtreatment in patients with clinically confined low-risk PCa that are surveilled, but curative treatment is still available when needed. Therefore, active surveillance should only be applied to very carefully selected low-risk patients, specifically with PSA levels below 10ng/mL, with a Gleason score lower than 7 and clinical stage of cT1c to cT2a [24, 26].

Watchful waiting comes from the knowledge that PCa is, usually, a slow progression disease. It is a strategy to avoid radical treatment in patients with serious co-morbidities from other diseases and/or less than a 5-year life expectancy [24]. Thus, tumor stage is of high importance, with tumor stage 3 having very low survival rates and stages 1 and 2 showing a better 10-year cancer-specific survival [24].

Concerning active treatment, radical prostatectomy (RP) and radiation therapy are the most common approaches for localized PCa. Active treatments are largely curative, despite the concern about overtreatment [24, 26].

RP is the surgical treatment for PCa. This surgery consists in removing the whole gland, the seminal vesicles and enough surrounding tissue to ensure negative margins. This procedure may be accompanied by bilateral pelvic lymph node dissection. In patients with localized disease and life expectancy higher than 10 years, the goal of this procedure is curative treatment with the preservation of continence and, if possible, potency. RP remains the only treatment for localized disease that showed cancer-specific benefit in comparison with watchful waiting in a prospective randomized trial. However, it must be taken into account that, so far, the benefit of RP over watchful waiting has only been observed in patients with intermediate or high-risk PCa [20].

Radiation therapy is a valid alternative to surgery as a curative therapy. External radiation beam therapy provides a quality of life comparable to surgery. Transperineal low-dose or high-dose rate brachytherapy is also widely used. Curiously, there have been no randomized studies comparing the radical prostatectomy with radiation therapy in localized disease [20].

Metastatic PCa has a poor prognosis, although it depends highly on the extent of the disease. Minimal metastatic disease has been defined as involvement of the axial skeleton and lymph nodes while extensive disease involves the viscera and/or appendicular skeleton [25].

For more than half a century, androgen deprivation therapy has been the standard treatment for patients with metastatic disease. This therapy originated from the demonstration by Huggins in 1941 that orchiectomy or estrogens could induce dramatic remissions in advanced PCa. Posteriorly, it was found that sustained LHRH administration leads to a down-regulation of LH secretion which in turn causes a decline in the levels of testosterone [25].

Nevertheless, the median duration of response to ADT is, approximately, 18-24 months, after which most cases progress to a form of disease called castration-resistant prostate cancer. This late form of the disease is extremely aggressive rendering a rather grim prognosis with some studies reporting that without treatment median survival time is about 9 to 21 months [27].

Due to the complexity of PCa as a disease and the multiple variables that affect its outcome, the prediction and adequate management of each patient is still one of the problems in clinical practice. An approach to this difficulty has been the development of several multivariable models for disease progression prediction, based in the clinico-pathological information available at the time of diagnosis [28]. Many of those models have been in the form of nomograms, one of the most prominent being CAPRA score [29].

Proposed by Cooperberg *et al.* in 2005, this nomogram aims to predict the pre-operative probability of biochemical-recurrence-free survival after radical prostatectomy in patients with clinically localized prostate cancer [29]. This model, based in the PSA level, Gleason score, clinical stage, percentage malignant cells in biopsy core samples and patients' age at diagnosis to generates a numerical value from 0 to 10. Values between 0-2, correspond to low-risk tumors, 3 to 5 intermediate and tumors above 5 are considered to have a high-risk for biochemical relapse [30].

Different validation studies have confirmed its ability to correctly predict biochemical recurrence at 3 years after radical prostatectomy. Some studies reported that this ability can be extended to 5 years, although this is still controversial as some authors argue that this model significantly under-predicts

biochemical recurrence 5 years after RP [29]. Moreover, CAPRA score has been also shown to accurately predict cancer-specific mortality and metastasis development in patients subjected to other therapeutic strategies, namely radiation therapy, androgen deprivation or even without treatment (watchful waiting /active surveillance) [30].

In an attempt to improve 5-year predictive value, additional predictive variables were added to the original nomogram. This revised score also includes surgical margins of the surgical specimen, seminal vesicle invasion, extracapsular extension and lymph node invasion [31]. This revised model appears to improve the original's prediction capabilities although further validation is needed [29].

3 Epigenetics and cancer

Epigenetics is currently defined as the mechanisms that initiate and maintain heritable patterns of gene function and regulation in a heritable manner without interference in the genome sequence, hence the name: *epi* – an ancient Greek word meaning "what stays beyond" – *genetics* [32]. These mechanisms contribute to the cell diversity observable inside a single organism, despite all cells keeping the same genetic information [33].

Currently, three epigenetic mechanisms are known: DNA methylation, histone covalent modification and non-coding RNAs. Together, these mechanisms constitute the epigenetic code, modulating the expression of the mammalian cell genome through developmental stages and various diseases, including cancer [4].

Epigenetics is an emergent field of research, which in the last two decades expanded rapidly, bringing new insights into cancer research [34]. Various discoveries *in vitro*, *in vivo* and human clinical and epidemiological studies have challenged the classical view of cancer as a genetic disease, and as such, epigenetic deregulations are now considered a hallmark of cancer [32, 35].

3.1 DNA methylation

The most widely studied epigenetic mechanism, DNA methylation was the first epigenetic alteration to be associated with cancer [32, 35]. In mammals, DNA methylation occurs mainly by the addition of a methyl group (CH_3) to the 5' carbon of a cytosine nucleotide preceding guanine, originating 5-methylcytosine (5mC) in

CpG dinucleotides [36]. Additionally, this modification may interfere with the binding of transcription factors [4].

The cytosine methylation reaction is catalyzed by the DNMT family of enzymes, particularly DNMT1, DNMT3A and DNMT3B being S-adenosyl-methionine the methyl group donor. It is known that DNMT3A and DNMT3B are responsible for establishing DNA methylation patterns during embryo development while DNMT1 is involved in the maintenance of these patterns [4].

CpG dinucleotides are not randomly nor evenly distributed throughout the genome. Instead, they tend to be concentrated either in short CpG-rich fragments or in long repeats called "CpG islands" or CGI [35]. There two criteria required to consider a certain DNA stretch as a CGI, namely to have more than 200 bases with a CG content of at least 50% and a ratio of expected CpG frequency of at least 0.6 [37]. About 60% of human gene promoters contain CGI and DNA methylation in CGI within gene promoters correlates with condensed chromatin structure leading to gene silencing either inhibiting transcription factor binding or by attracting DNA-binding proteins which in turn recruit repressive complexes [38]. However, methylation within the gene body does not block gene's transcription but instead appears to promote transcription elongation and impacts in splicing. Moreover, methylation in repetitive regions, such as centromeres, is crucial for chromosomal stability. Strikingly 70-80% of these regions are methylated in the human genome [39].

During the development and differentiation stages, CpG islands are not methylated. However, in normal, mature tissues, some CpG islands in promoter regions can be methylated leading to permanent silencing of target genes. Therefore, the methylation pattern of a given tissue is acquired during differentiation and this pattern is tissue-specific [33, 35].
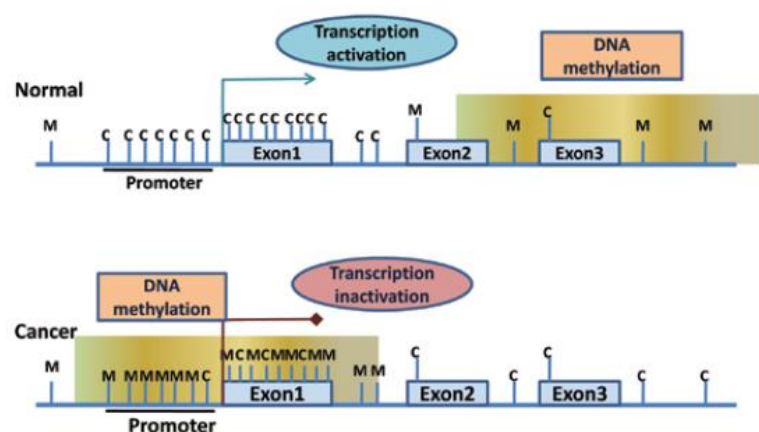

Figure 2 - Global changes in DNA methylation from normal to cancer cells. In normal conditions, gene promoters usually are not methylated allowing gene expression. In cancer, methylation patterns are altered, with promoter hypermethylation and hypomethylation of gene bodies and noncoding regions. Adapted from [4].

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These specific methylation patterns may change in disease. These changes can be divided in into three categories: promoter-specific hypermethylation, global hypomethylation and loss of imprinting when comparing a pathological tissue with its normal counterpart [32].

DNA hypermethylation is generally the gain of methylation in regions where, in normal conditions, there is no methylation and has been associated with loss of gene expression, occurring fundamentally on tumor suppressor genes [32].

Hypomethylation is the loss of methylation and happens mainly in genome-wide regions, being a well-established trait of cancer cells. DNA hypomethylation in cancer was discovered before DNA hypermethylation and its effects have greater impact [36]. It occurs in many gene-poor areas containing repetitive elements, retrotransposons leading to genome instability and causing translocations and chromosome rearrangements [32, 36].

Loss of imprinting consists in the loss of allele-specific monoallelic expression of particular genes because of aberrant hypomethylation in one of the two parental alleles [32]. A classic example is the loss of imprinting at the *IGF2* locus in colorectal carcinoma [40].

3.1.1 DNA methylation in prostate cancer

Aberrant methylation has been established as a hallmark of cancer, both at individual genes and at a global genomic scale [41, 42]. The decreased levels of methylation in tumors comparatively to their normal-tissue counterparts was one of the first epigenetic alterations discovered in human cancer. It is also known that, during the progression of a neoplasm from a benign proliferation of cells to a malignant and invasive cancer, the degree of genomic hypomethylation increases [36]. In turn, these changes may favor mitotic recombination causing deletions and translocations, and promote chromosome recombination [43].

As previously stated, hypermethylation of CpG islands in the promotor region of a gene may lead to its inactivation. In many cancers, the inactivation of tumor-suppressor genes by this mechanism is a major event. However, hypomethylation is a general feature of cancer, whereas CGI hypermethylation is highly specific to each cancer type, to the point that every tumor type may be assigned a specific DNA "hypermethylome". Inactivation of tumor-suppressing genes by methylation occurs both in sporadic as well as in hereditary cancers where hypermethylation may be the second hit in Knudson's two-hit model [43].

So far, the reason why some CGIs are methylated in some cancers and not in others remains unknown. Some DNA sequences show "opposite" methylation levels depending of the type of cancer such as *NBL2* and *D4Z4*, two DNA repeats that are hypermethylated in certain cancers and hypomethylated in others. Another case is the hypomethylation of the justacentromeric *Sat2* repeat, which is observed in gastric and breast cancer but not in colon cancer [36].

Concerning PCa, several methylation abnormalities have already been reported. Global hypomethylation has been observed in PCa which may have the already described effects in these cancer cells. Moreover, this hypomethylation is considerably higher in patients with metastatic disease when compared with non-metastatic neoplasms [4]. Gene specific hypomethylation has also been described in PCa affecting genes involved in a wide range of cell functions: from invasion, to metastasizing properties, cell cycle control and activation of carcinogens [4].

Gene hypermethylation has been widely studied and found to occur at a large scale. Hypermethylated genes in PCa are involved in many biological processes such as DNA repair (*GSTP1*), signal transduction (*RASSF1A*), cell adhesion (*E-cadherin*, *CD44* and *galectins*), hormonal response (*RARβ* , *AR* and *ER*), apoptosis (death-associated protein kinase), invasion, metastasis and cell cycle control [4].

3.2 Covalent modifications of histones

In the eukaryotic cell, DNA is packed in the nucleus. However, it must be tightly regulated and then copied during cell division. Vital to both packaging and overseeing DNA stability are the histones. About 147 base pairs of DNA wrap around an octamer of histones which consist of two copies of H3, H4, H2A and H2B, forming the building block of chromatin, the nucleosome. Another histone protein, H1, binds to both the nucleosome and to the "linker DNA" thus keeping in place the DNA wrapped around the nucleosome [44].

Chromatin is a dynamic macromolecule with two distinct domains: heterochromatin and euchromatin, defined by the level of compaction and consequent genomic functions. Therefore, euchromatin is a more loose conformation allowing a higher level of transcription whereas heterochromatin (either constitutive or facultative) is the more condensed form and is typically transcriptionally repressive [44].

Chromatin organization and regulation is achieved by numerous factors. Of particular interest are histone post-translational modifications. Typically, these occur in the C- and N- terminal domains, which protrude from the core of the nucleosome. However, a significant amount occur in the fold or globular domains of the protein thus interfering in the histone-histone and histone-DNA interactions. Several post-transcriptional modifications have been reported such as methylation, acetylation, phosphorylation, deamination, ubiquitylation, glycosylation and sumoylation [45].

Globally, each post-transcriptional modification confers different effects. Particular marks, such as acetylation traditionally leads to transcriptional activation [46]. Histone methylation, for instance, is a more complicated field than acetylation leading either to repression or promotion of gene transcription depending of the target residue and even of the degree of its methylation [47]. Behind all these marks and their effects is the cellular machinery that creates and maintains these patterns. The enzymes that constitute this machinery are usually classified into writers, laying the marks on the histones; readers, which recognize the marks; and erasers, the ones that remove the marks. The distinct patterns of histone post-translational modifications is called the "histone code" and, together with DNA methylation, plays a huge role in gene transcription [48].

Table 2 - Examples of histone marks, their enzymes and their effect. Lysine (K), arginine (R), serine (S), threonine (T). Transcriptional activation (Ta), transcriptional repression (Tr), DNA repair (R), and DNA replication (Rep). Adapted from (48).

Modification	Histone	Residue	Enzyme	Possible role
Acetylation	H2A	K5	Tip60, Hat1, P300/CBP	Ta, R
	H2B	K12	ATF2, P300/CBP	Ta
		K20	P300	Ta
	H3	K9	Gcn5, SRC-1	Ta, R
		K14	Gcn5, PCAF, Tip60, SRC-1, hTFIIIC90, TAF1, p300/ Gcn5, Esa1, Elp3, Hpa2, TAF1, Sas2	Ta, R, Rep
		K18	P300, CBP/Gcn5 (SAGA)	Ta, R
		K23	P300, CBP/Gcn5 (SAGA), Sas3	Ta, R
		K27	Gcn5	Ta, R
	H4	K5	Hat1, Tip60, ATF2, p300/Hat1, Esa1, Hpa2	Ta, R, Rep
		K5	Gcn5, PCAF, Tip60, ATF2, p300/Esa1, Elp3	Ta, R, Rep
		K12	Hat1, Tip60/Hat1, Esa1, Hpa2	Ta, R, Rep
		K16	MOF, Gcn5, Tip60, ATF2/Gcn5, Esa1, Sas2	Ta, R
Methylation	H1	K26	EZH2	Tr
	H3	K4	MLL4, SET1, MLL1, SET7/9, MYD3/Set1	Ta
		K9	SUV39H1, ESET/SETDB1	Tr
		K27	EZH2, G9A	Ta, Tr
		K36	HYPB, NSD1/Set2, S.c.	Ta
		K20	PR-SET7, SUV4-20/SET9	Ta, Tr, R
Phosphorylation	H2A	T119	NHK-1	Tr, R
	H2B	S14	Mst1	R
	H3	S10	TG2, Aurora B, MSK1, MSK2/Snf1	Ta
		T11	Dlk/ZIP	Ta
	H4	S1	CK1	R
Ubiquitination	H2A	K119	Ring 1b	Ta, R
	H2B	K120	RNF 20/40	Ta, R

3.3 MicroRNAs

MicroRNAs (also called miRs or miRNAs) are a class of small non-coding RNAs with a length of 19-25 nucleotides [49]. They are encoded in the genome in diverse contexts, either expressed in intronic or intergenic transcripts that encode a single microRNA hairpin precursor or in clusters of multiple precursors [50].

These small sequences are transcribed, mostly, by RNA polymerase II as long primary transcripts characterized by hairpin structures (pri-microRNAs) and then processed in the nucleus by RNAse III Drosha into 70-100 nucleotides long pre-microRNAs. These molecules are then exported to the cytoplasm by an Exportin 5-mediated mechanism. There, another RNAse III, Dicer, acts in complex with TRBP, generating a dsRNA with approximately

Figure 3 – MicroRNA biogenesis and effects. The mature microRNA is incorporated in the RISC complex and exerts its function by translational repression, mRNA cleavage or translational activation. Adapted from [3].

22 nucleotides of length, called miRNA/miRNA*, including the mature miRNA guide, and the complementary passenger strand, the miRNA*. Many publications refer to these two strands as miR-5p and miR-3p, respectively. According to thermodynamic properties, one of the strands is selected as guide strand, whilst the other is usually degraded. Recent evidence suggests that miRNA* or miR-3p may not be just a byproduct of microRNA biogenesis but may also be selected as the functional strand and therefore play a biological role [3]. Finally, the mature microRNA is incorporated into RISC. It is through this complex that microRNAs exert their function upon their target mRNAs [51].
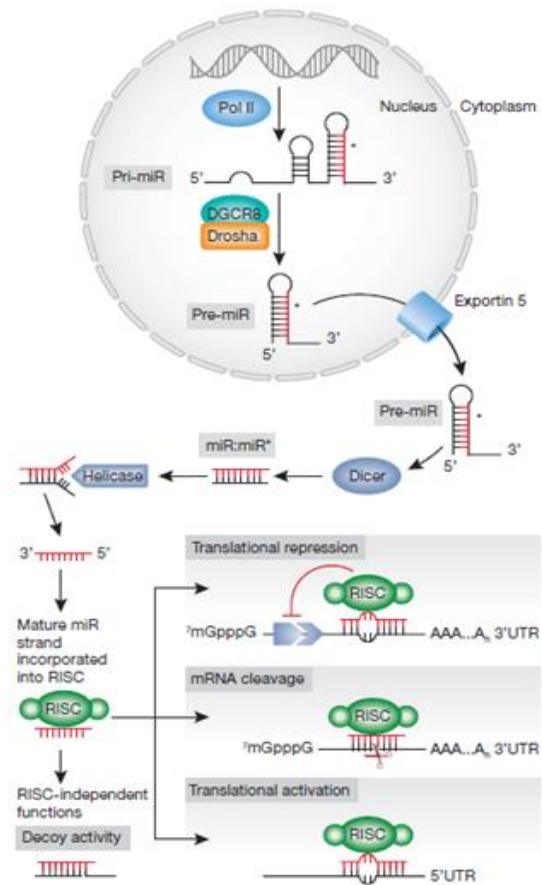
The partial or total match of a microRNA with the 3' untranslated region of its target mRNA leads to its post-transcriptional inactivation and/or degradation. It is estimated that about 30% of human genes are direct microRNA's targets which

implies that microRNAs are involved in mostly all cellular processes including cell cycle regulation, cell growth, cellular differentiation and apoptosis [4].

Similarly to protein-coding genes, in a normal cell, microRNAs need to be tightly regulated in order to maintain a distinct transcriptome signature of a particular cell. The loss of this tight regulation has been described in disease, including cancer [4]. In the last few years microRNAs became a major focus in cancer research with many studies demonstrating their importance in cancer biology and the impact of their deregulation in tumor growth, invasion, angiogenesis, apoptosis and immune evasion [50].

Interestingly, such as tumor suppressing genes and oncogenes, the location of a particular microRNA in the genome has consequence on its role in cancer. It has been found that many microRNAs are located in chromosomal *loci* prone to deletions or amplifications. As such, a microRNA that negatively regulates a tumor suppressor gene may be amplified causing silencing of its target. Conversely, microRNAs that repress oncogenes tend to be located in fragile *loci* and their mutation, methylation or deletion results in reduced microRNA expression and overexpression of respective target oncogenes [3]. Different tumors appear to have particular microRNA profiles and some of these were already reported as being able to discriminate tumor subtypes and to correlate with survival and treatment response [50].

Although the whole mechanism underlying microRNA deregulation in cancer is not fully understood, it is known that DNA methylation is involved in the regulation of microRNA expression. New technologies have enabled comprehensive analysis of the epigenome and as a consequence, the list of microRNAs silenced by methylation in cancer is growing rapidly. Indeed, many families of microRNAs have already been described as being silenced by methylation, including the miR-124, miR-34, miR-9, miR-200 and miR-205 families [52]. In PCa, several microRNAs are known to be hypermethylated. Among them is the miR-34 family, miR-126, miR-193b, miR-145, miR-205, miR-21, miR-615 and miR-196b. MiR-34a appears to play an important role in metastasis and is also a target of p53. MiR34b/c targets *DNMT1* and several histone deacetylases, besides *MYC*, *CDK4*, *CDK6* and *MET* (a proto oncogene). MiR-145 is downregulated in about 81% of PCa. Its methylation has been shown to prevent p53 from binding to miR-145 promoter and regulate this microRNA expression [40].

4 Biomarkers in prostate cancer

The introduction of biomarkers for cancer diagnosis and disease management has revolutionized modern oncology. Biomarkers are molecules whose detection or quantitation may be used to interpret the disease and its behavior, beyond the traditional clinical parameters [53, 54]. Proteins, metabolites, DNA, RNA and epigenetic alterations are all potential candidates to become disease biomarkers [54].

Biomarkers may serve different roles. Currently, there are broadly seven different roles for biomarkers, depending on their use and the kind of questions they may help answering: disease predisposition, screening, diagnostic, prognostic, predictive, monitoring and pharmacogenomics. Concerning this work and PCa management current issues, two roles are of particular interest: diagnostic and prognostic. Biomarkers that are included in the former role help distinguishing patients that have cancer from those who do not harbor this malignancy. On the other hand, prognostic biomarkers assist in foreseeing the clinical outcome of a patient if therapy is not administered and therefore are useful in disease management decisions [53].

The ideal marker is defined by its specificity to a given kind of pathological entity and its high sensitivity while providing advanced warning, ahead of an eventual clinical diagnosis. Also, the detection test should be cheap and non-invasive in order to allow patient screening and be acceptable by the majority of the patients [55]. The biomarker should also improve decision-making abilities in conjunction with clinical and pathological parameters. It would be ideal to find a biomarker that fulfills all of the aforementioned requirements, however, in order to cover screening, diagnosis, prognosis and prediction to treatment, multiple biomarkers are typically required [53].

The use of biomarkers in PCa management, in contrast to most other types of cancer, has a long history. The first biomarker found was prostatic acid phosphatase which was noted, in 1930, to be elevated in the serum of patients with metastatic PCa. PAP remained in use until the 1980's when it was replaced by Prostate specific antigen [53].

PSA has been the most commonly used marker for diagnosis and follow-up. Until recently, this marker was considered as the most reliable to predict PCa [55].

The introduction of the PSA test, a highly accessible blood test, has revolutionized PCa diagnosis in the last three decades [21]. PSA is a kallikrein-related serine protease produced in normal prostate secretions [21]. In normal conditions, only low levels of PSA can be detected in blood and increased levels are associated with abnormalities in the normal architecture of the gland. A blood PSA level higher than 4.0ng/mL is considered an indication for PCa [53, 55]. Since the advent of PSA, PCa prevalence in the USA has more than doubled [24].

However, PSA screening has fallen under controversy since it detects 30-50% of BPH but just about 20% of the PCa cases. At the same time, evidence has arisen that patients harboring PCa may present PSA levels below 4.0ng/mL and that PSA levels may rise with other afflictions such as prostatitis and urinary tract infection. Also, some drugs used to treat BPH are known to cause a reduction of PSA in the blood [55].

One of the hard lessons in biomarker design learned with the popularization of PSA as a screening test is that biomarker design requires *a priori* deliberations of the intended role [53]. PSA was, initially, thought only as a monitor for PCa recurrence but its use was quickly extended to the screening of asymptomatic men resulting in overdiagnosis and overtreatment of potentially indolent cancers [53, 55]. Nevertheless, PSA testing remains in use, since it is inexpensive and sensitive (but not specific) for disease detection as well as for monitoring progression and recurrence after curative therapy of localized disease [53].

Due to the aforementioned limitations of PSA, the search for additional biomarkers that supplement or substitute PSA is a very active field of research. This is reflected by the number of currently available biomarkers in addition to the number of ongoing studies in this field that constantly bring up new potential candidates.

4.1 FDA-approved PCa biomarkers

Currently, two FDA-approved PCa biomarkers are available: Prostate Health Index (*phi*) and Progensa *PCA3* assay. The Prostate Health Index consists in a mathematical formula, -(p2PSA/fPSA) × PSA½, of three biomarkers: the [2] Pro-PSA (p2PSA), free PSA (fPSA) and PSA. p2PSA is a molecular form of free PSA suggested to be associated with PCa and to be more specific allowing for a better discrimination of PCa from BPH than PSA levels [56]. The main intention of this test

is to distinguish PCa from benign prostatic conditions in men with more than 50 years of age and a total serum PSA between 4.0 and 10.0 ng/mL with negative DRE. This test also appears to be an independent predictor of biochemical relapse [57]. Lazzeri *et al.*, with a small cohort, indicated that *phi* outperformed tPSA and %fPSA with AUC values of 0.73, 0.55 and 0.60, respectively [58].

Regarding *PCA3*, the other FDA-approved PCa biomarker, is a long non-coding RNA whose expression has been found to be elevated in 90% of PCa tissues, but is only slightly expressed in normal or BPH tissues and, contrary to PSA, its values are not influenced by the size of the gland [53]. The Progensa *PCA3* assay uses post DRE urine specimens and consists in an *in vitro* amplification test measuring the concentration and then calculating the ratio of *PCA3* RNA molecules to PSA RNA molecules originating the *PCA3* score. Since its approval by FDA in 2012 it has been used as a diagnostic test for PCa in the setting of suspect PSA levels, negative DRE and/or a previous negative prostate biopsy [53]. Different studies provided AUC values ranging from 0.66 to 0.75, sensitivity from 53-69% and specificity between 71-83% [56].

4.2 Laboratory-developed tests

The following tests are not yet approved by the FDA but are offered under a laboratory's CLIA certificate meaning that these tests are required to demonstrate certain performances from an analytical standpoint but their validation is far more limited than what is required for regulatory approval. Therefore, validation extent of the exposed tests is highly variable [56].

The advances of DNA sequencing and transcriptome profiling shifted biomarker research towards these "-omics" methods. A promising biomarker in this field is the presence of *TMPRSS2-ERG* gene fusions. These genetic events are among the most common alterations in PCa, accounting for 90% of all gene fusions. These fusions are highly specific (93%) and can even be detected in precursor lesions such as PIN. However, *TMPRSS2-ERG* is absent in about 50% of PCa cases leaving its potential use reduced and only being useful in conjunction with other biomarkers as in Mi-Prostate Score [53, 54]. Mi-Prostate Score is an attempt to combine the high specificity of *TMPRSS2-ERG* fusions with *PCA3* and PSA measurements [56]. A study by Salami *et al.* although with a limited series of patients showed an AUC value of 0.88, 90% specificity and a 80% sensitivity [59].

Oncotype DX is a multi-gene assay designed to be used in small FFPE samples obtained by needle-biopsy. This assay measures the expression of 12 genes, representing four different biological pathways: androgen pathway (*AZGP1*, *KLK2*, *SRD5A2*, and *RAM13C*); cellular organization (*FLNC*, *GSN*, *TPM2*, and *GSTM2*); proliferation pathway (*TPX2*); and stromal response (*BGN*, *COL1A1* and *SFRP4* in addition to five reference genes [56]. The expression levels of these genes are then combined to calculate the Genomic Prostate Score which is an independent predictor of aggressive disease and, recently, showed promise by independently predicting biochemical relapse [60, 61].

The Prolaris test measures the expression of 31 cell cycle progression genes and 15 housekeeping genes to stratify disease risk of progression [56, 62]. It was designed to be used in paraffin-embedded specimens and it was found that lower expression levels are associated with low risk of disease progression and higher levels with increased risk [56].

Another multi-gene assay is Decipher, which was developed to assess disease progression risk after RP. This assay evaluates the RNA expression levels of 22 genes involved in multiple biological pathways implicated in the development and progression of PCa. Four studies reported the usefulness of this gene panel to predict biochemical recurrence, metastatic progression and disease-specific survival after RP [62]. Moreover, in a study using multivariate model this panel was the only parameter which correlated with metastatic progression [62].

Prostarix is a test designed to aid clinicians in deciding to perform a biopsy in patients with a negative DRE and modestly elevated serum PSA levels. It measures four metabolites (sarcosine, alanine, glycine, and glutamate) present in urine. The urine samples must be collected after a vigorous DRE and liquid chromatography–mass spectrometry is performed to acquire the metabolite signature which is different between cancer-free tissue and PCa [56].

The 4K Score measures PSA isoforms (total PSA, free PSA and intact PSA) in addition to hK2. Without hK2, this test uses practically the same isoforms of Prostate Health Index [56, 63]. It has been indicated that this score may be useful to distinguish indolent from aggressive neoplasms, thus reducing unnecessary biopsies and that 4K Score has a better diagnostic performance than PSA (AUC=0.83 *vs* AUC=0.68, respectively) [56].

Some studies have also provided whole-genome information about PCa bringing to light some chromosome alterations. Among these, relative 8q gain has been proposed as a biomarker for aggressive disease. Indeed, patients with relative 8q gains had worse prognosis compared with patients harbouring tumors without that genetic alteration. Particularly, when stratifying patients according to tumor grade or stage, relative 8q gains allowed to discriminate those with poorer outcome [54, 64].

Another interesting biomarker is α-methylacyl-coenzyme A racemase, currently used as a diagnostic ancillary tool in immunohistochemistry. However, its prognostic value has not been established so far [54]. Its sensitivity and specificity is quite high (>90%) when used as a tissue marker in prostate biopsy. On the other hand its potential remains uncertain since it is also significantly expressed in high-grade prostatic intra-epithelial neoplasia[54].

Other potential biomarkers reported by high-throughput proteomics include prostate-specific membrane antigen, prostate-specific cell antigen and early prostate cancer antigen [53].

4.3 Epigenetic biomarkers in PCa

As previously exposed, epigenetic alterations are a common trait in PCa lesions and are thought to be highly involved in disease onset and progression. Despite their exact roles and full involvement not being yet fully understood, the fact that they occur at a higher rate and at an earlier point than mutations makes them very attractive biomarkers for diagnosis, prognosis and treatment response [65].

4.3.1 DNA methylation biomarkers in PCa

Since DNA methylation is a tissue-specific trait as well as a tumor-specific one, its potential as a biomarker has also been extensively explored. Contrarily to protein biomarkers, methylated genes appear to have a higher specificity for cancer. This superior specificity is even more noticeable when using blood or other biological fluids that are the most attractive clinical samples to be analyzed using biomarkers [66].

From a practical standpoint, DNA methylation is an interesting alteration for laboratory testing since it is far more stable and easier to work with than RNA and its isolation and detection is rather straightforward. It can be isolated from most

specimens used in the clinic: from formalin fixed tissues to biological fluids that can be easily collected, such as urine and blood [67]. Its analysis is possible by digestion with restriction enzymes or bisulfite treatment. The latter in particular opens many possibilities by PCR based techniques like MSP, either using methylation-specific primers or methylation-specific probes; Pyrosequencing and bisulfite sequencing [67].

The most well studied methylation biomarker in PCa is *GSTP1* [68]. This was one of the first genes found to be silenced in PCa. *GSTP1* encodes an enzyme that catalyzes the conjugation of hydrophobic and electrophilic compounds with reduced glutathione, acting as a detoxifying agent. Promoter hypermethylation of this gene does not occur in normal prostatic tissue nor in BPH. On the other hand, it has been detected in PIN lesions [40]. *GSTP1* is methylated in more than 90% of PCa cases being a particularly promising biomarker for early detection. Assays have been developed achieving high specificity for PCa (86-100%). However, sensitivity is highly variable depending both on the detection method used and the sample type: urine (19-83%) and serum or plasma (13-72%) [69]. Other interesting methylation biomarkers in PCa include promoter hypermethylation of *APC*, *RASSF1A* and *RARβ2* [65, 70].

Besides being involved in familial adenomatous polyposis, *APC* is also a well-known tumor-suppressor gene involved in the Wnt signaling transduction pathway and cellular adhesion. This gene is a well-known gatekeeper, preventing the transcription of products that lead to cell proliferation and survival. Hypermethylation of this gene causes the cell to become more vulnerable to additional epigenetic and genetic changes. Although *APC* promoter methylation is common in many malignancies, particularly in colon cancer, previous studies have found this gene to be involved in PCa progression and it is hypermethylated in 22-100% of PCa cases but only in 5-6% of noncancerous prostate tissues [71]. Moreover, it was demonstrated to be an independent prognostic factor for worse outcome [72].

The *RASSF1A* gene belongs to the RAS proto-oncogenes family. Although these are well-known to be involved in signal transduction pathways involved in cell proliferation and survival, *RASSF1A* is a tumor-suppressor gene, known by its association with DNA repairing proteins and apoptotic effect [71]. Initially, no promoter methylation of *RASSF1A* was found in benign prostate tissue. More recently, it has been found in PIN and benign prostatic epithelium [68]. *RASSF1A*

promoter hypermethylation has been observed in 60-74% or 23-99% of PCa tissues, according to different sources, besides being clearly associated with aggressive cancers [68, 71].

Another interesting biomarker for prostate cancer is promoter hypermethylation of *RARβ2*, a tumor-suppressor gene. Its expression is downregulated or absent in many tumor tissues and its promoter is hypermethylated in many tumors as well, including those of the prostate. Methylation frequencies in PCa vary according to different studies, ranging from 22% to 98%. However, it is rarely hypermethylated in normal tissue or in BPH [73]. Interestingly, frequencies of 34-62% were observed in urine, and 39-70% in blood samples, making it an interesting non-invasive biomarker for PCa [71]. A high methylation level of this gene has also been reported as is associated with a biochemical recurrence after radical prostatectomy [74].

Panels comprising two or more methylated genes have been proposed. For instance, Hoque *et al.* proposed a panel with methylation status from *RASSF1A*, *RARβ2* and *APC*, in conjunction with *GSTP1* [66]. Moreover, methylation-based laboratory-developed tests, not FDA-approved, are currently available. ConfirmMDx uses methylation analysis of *GSTP1*, *APC* and *RASSF1A* to detect an epigenetic field [62]. Its aim consists in distinguishing patients with a true negative biopsy from those who may have occult cancer [56]. This test achieved NPV of 90% and 88% in two different studies and showed to be the most significant predictor of biopsy results [62].

Another proposed test that comprises methylation analysis of a panel of 3 genes is ProCaM, developed by Baden *et al.* In this study, *GSTP1*, *RARβ2* and *APC* promoter methylation levels were analyzed in urine samples of patients with serum PSA levels between 2.0 and 10.0 ng/ml. AUC values for each marker ranged from 0.63 to 0.68. When used in conjunction, the three markers yielded an AUC value of 0.73, sensitivity of 60% and specificity of 80% [75]. Importantly, this assay displayed higher predictive accuracy than currently used parameters to decide a prostate biopsy and identifies a larger number of cases with high Gleason score [75].

As was previously mentioned, microRNAs are known to be silenced by DNA methylation in PCa. Therefore, the utility of microRNA promoter methylation as potential PCa biomarker has been also studied. A study by Shimizu *et al.* sought to determine a panel of four methylated microRNAs to detect bladder cancer with

interesting results: 81% sensitivity and 89% specificity and AUC=0.916 [76]. Moreover, it was reported that hypermethylation of miR-34b/c in normal gastric mucosa was an independent risk predictor of metachronous gastric carcinoma [77], whereas miR-148a was found to be an independent prognostic/predictive biomarker in advanced colorectal carcinoma treated with conventional chemotherapy [78]. In PCa, hypermethylation of the *GABRE*~miR-452~miR-224 locus was able to discriminate normal tissues adjacent to tumors from prostatic tumors with 94.3% specificity and 95.5% sensitivity (AUC=0.98) [79]. MiR-205 is another microRNA that is known to be hypermethylated in PCa. Hulf *et al.* explored its role in PCa carcinogenesis as well as its potential as a biomarker reporting that it is a significant predictor of biochemical relapse in patients with low preoperative PSA levels [80].

4.3.2 Histone modifications as biomarkers in PCa

Similarly to other epigenetic mechanisms, aberrant patterns of histone modifications are a hallmark of cancer and as such, their potential as disease biomarkers has started to be unveiled. For instance, in PCa altered levels of dimethyl-K4 and acetyl-K18 of histone H3 have been proposed as markers of high-risk of recurrence [81]. Barlesi *et al.* reported that high dimethyl-H3K4 or low acetyl-H3K9 levels have been related with better survival in non-small cell lung carcinoma [82]. Also, Barbisan *et al.* used global acetyl-H3K9 levels to identify patients with low-grade bladder cancer who experienced disease recurrence after transurethral resection of the bladder [83].

Because histone modification patterns are altered in cancer in general, and in PCa in particular, the enzymes that establish and maintain these patterns it is reasonable to consider that these are also deregulated in malignanat cells [84, 85]. The best demonstration is provided by EZH2, a methyltransferase, which in PCa was shown to better predict tumor progression than serum PSA or Gleason score. Moreover, its expression in organ-confined PCa was reported to correlate with risk of biochemical recurrence [85]. LSD1, which is a demethylase, is another histone modifier with prognostic value, since higher levels were associated with shorter time to cancer relapse [86]. However, the role of histone modifications in PCa is still not fully understood and their potential as prognostic and diagnostic biomarkers is not fully explored.

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4.3.3 MicroRNAs as biomarkers in PCa

An emerging and exciting field in prostate carcinogenesis field of research, different studies showed that microRNAs may be potential diagnostic and prognostic biomarkers for PCa. These non-coding RNAs are tissue and tumor-specific, stable and detectable in body fluids [56]. Several microRNAs have been described as being altered in PCa and different expression profiles allow differentiation between benign and malignant conditions [87].

For instance, miR-141 expression levels were found to be highly increased in the sera of men harboring metastatic PCa, predicting the presence of neoplasms with 60% sensitivity and 100% specificity. Different studies confirmed this finding and thus miR-141 appears to be a suitable biomarker of metastatic progression [88, 89]. MiR-21 showed higher expression levels in patients with hormone-resistant PCa and patients with androgen-sensitive metastatic disease. Moreover, in the hormone resistant group, miR-21 levels were higher in patients with docetaxel resistance [88]. In another study, miR-375 and miR-141 were found to be associated with pathological stage and Gleason score [54, 65].

Several other studies have reported some microRNAs with diagnostic and prognostic value most of which require validation in larger cohorts. Due to their interesting performances so far, stability and ease of collection, we may admit that microRNA biomarker potential is yet to be fully realized [79].

Aims of the study

The PSA-era has brought lasting changes in the study of how biomarkers should be designed and used. Although an early diagnosis of PCa is important, from a clinical point of view, the high prevalence of indolent cancers detected by PSA testing requires surrogate biomarkers able to define clinically significant disease. Ideally, PCa biomarkers should reduce the need for biopsy while helping to stratify patients according to disease significance which, in turn, may lead to a reduction in radical prostatectomies and radiotherapy.

DNA methylation is a highly specific tissue trait. Therefore, it encompasses a promising value as a cancer biomarker, including PCa. MicroRNA deregulation is known to be an early event in carcinogenesis and one of the mechanisms underlying this deregulation is aberrant DNA methylation. Moreover, since epithelial prostatic cells, normal or neoplastic, are often shed in urine, PCa-specific biomarkers can be easily tested in that bodily-fluid obtained by non-invasive procedures.

This study is integrated in a broader project which aims to uncover how microRNAs' deregulation contributes to prostate carcinogenesis. Therefore, its design was based on preliminary results obtained in that context.

Specifically, the aims of this master's dissertation were:

- To assess promoter methylation status of newly identified microRNAs in prostate cancer to determine:
  a) Its potential as prostate cancer biomarkers in tissue samples;
  b) Its performance as biomarkers for early detection in urine samples;
  c) Its prognostic value in biopsy samples.

Materials and methods

1 Preliminary data

1.1 Identification of new putative microRNAs regulated by methylation

As part of another study of our group, epigenetically regulated microRNAs were previously investigated. For this, tissue samples (5 controls and 25 tumors) were used for gene methylation profiling using HumanMethylation450 BeadChip (Illumina, USA).

DNA samples were assessed for integrity, quantity and purity by electrophoresis in a 1.3% agarose gel, PicoGreen quantification assay and nanodrop measurements.

All samples were distributed into 96-well plates. Bisulfite conversion of 500 ng of genomic DNA was performed using EZ-96 DNA Methylation-Gold Kit (Zymo Research, USA) following manufacturer's instructions. About 200ng of bisulfite-converted DNA was used for hybridization using HumanMethylation450 BeadChip (Illumina, USA). Briefly, samples were whole genome amplified followed by an enzymatic end-point fragmentation, precipitation and resuspension. The resuspended samples were hybridized onto the BeadChip for 16h at 48°C and washed. A single nucleotide extension with labeled deoxy-nucleotides was performed, and repeated rounds of staining were applied with a combination of labeled antibodies differentiating between biotin and 2,4-dinitrophenol. Color balance adjustment and quantile normalization were performed in order to normalize the samples between the two color channels. DNA methylation level is displayed as beta-values ranging from 0–1. Beta-values with detection P-value > 0.01 are considered to fall below the minimum intensity and threshold and were consequently removed from further analysis.

To identify consistently differentially methylated CpG sites Wilcoxon rank sum paired test was performed for normalized beta-values. The p-values were adjusted using false discovery rate, and those CpGs with p-values <0.05 were selected.

2 Patients and sample collection

2.1 Tissue samples

For this study, 180 PCa samples were prospectively collected from patients with clinically localized disease consecutively diagnosed and submitted to radical prostatectomy with curative intent from 2001 to 2006, in IPO-Porto Francisco Gentil. Control samples, totaling 15, were collected from cystoprostatectomy specimens from patients with bladder cancer not harboring PCa. After surgical resection and examination, samples were immediately frozen at -80°C.

Histological examination of the specimens was performed by a pathologist in histological slides obtained from formalin-fixed paraffin-embedded tissues of these specimens for assessment of Gleason Grade and TNM staging. Other relevant clinical data was collected from clinical charts.

Furthermore, samples from prostatic biopsies from 74 patients were collected from individuals referred to IPO-Porto Francisco Gentil due to elevated PSA levels, from 2001 to 2003. In addition to the standard diagnostic cores, two tissue core samples were collected from the most suspicious areas and fresh-frozen at -80°C. Frozen sections with 5 μm were cut and stained and histological evaluation was performed by an experienced pathologist.

2.2 Urine samples

Urine samples were collected from 95 patients diagnosed with PCa, before being submitted to radical prostatectomy, from 1999 to 2002. Control samples were collected from 46 healthy donors.

Collected samples were centrifuged at 4,000 rpm for 20 minutes and washed in PBS 1X. The supernatant was discarded and the procedure was repeated. Pellets were re-suspended in 1mL of PBS 1X and centrifuged again at 4,000rpm for 5 minutes. The pellets were then frozen at -80°C.

All patients enrolled signed an informed consent and this work was approved by the institution review board (CES-IPOFG-EPE 019/08 and CES-IPOFG-EPE 205/2013) of IPO-Porto Francisco Gentil.

3 DNA extraction

DNA was extracted by phenol-chloroform method, with minor variations between fresh-frozen tissues and urine pellets. From the fresh-frozen tissues, 15 µm sections were cut and transferred to 15mL tubes. Then 2,700 mL of SE buffer (75mM NaCl and 25 mM EDTA), 300 µL of 10% SDS and 25 µL of proteinase K (Genaxxon bioscience, Germany) were added. Samples were incubated overnight at 55°C. After this period, digestion was prolonged according to necessity with subsequent addition of Proteinase K at every 12h until complete digestions was achieved.

Urine samples were first centrifuged at 13,000 rpm for 5 minutes after which any remaining supernatant was removed and the pellet re-suspended in 500µL of SE buffer. Digestion was achieved by further adding 30µL of SDS at 10% and 15µL of Proteinase K at 20 mg/mL and incubating samples at 55°C until digestion was complete.

Following digestion, all samples were transferred to Phase Lock Light 2mL tubes (5 Prime, Germany) previously centrifuged at 12,000 rpm for 5 minutes, and mixed with 500µL of PC8 (Sigma-Aldrich, USA).

After centrifugation, at 13,000 rpm for 15 minutes, the aqueous phase was transferred to new Phase Lock Light 2mL tubes previously centrifuged and 500µL of PC8 were added followed by another centrifugation.

DNA precipitation was accomplished by transferring the aqueous phase to 2 mL tubes and adding 1 mL of cold absolute ethanol, 135 µL of ammonia acetate at 7.5M and 2 µL of glycogen. After mixing, samples were left at -20°C overnight.

Posteriorly, samples were washed in ethanol 70%, the pellets air dried and then eluted in 30 µL (tissue samples) and 20 µL (urine samples) of sterile distilled water. DNA concentration and purity were assessed using NanoDrop Lite Spectrophotometer (Nanodrop Technologies, USA) and stored at -20°C until further use.

4 Bisulfite modification

Sodium bisulfite modification of DNA is the basis of methylation studies involving sequencing and PCR. By this process, DNA is firstly denatured and, as displayed in Figure 4, unmethylated cytosines are sulfonated giving origin to a cytosine sulfonate, then deaminated and finally desulfonated, thus losing the bisulfite group and, finally, becoming uracils. Since methylated cytosines resist this modification, discrimination between methylated and unmethylated cytosines becomes possible [2].
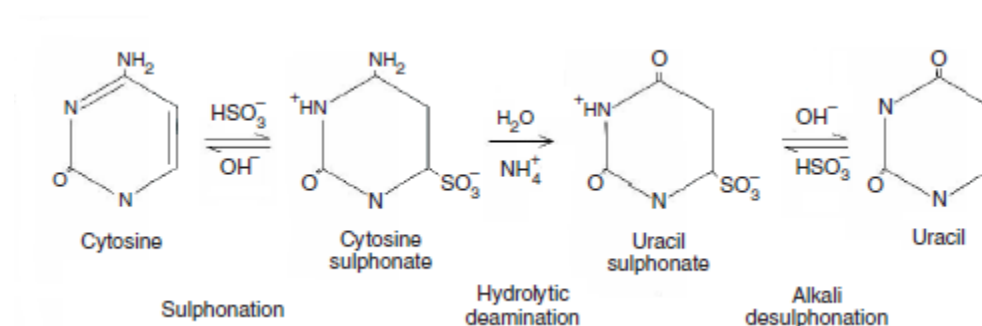

Figure 4 - DNA modification by sodium bisulfite. Cytosine is deaminated becoming uracil while methylcytosine resists this treatment. Adapted from [2].

Genomic DNA from all samples used was modified by sodium bisulfite using EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) according to the manufacturer's guidelines. From DNA obtained from frozen tissues, 1 µg of DNA was used. Due to the scarcity of the material, the used quantity of DNA extracted from urine samples was adjusted to 500 ng.

The required volume of DNA was diluted in sterile water to a total volume of 20 µL in a PCR tube, according to the specified concentration of each sample. To each sample, 130 µL of CT Conversion Reagent was added and then incubated in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, USA) at 98°C for 10 minutes and then at 64°C for 3 hours.

Once finished the incubation, samples were transferred to a Zymo-Spin IC column with 600 µL of M-binding buffer and centrifuged at 10,000 rpm for 30 seconds. After being washed with 100µL of M-Wash buffer and again centrifuged at 10,000 rpm for 30 seconds, desulphonation was achieved with an incubation at room temperature with 200 µL of M-Desulphonation buffer for 20 minutes. M-

Desulphonation buffer was discarded following a centrifugation at 10,000 rpm for 30 seconds. Then two washings were carried out with 200 μL of M-Wash buffer followed by centrifugations at 10,000 rpm for 30 seconds.

Finally, the collumn was placed in a 1.5mL tube and DNA was elluted by incubating with 60 μL of M-Elution buffer for 5 minutes at room temperature followed by a centrifugation at 10,000 rpm for 30 seconds. The modified DNA was stored at -80°C until further use.

One μg of CpGenome™ Universal Methylated DNA (Millipore, USA) was also modified, according to the method described above and eluted in 30μL of M-elution buffer.

5 Validation of new putative microRNAs regulated by methylation using qMSP

To assess methylation levels, quantitative real-time methylation specific PCR was performed in all samples using KAPA SYBR FAST qPCR Kit Master Mix. The modified DNA was used as template and samples were submitted to reactions with the target genes: miR-34b/c, miR-129-2, miR-152, miR-193b, miR-663a and miR-1258; as well as the reference gene, *ACTβ*.

Reactions were carried out in 384-well plates using LightCycler 480 II (Roche, Germany). Briefly, per each well 1 μL of modified DNA and 5 μL of 2X KAPA SYBR FAST qPCR Master Mix were added. The volume of primers used varied (according to Table 3) and sterile distilled water was added in order to total 10 μL of reaction volume.

Table 3 - Primers sequences used and qMSP conditions for each of the tested candidates.

Gene	Forward (F)	Reverse (R)	Annealing T°C	Volume per reaction (F+R)
ACTβ	TGGTGATGGA GGAGGTTTAG TAAGT	AACCAATAAAA CCTACTCCTCCC TTAA	60	0.4 µL
miR-34b/c	GTTTAGTTACG CGTGTTGTGC	CGAAAAACGCC CTACCATA	60	0.3 µL
miR-129-2	CGGCGAATCG AAGAAGTC	TACGCCCTCCG CAAATAC	62	0.4 µL
miR-152	TCGTCGTTCG GGATTTTC	ACTAACCACGT CCGCACC	62	0.4 µL
miR-193b	AGCGGGGTGT TTGTGTTC	AAACATAAACG ACGCATTCCA	62	0.2 µL
miR-663a	GGGATAGCGA GGTTAGGTC	CATTCGTAACG AATAAAACCC	60	0.3 µL
miR-1258	TCGGTATATTT GGCGGAGTC	TCCGACGAAAA TAAACCCC	60	0.3 µL

The PCR program consisted of a period of 3 minutes at 95°C for enzyme activation followed by 45 cycles with 3 seconds at 95°C (for DNA denaturation) and 30 seconds at 60°C (for annealing, extension and data acquisition).

All samples were run in triplicates and in each plate one negative template control was run. Modified CpGenome™ Universal Methylated DNA was used to create five serial dilutions by a 5x dilution factor. These serial dilutions were run in each plate and were used to generate a standard curve thus allowing absolute quantification as well as ascertaining PCR efficiency. All plates had an efficiency between 90-100%.

Methylation levels were calculated as a ratio between the target gene mean quantity and *ACTβ* mean quantity:

$$\text{Methylation level} = \frac{\text{Target gene mean quantity}}{\text{ACTβ mean quantity}}$$

6 Statistical analysis

Non-parametric tests were performed to determine statistical significance in all the comparisons made. In particular, Kruskall-Wallis test was used in comparisons between 3 or more groups, whereas Mann-Whitney U test was used for comparisons between two groups. Survival function was performed to evaluate correlation between methylation levels and disease specific survival. Logistic regression models were also built in order to evaluate the potential of using the targets as a panel to increase performance. Spearman nonparametric correlation test was performed to correlate methylation levels with patients' serum PSA. Statistical significance was set at $P<0.05$. Bonferroni's correction was applied to pairwise comparisons.

When assessing the targets' performance as biomarkers, ROC curves were built. Moreover, biomarker parameters [specificity, sensitivity, positive predictive value, negative predictive value and accuracy] were determined using the formulas provided in Table 4. For this, the cut-off established was the highest value obtained by the ROC curve analysis (sensitivity + (1-specificity)).

Statistical analysis was performed using SPSS Statistics 20 (IBM, USA). Graphics were assembled using GraphPad 5 Prism (GraphPad Software, USA).

Table 4 - Formulas used for biomarker parameters calculation.

Tumors vs Controls				Sensitivity (%)	(C/A) x100
	Tumors	Controls		Specificity (%)	(F/B) x 100
Total	A	B		PPV (%)	(C/(C+D)) x 100
> cut-off	C	D		NPV (%)	(F/(E+F)) x 100
< cut-off	E	F		Accuracy (%)	((C+F)/(A+B)) x 100

Results

1 Clinical samples

For the purposes of this study, clinical samples (tissues and urine) from different patient cohorts were collected. The relevant clinical and pathological data are depicted in Tables 5 and 6.

Table 5 – Clinical and pathological data of all the PCa samples used in this study.

	Prostatectomies		Urine sediments		Prostate biopsies
Clinical and pathological data	**MNPT**	**PCa**	**Healthy donors**	**PCa**	**PCa**
Patients, *n*	15	180	46	95	74
Median age, *years* (range)	63 (45-80)	65 (49-74)	61 (58-77)	64 (45-80)	68 (49-85)
Median PSA (*ng/mL*) (range)	-	8.30 (3.4-23)	-	8.80 (3.5-20.4)	18.22 (4.52-542)
Pathological stage					**Clinical stage**
T2 (%)	-	96 (53.3)	-	46 (48.4)	48 (64.9)
T3 (%)	-	84 (46.7)	-	49 (51.6)	12 (16.2)
T4 (%)	-	-	-	-	14 (18.9)
Gleason score					
<7 (%)	-	56 (31.1)	-	37 (39.0)	30 (40.5)
=7 (%)	-	108 (60)	-	50 (52.6)	33 (44.6)
>7 (%)	-	16 (8.9)	-	8 (8.4)	11 (14.9)
Follow up					
Biochemical recurrence	-	50 (28)	-	24 (25)	32 (43)
Death due to PCa	-	9 (5)	-	4 (4)	17 (23)

MNPT - morphologically normal prostatic tissue; PCa - prostate carcinoma

Table 6 - Number, age and gender of vesical and renal tissue samples used as controls in this study.

Tissues	**NB**	**BlCa**	**NK**	**RCT**
Patients, *n*	9	43	9	50
Mean age, *years* (range)	61 (52-75)	63 (43-83)	64 (20-83)	63 (37-83)
Gender, *n* (%)				
Male	9 (100)	36 (83.7)	6 (66.7)	29 (58)
Female	-	7 (16.3)	3 (33.3)	21 (42)

NBl – normal bladder; BlCa - bladder carcinoma; NK - normal kidney; RCT - renal cell tumor.

2 Preliminary results

2.1 Identification of new putative microRNAs regulated by methylation

As part of an ongoing PhD thesis project, aiming at the discovery and characterization of new epigenetically regulated microRNAs in PCa, results from the analysis of a HumanMethylation450 BeadChip array were used. This array is designed to evaluate thousands of methylation sites across the genome. The most differentially methylated CpGs observed in known microRNA promoter regions were depicted for miR-34b/c, miR-129-2, miR-152, miR-193b, miR-663a, and miR-1258 (Figure 5).
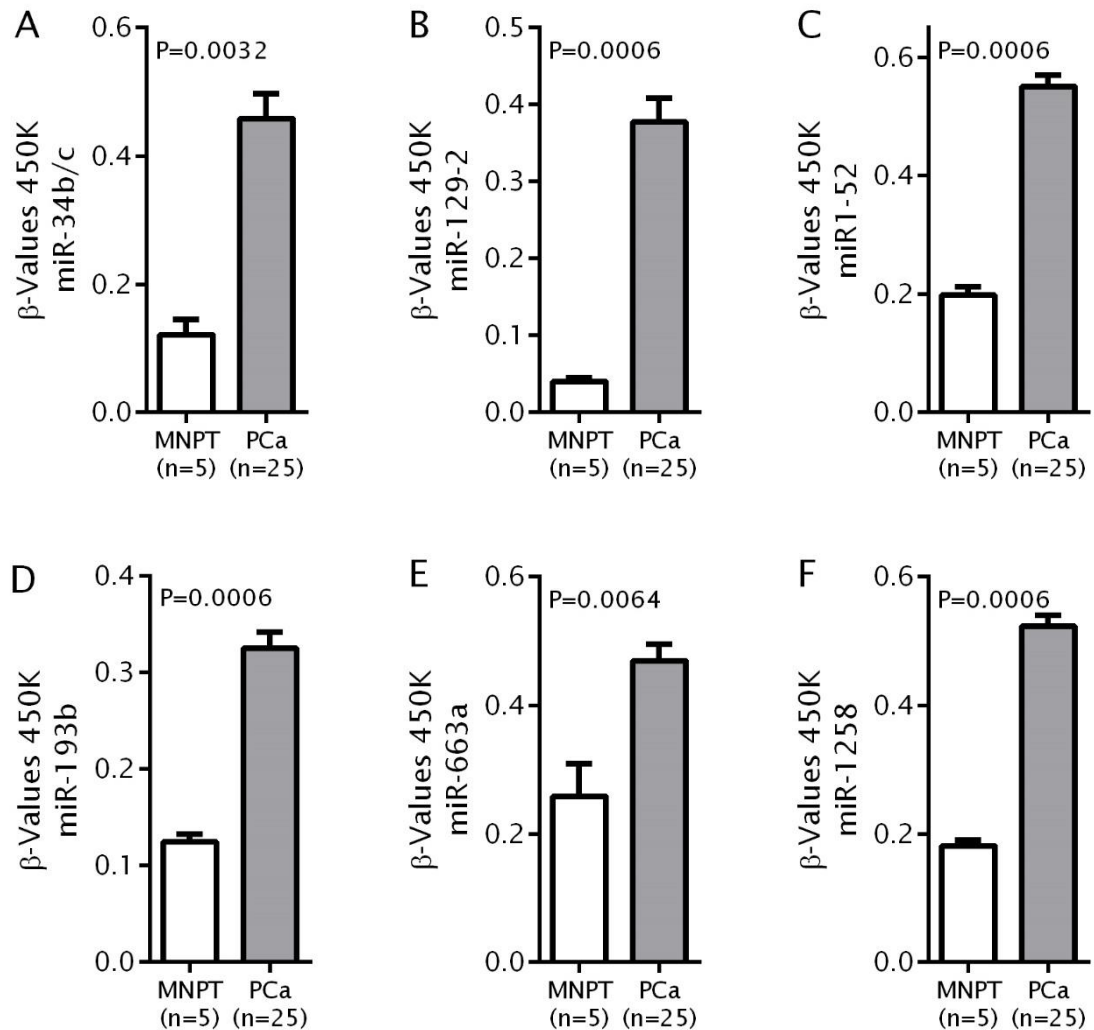

Figure 5 - HumanMethylation450 BeadChip results. The microRNAs displayed showed the most significant differences between MNPT and PCa samples were selected for further analysis.

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3 Validation of differentially methylated microRNAs in tissue samples

After primer design and optimization, validation of the selected candidate microRNAs was achieved using qMSP assays to assess the methylation levels in MNPT and PCa samples for each.

In accordance with the array's results, all microRNAs tested showed higher promoter methylation levels in PCa compared to MNPT. Because our first goal was to discover novel microRNAs fit for PCa detection, ROC curve analysis was conducted in the validation cohort (Figure 6) and an empirical cut-off value was set for each microRNA.

E miR-152

F miR-152

G miR-193b

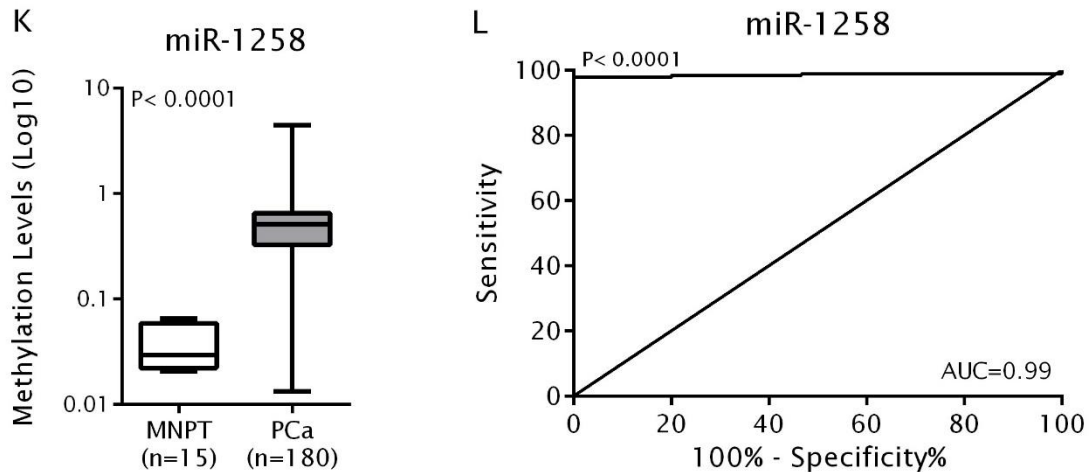H miR-193b

I miR-663a

J miR-663a
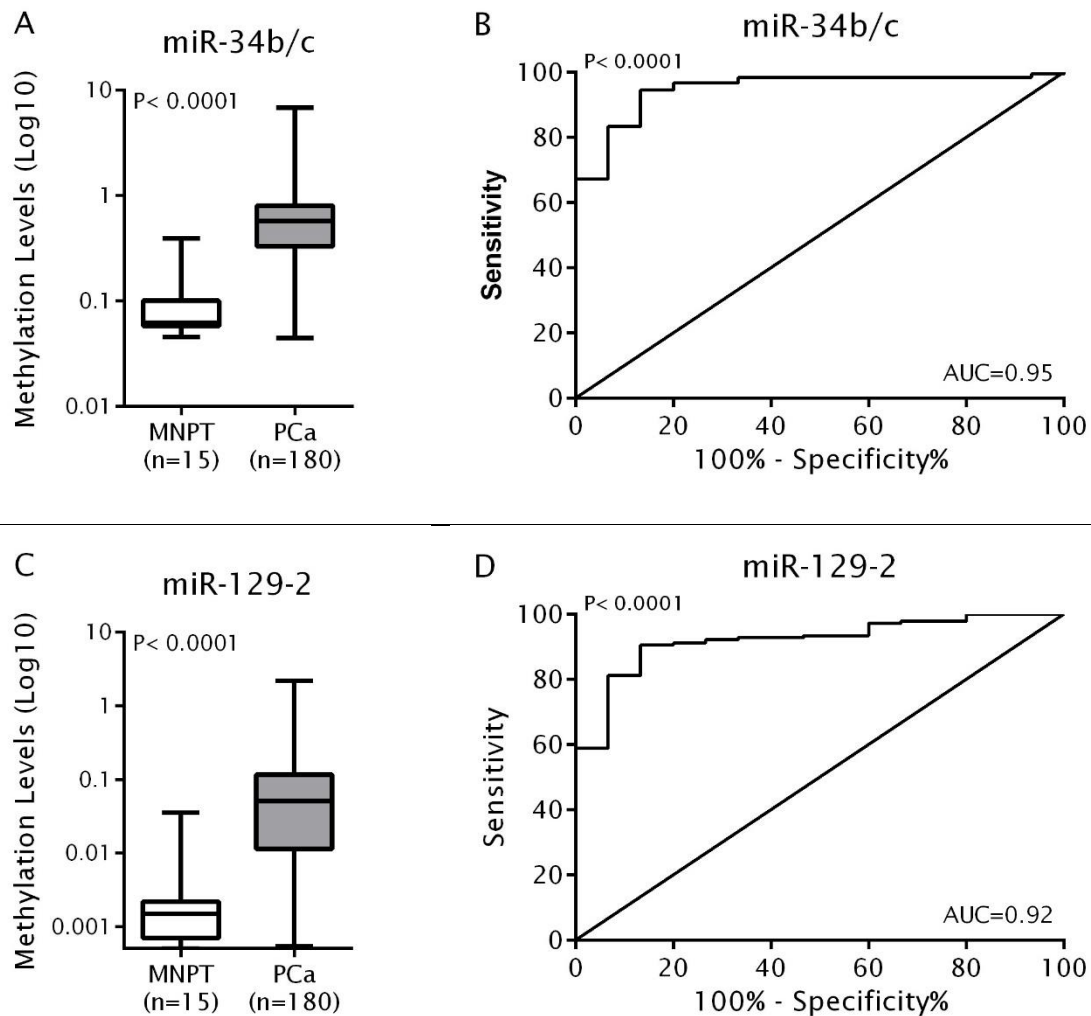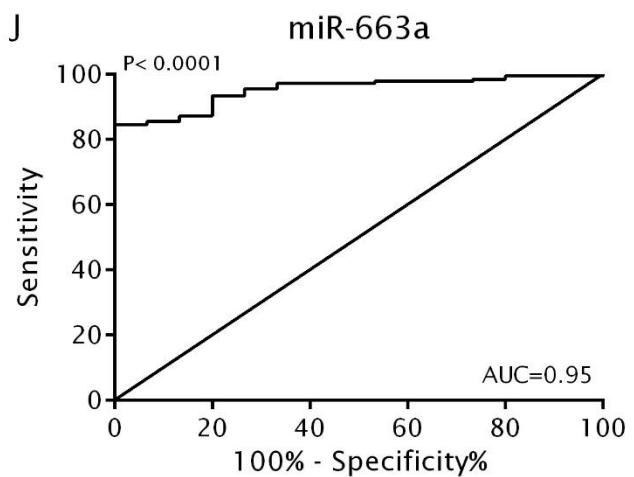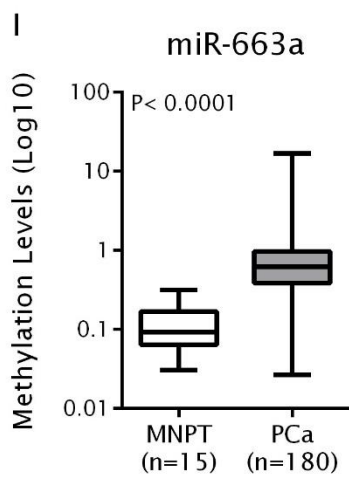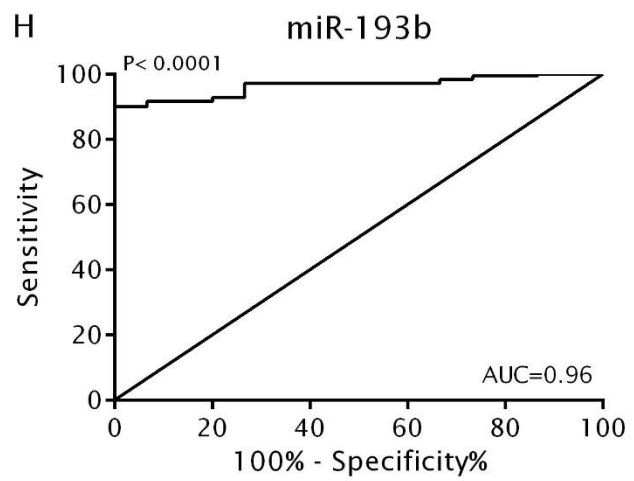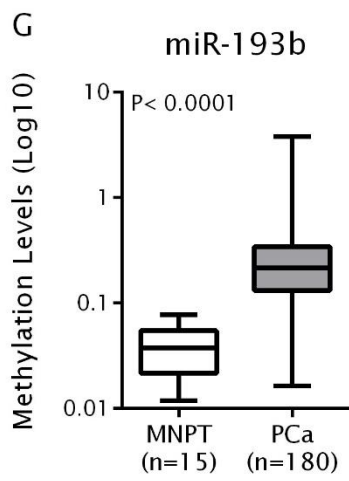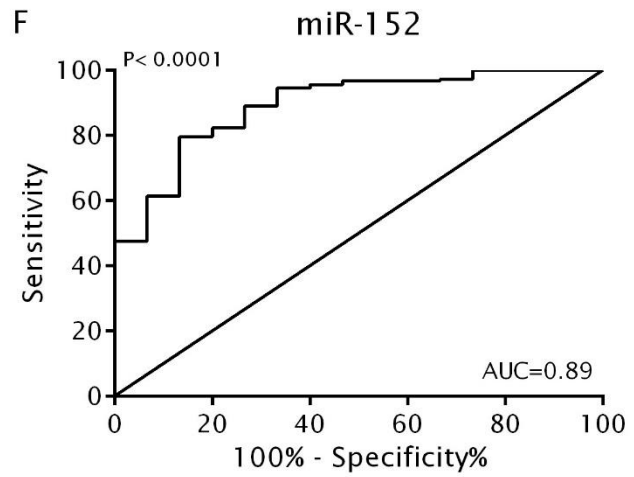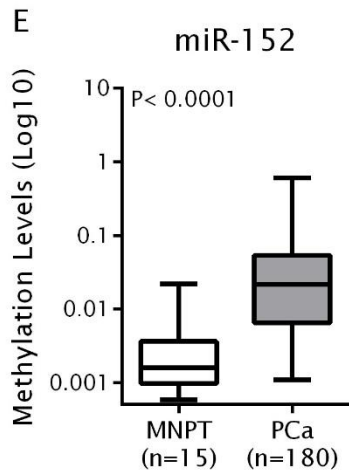
Figure 6 - Box-plots (left panel) depicting higher promoter methylation levels in PCa tissues compared to normal prostatic tissues and ROC curves (right panel) displaying high biomarker performance, in all candidates tested.

The standard validity and information estimates for each microRNA were also calculated to further characterize its performance as PCa biomarkers (Table 7).

Table 7 - Performance of epigenetic biomarkers for the detection of PCa in RP specimens.

microRNA	Sensitivity % (n positive/n total)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR–34b/c	94.4 (170/180)	86.7	98.8	56.5	93.8
miR–129–2	90.6 (163/180)	86.7	98.8	43.3	90.3
miR–152	79.4 (143/180)	86.7	98.6	26.0	80.0
miR–193b	90.0 (162/180)	100.0	100.0	45.5	90.8
miR–663a	84.4 (152/180)	100.0	100.0	34.9	85.6
miR–1258	97.8 (176/180)	100.0	100.0	78.9	97.9

Notably, all candidate microRNAs, except miR-152, displayed AUC values above 0.90. In the testing cohort series (tissue samples from prostatectomy specimens), miR-1258 showed the best performance with AUC=0.99, 97.8% sensitivity and 78.9% NPV, followed by miR-193b, with AUC=0.96 and then miR-34b/c and miR-663a, both with AUC=0.95, although miR-34b/c showed higher sensitivity, accuracy and NPV.

Additionally, for each microRNA, promoter methylation levels were found to be associated with the standard clinicopathologic parameters. As depicted in Figure 7, higher methylation levels of miR-34b/c, miR-129-2, miR-663a and miR-1258

were significantly associated with higher pathological stage. Moreover, increased methylation levels of miR-129-2 were also found in less differentiated tumors (Gleason Score ≥ 7). Owing to the inferior biomarker performance of miR-152, it was not further tested.
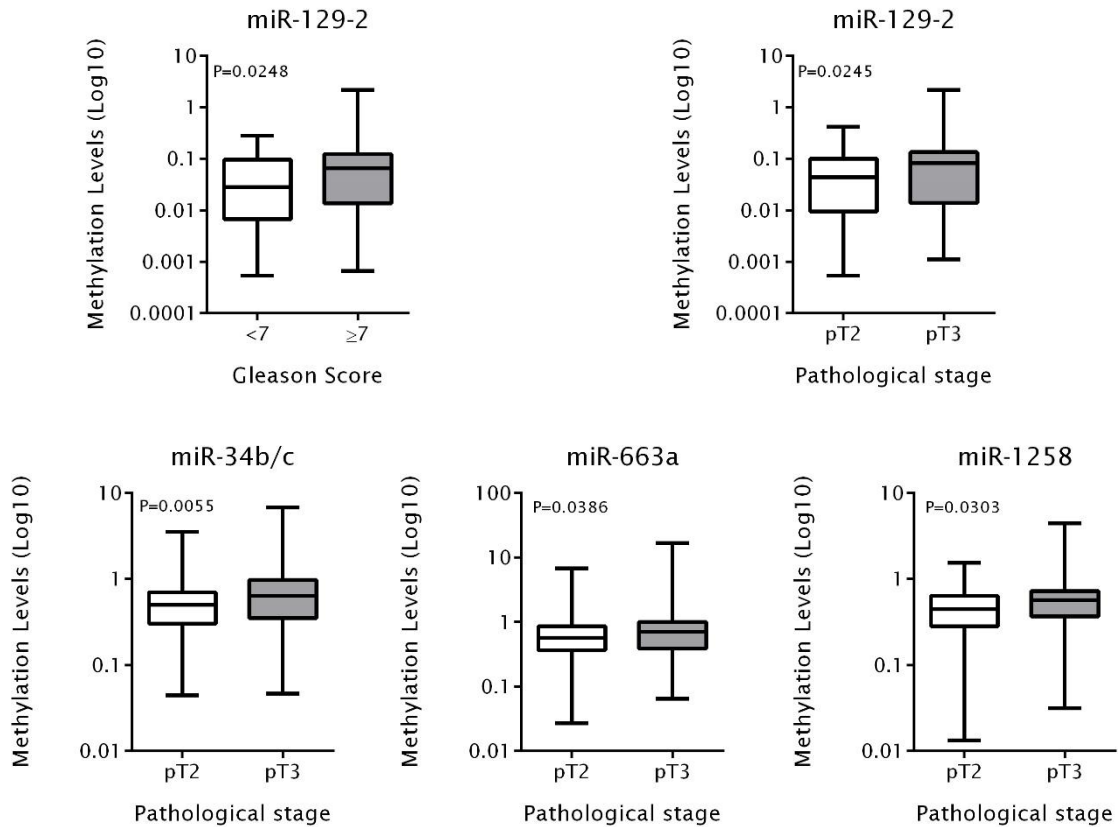

Figure 7 - Distribution of methylation levels according to Gleason score and pathological stage. The candidates displayed had statistically higher methylation levels in samples with higher Gleason score and pathological stage.

4 Assessment of methylation levels in tissue samples from the urinary tract

To verify whether the candidate microRNAs were prostate cancer-specific, the methylation levels of each microRNA were evaluated in tissue samples from other urinary organs tract, namely bladder and kidney, both normal and neoplastic.
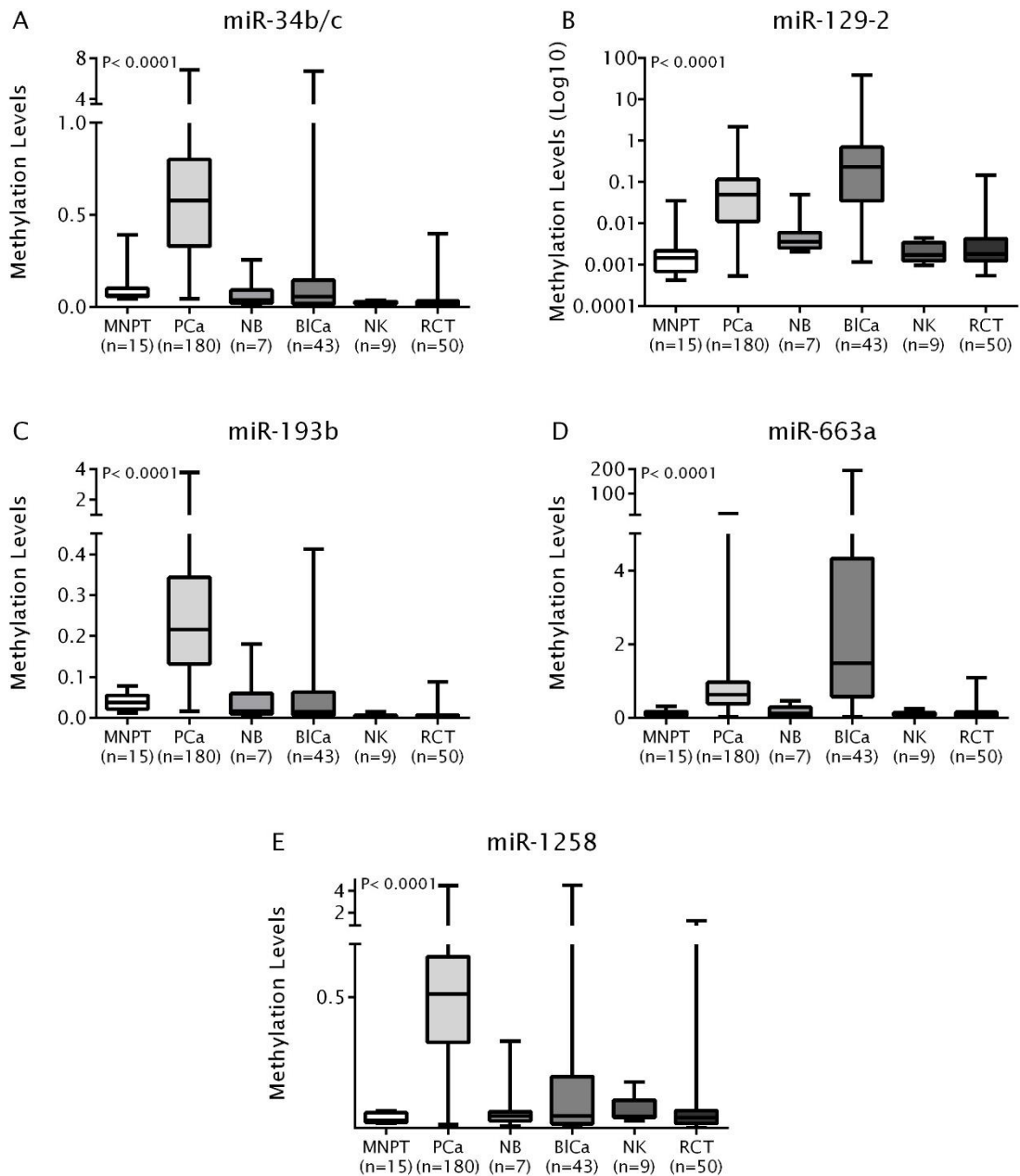

Figure 8 - Distribution of microRNAs' promoter methylation levels in prostatic, vesical and renal tissues. MNPT - morphologically normal prostatic tissue; PCa - prostate carcinoma; NBl - normal bladder; BlCa - bladder carcinoma; NK - normal kidney; RCT – renal cell tumor.

Since the ultimate goal of this study was to develop an assay powerful enough to detect PCa in bodily fluids, namely in urine samples and because both kidney and bladder exfoliate cells for urine, it was critical to ensure that methylation levels of cells (both normal and neoplastic) shed from those organs would not confound our assay.

Globally, promoter methylation levels of all microRNAs were lower in renal tissues (both normal and malignant) compared to PCa samples. However, methylation levels of miR-129-2 and miR-663b were significantly higher in BlCa than in PCa. Thus, miR-34b/c, miR-193b and miR-1258, were selected for further testing in urine sediments as its promoter methylation was shown to be PCa-specific.

5 Evaluation of diagnostic performance in urine sediments

Validation of miR-34b/c, miR-193b miR-1258 was subsequently assessed in urine sediments collected from both healthy donors and PCa patients.

Whereas promoter methylation levels of miR-34b/c and miR-193b were significantly higher in urines from PCa patients compared to those from healthy donors, the opposite was observed for miR-1258 methylation levels.
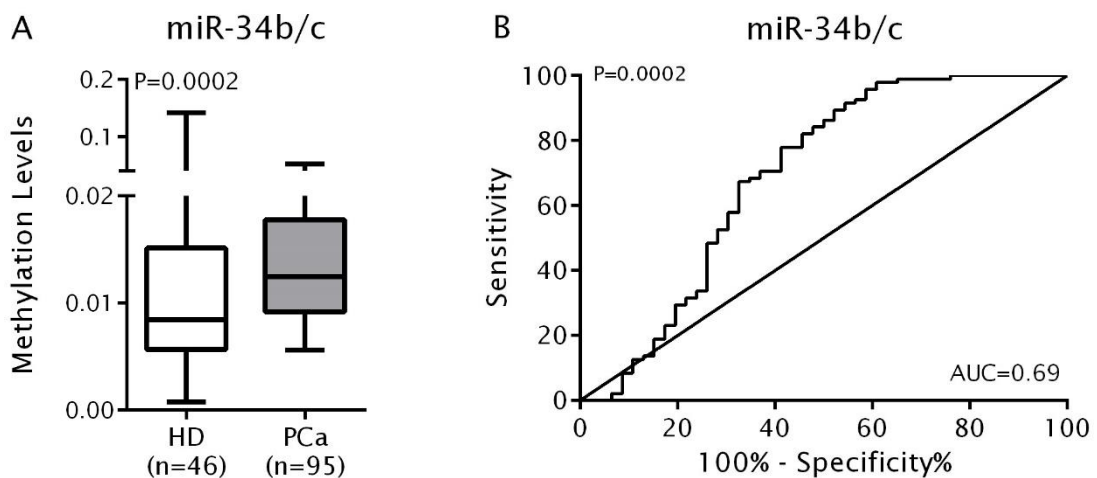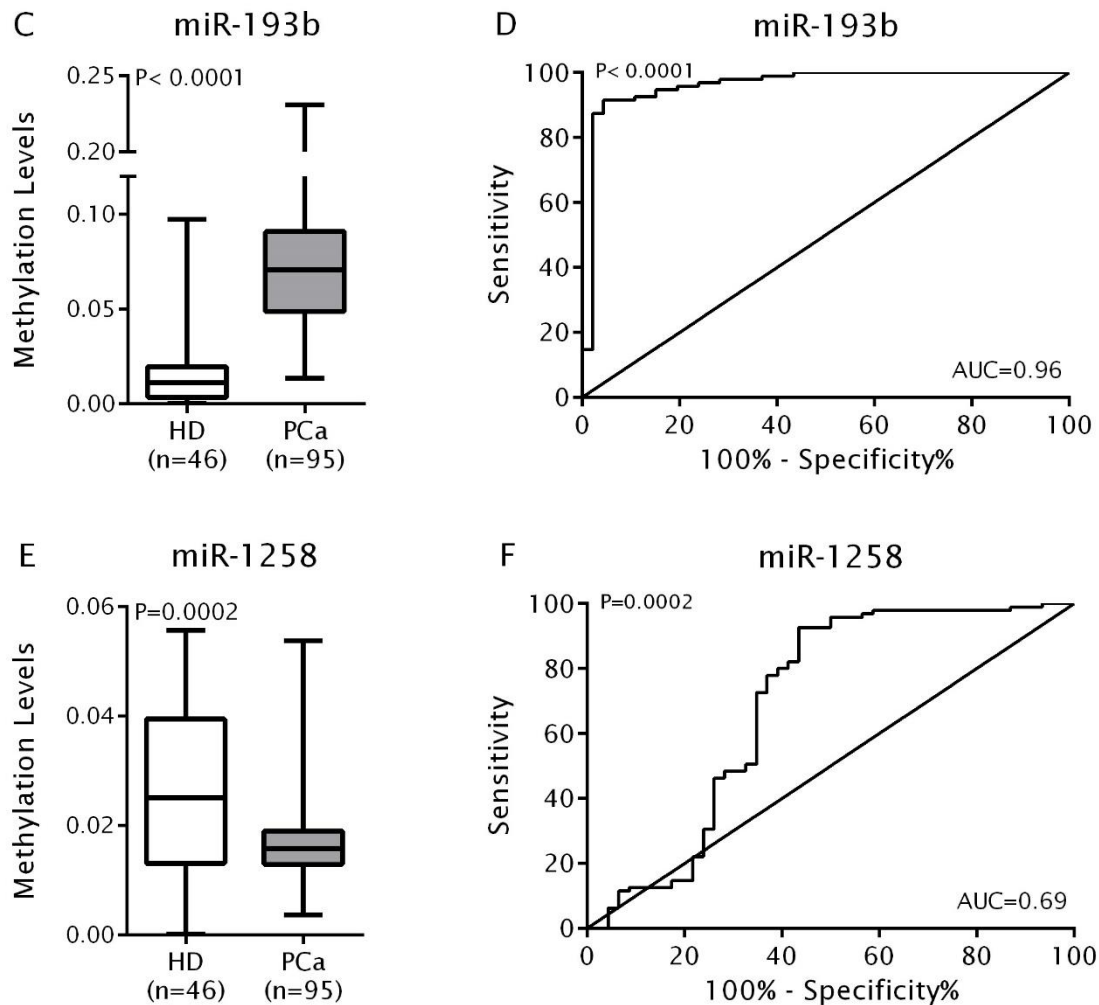
Figure 9 - Box-plots (left panel) depicting higher promoter methylation levels in PCa patients for miR-34b/c and miR-193b (but not for miR-1258) and ROC curves (right panel) displaying miR-34b/c, miR-193b and miR-1258 biomarker performance across urine sediments.

Table 8 - Performance of epigenetic biomarkers for the detection of PCa in urine sediments.

microRNA	Sensitivity % (n positive/n total)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR−34b/c	89.5 (85/95)	47.8%	78.0%	68.8%	75.9%
miR−193b	91.6 (87/95)	95.7%	97.8%	84.6%	92.9%
miR−1258	7.4 (7/95)	43.5%	21.2%	18.5%	19.1%

ROC curve analysis, revealed that miR-193b allowed for a better discrimination of PCa from normal samples, compared to miR-34b/c, with AUC values of 0.96 vs. 0.71, as well as 91.6% sensitivity and 95.7% specificity vs. 89.5% and 47.8% respectively. Although the sensitivity of miR-34b/c was about 90%, specificity was below 50%. Remarkably, PPV and NPV values for miR-193b were

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97.8% and 84.6%, respectively. No statistically significant association between microRNAs promoter methylation levels and clinicopathological parameters was depicted, in urine samples.

6 Exploring microRNA promoter methylation as prognostic biomarker

The last aim of this Master thesis consisted on the assessment of the prognostic value of microRNA promoter methylation in prostate biopsy tissue cores. The median follow-up in this PCa patient cohort was 104.04 months (range: 9.11–170.10 months). At the time of the last follow-up, 17 patients (22.97%) had died from PCa and 32 out of of 74 (43.24%) developed biochemical recurrence. In 3 patients, serum PSA levels >0.2 ng/ml persisted following treatment and these were not further considered for disease-free survival analysis.

Hence, disease-specific survival (DSS) and disease-free survival (DFS) curves were constructed based on clinical variables, namely clinical stage, Gleason score, PSA levels and CAPRA Score, to validate our data. Since CAPRA score values range from 1 to 10, for the purpose of this analysis this variable was categorized as 0-2 (low-risk tumors), 3-5 (intermediate risk) and 6-10 (high-risk tumors) [30]. For statistical purposes, microRNA methylation levels were dichotomized using the percentile 75 as threshold value. Except for serum PSA, all clinicopathologic parameters tested and miR-129-2, but not miR-34b/c, methylation levels, associated with DSS in univariate analysis.

Figure 10 – Higher clinical stage (upper left), Gleason Score (upper right) CAPRA Score (lower left) and miR-129-2 methylation levels (lower right) associated with worse disease-specific survival curves on a biopsy cohort of 74 PCa patients.

Moreover, a Cox regression analysis was also computed to assess the potential of both clinicopathological and epigenetic variables in predicting DSS (Table 9). Of the clinicopathological variables, only clinical stage was statistically significant. Interestingly, high miR-129-2 promoter methylation levels also independently predicted shorter DSS, although with a lower hazard ratio.

Table 9 - Cox regression assessing the potential of clinical and epigenetic variables in the prediction of disease-specific survival for 74 PCa patients.

Disease-specific survival – Cox regression

Variable	HR	CI (95%)	*P*
Clinical stage			
II	1		
III/IV	8.12	2.63-25.1	<0.001
miR-129-2			
≤P75	1		
>75	3.30	1.26-8.61	0.015

Because biochemical recurrence is an important primary endpoint in PCa, we further tested the prognostic value of standard clinical variables and methylation-based markers in this setting.
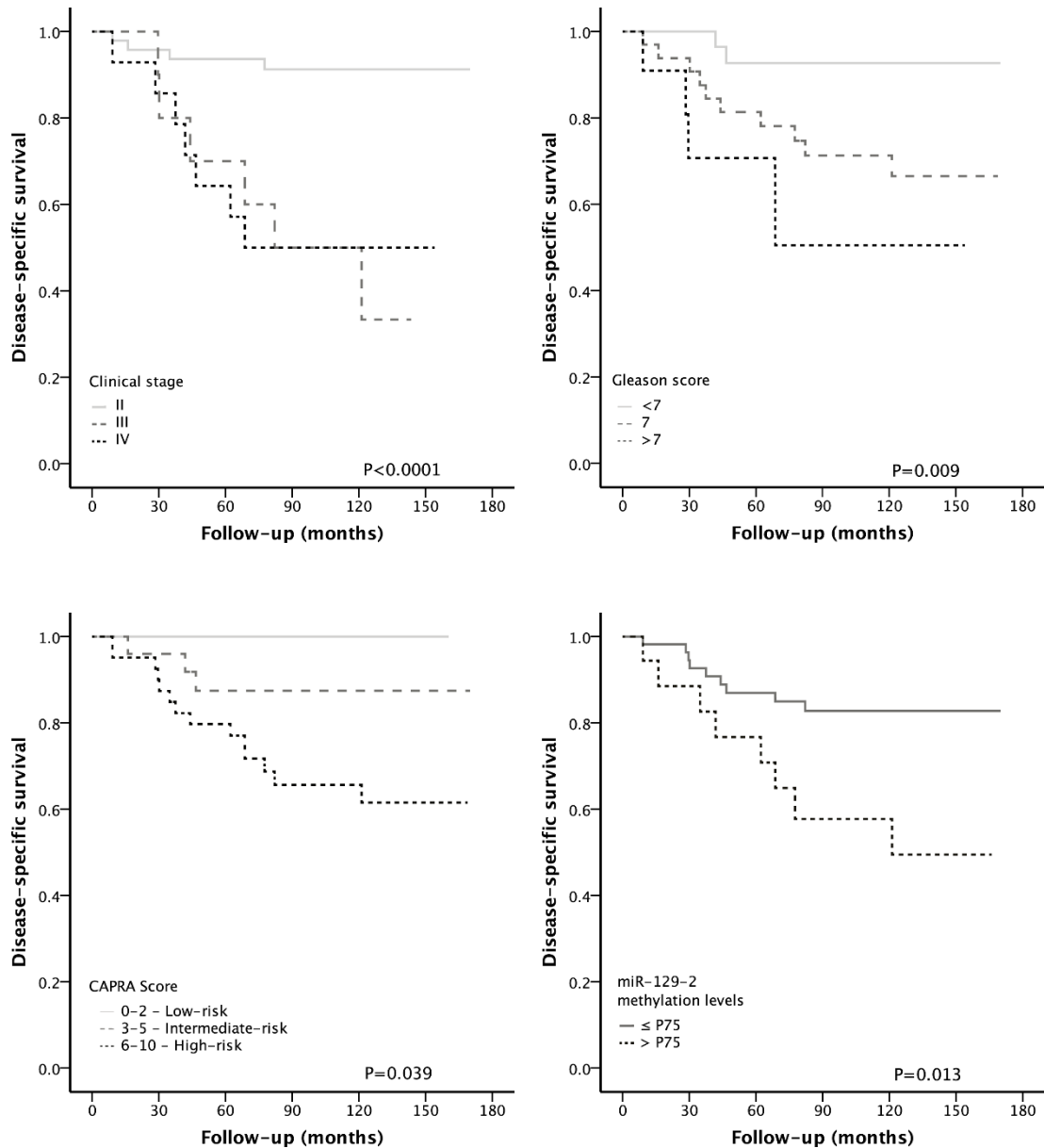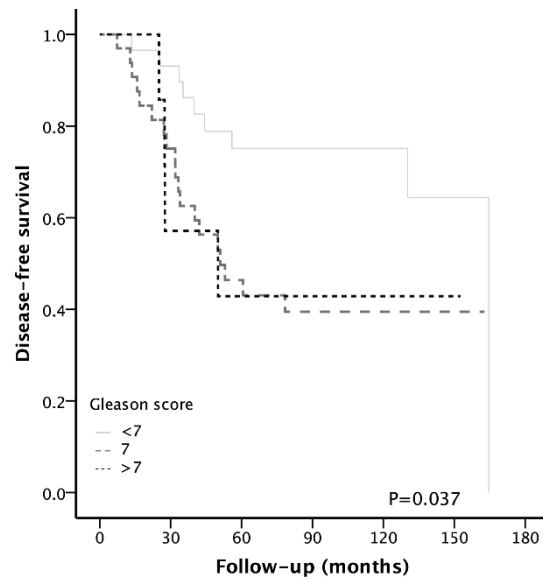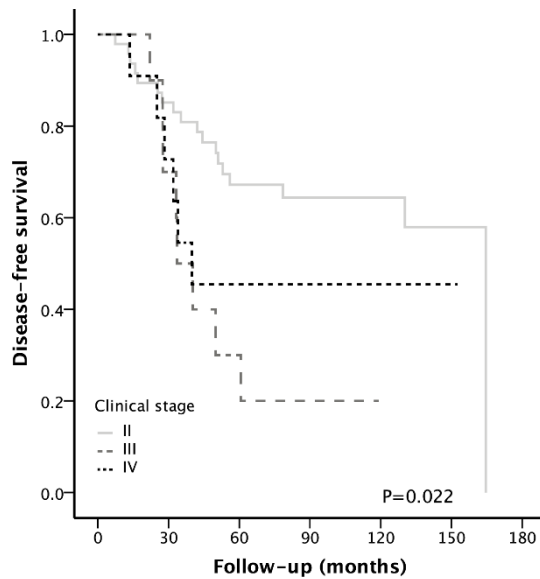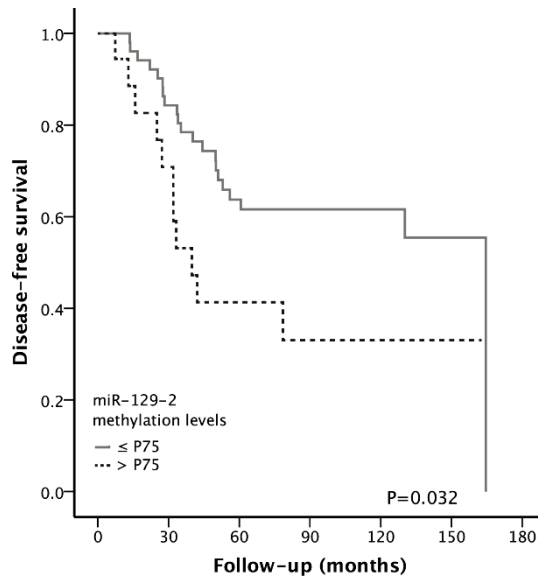
Figure 11 – Higher clinical stage (upper left panel), Gleason score (upper right panel) and miR-129-2 methylation levels (lower) associated with poorer disease-free survival in a biopsy cohort of 71 PCa patients.

PSA levels and CAPRA score did not associate with DFS in univariate analysis. Nonetheless, higher clinical stage, Gleason score and miR-129-2 promoter methylation levels statistically associated with worse DFS. MiR-34b/c methylation levels displayed a trend similar to that of miR-129-2, but did not reach statistical significance (P=0.06).

Similar to DSS, only higher clinical stage and increased miR-129-2 promoter methylation levels independently predicted shorter DFS, in multivariate analysis (Table 10).

Table 10 - Cox regression assessing the potential of clinical and epigenetic variables in the prediction of disease-free survival for 71 PCa patients.

Disease–free survival – Cox regression			
Variable	HR	CI (95%)	*P*
Clinical stage			
II	1		
III/IV	2.46	1.20-5.03	0.014
miR–129–2			
≤P75	1		
>75	2.26	1.08-4.73	0.031

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Discussion

PCa remains one of the most prevalent neoplasms worldwide and constitutes a leading cause of morbidity and mortality by cancer in men. Due to PSA screening, improvements have been achieved in clinical management of this malignancy. Indeed, its introduction, not only has led to a lower median age of diagnosis, but also to a decrease in the number of men presenting with metastatic disease at the time of diagnosis. Conversely, more men have been diagnosed with PCa, most of which with indolent tumors, and more men with benign conditions such as BPH and prostatitis have been biopsied. Thus, the potential benefits of PSA screening have been challenged by overdiagnosis of prostate carcinomas that would not cause harm or death if left undiagnosed and untreated [65]. Thus a strong recommendation against PCa screening through serum PSA levels has been issued recently, prompting the search for more effective biomarkers that allow for patients' risk stratification. In this project, we aimed to contribute to this relevant scientific and clinical quest through the discovery of new biomarkers for PCa detection and prognostication. For that purpose, we performed DNA methylation analysis in multiple patient cohorts and sample types for identification and validation of novel PCa biomarkers with improved performance.

Deregulation of epigenetic mechanisms is a key factor in carcinogenesis. Besides providing insight into the complex process that is prostate carcinogenesis, DNA methylation analysis has also brought forward valuable detection biomarkers for this disease, such as *GSTP1*, *APC*, *RARß2* and *RASSF1A* [69]. Indeed, epigenetic silencing of cancer-related genes by aberrant promoter methylation is now recognized as a key event in prostate carcinogenesis and a promising tool for screening, early diagnosis and prognostication of PCa [40]. Besides gene promoter methylation, microRNA deregulation is an acknowledged epigenetic feature of cancer. Aberrant microRNA promoter methylation has been recognized as an underlying cause of this deregulation in several neoplasms, including PCa [90].

Owing to our previous experience in DNA methylation analysis [70, 72, 73], we decided to seek for altered methylation patterns at the promoter regions of microRNAs deregulated in PCa. This information was then used to develop novel biomarkers, instead of microRNA expression levels, as previously attempted by others [91-93]. Indeed, DNA methylation is easier to assess than microRNA expression, it is more specific and, importantly, more stable. Moreover, because microRNAs downregulation in cancer is more common than upregulation, it

seemed likely that aberrant promoter methylation might constitute an underlying mechanism, similar to protein-coding genes [52].

Although several strategies might be used to identify microRNAs putatively downregulated due to promoter hypermethylation, high-throughput technologies such as methylation-array analysis is able to simultaneously pinpoint putative candidates [94] and the reliability of the results might be readily assessed through the analysis of well-known hypermethylated loci. Indeed, the results of the methylation array experiments that gave rise to this dissertation confirmed the high prevalence of *GSTP1* and *APC* promoter methylation (data not shown). To increase the likelihood of finding robust candidate biomarkers, we used stringent conditions based on high fold-variation of methylation levels between cancerous and non-cancerous tissue samples. Nevertheless, subsequent validation of the results from the array are mandatory to definitively assess the performance of the candidate biomarkers.

From methylation-array analysis, six candidate microRNAs, putatively deregulated by promoter hypermethylation were identified. Among those, miR-1258 was the most promising due to very high sensitivity, specificity and accuracy in tissue samples. Moreover, it demonstrated substantial PCa-specificity compared with other tumors from the urinary tract, a feature that would make it the most attractive candidate to test in bodily fluids. MiR-193b was also a very promising candidate with a very good performance. Like miR-1258, its methylation seems to be very specific for PCa. Finally, miR-34b/c was also considered for testing in urine samples since it showed interesting results albeit not as promising as the previous two. Although it had better sensitivity and accuracy than miR-193b, its specificity was lower than that of miR-193b. Nevertheless, it was also very specific for PCa when compared with bladder and renal tissues, either cancerous or non-cancerous. MiR-129-2 and miR-663a showed modest results as diagnostic biomarkers but their inability to distinguish PCa from bladder cancer rendered them unsuitable to be tested in urine samples.

Correlation between the methylation levels measured by qMSP in the tumor tissue samples and standard clinicopathological variables (serum PSA at diagnosis, pathological stage, Gleason score) was also assessed for all candidates. In this analysis, increased promoter methylation levels of miR-129-2 were associated with higher Gleason score and stage, indicating that this microRNA could be a putative prognostic biomarker. MiR-34b/c, miR-663a and miR-1258 methylation levels also

correlated with pathological stage, but its higher diagnostic performance underscores the potential for detecting PCa at early stages instead of prognostic assessment, as we previously reported for *EFEMP1* promoter methylation [95]. Importantly, these results are in line with previous observations by our research team and others concerning the association of higher gene promoter methylation levels with clinicopathological features of more advanced and aggressive disease.

The next step consisted in testing the best performing diagnostic candidates in urine samples. Urine is a very attractive clinical sample to evaluate DNA methylation biomarkers for PCa. It is easily obtainable and biomarkers are diluted to a smaller extent than in plasma, providing higher sensitivity [96]. Nevertheless, the amount of DNA potentially deriving from prostatic cells is rather variable, usually low, entailing the need to use a panel with limited number of biomarkers. Thus, only miR-34b/c, miR-193b and mir-1258, were tested in urine samples obtained from healthy donors and patients harboring PCa. Mir-193b displayed the best results in this assessment, with high AUC, sensitivity, specificity and PPV, whereas miR-34b/c performance was modest. Intriguingly, miR-1258, which showed the best performance in tissue samples, displayed a strikingly different result in urines as its methylation levels were higher in healthy donors' samples than those from PCa patients. The reason for this discrepant result is not immediately apparent, but it could be due to high miR-1258 promoter methylation in non-epithelial cells, such as leucocytes that might be relatively more abundant in urine than in tumor tissue samples. Moreover, median miR-1258 promoter methylation levels in urines from PCa are substantially inferior to those of miR-193b, thus, impairing the robustness of the qMSP assay. It should be recalled that, contrarily to other studies, the urine samples we used were not collected following DRE or prostatic massage, which are usually employed in an attempt to yield a more representative sample to increase sensitivity. Studies dealing with PCa biomarkers in urine vary in the method of urine collection and the real impact of prostatic massage has never been evaluated [97]. It could be argued that the distance from the peripheral zone to the urinary tract flow may render urinary based tests less sensitive, which would be an important issue since most malignancies arise from this zone. Nevertheless, studies on *PCA3* did not find a difference in the levels of this biomarker between patients with peripheral versus transitional zone PCa [98, 99].

Interestingly, studies using post-DRE collected urine samples have reached different levels of success. *GSTP1*, *RARβ2* and *APC* promoter methylation levels assessed in urine samples collected after DRE showed modest performance as biomarkers for PCa detection (AUCs varied from 0.63 to 0.68) [75]. Even *GSTP1* promoter methylation sensitivity in urine differs among reports, from 21.4% to 38.9%, depending on the assay used, although it is improved by prostatic massage to 75% [100]. Moreover, in our study, miR-34b/c and miR-193b had higher methylation levels in urine samples from patients with PCa, as would be expected, with miR-193b showing better results than those mentioned above for *GSTP1*, *RARβ2* and *APC*. In another study using urine samples following prostatic massage, Rouprêt *et al* assessed methylation levels of several genes (including *GSTP1*, *RASSF1A*, *CDH1*, *APC*, *DAPK*, *MGMT*, *p14*, *p16*, *RARβ2*, and *TIMP3*) by qMSP. Of these *GSTP1*, *RASSF1A*, *APC* and *RARβ2* were those that best discriminated malignant from non-malignant cases, with AUC values ranging from 0.74 to 0.86. The combination of these four genes yielded the greatest discriminatory power with 86% sensitivity and 89% overall accuracy [101]. Importantly, the diagnostic performance of miR-193b in urine compares favorably with the aforementioned biomarkers, but it should be tested in a larger and independent dataset.

Notwithstanding, the performance of miR-193b in urine samples needs to be compared with that of serum PSA and urinary *PCA3*, as these are the only biomarkers approved for clinical use. As previously mentioned, the performance of serum PSA as a PCa biomarker is rather modest, with AUC ranging from 0.54 to 0.70 [53, 100]. Even other serum PSA-derived measurements, like PSA-density, free PSA percentage and PSA-velocity have not improved this value above that interval [53]. On the other hand, *PCA3*, which is currently the most widely used non-PSA based first-line test, performed better that serum PSA, both in urine and ejaculates, with AUC varying from 0.66 to 0.79 [53, 100, 102, 103]. Once again, miR-193b performance compares well with those two PCa biomarkers and might constitute a promising tool for early non-invasive detection of PCa, if these results are independently confirmed. It should be emphasized that combining miR-193b with miR-34b/c did not increase the diagnostic performance of the assay (data not shown).

The last aim of this study consisted on the determination of the prognostic value of the candidate microRNAs. For this purpose, miR-34b/c and miR-129-2 promoter methylation levels were analyzed in set of prospectively collected

prostate biopsies. Because the major goal was to discriminate the clinically aggressive PCa from those that do not pose a threat to the patient's life and might be left untreated, it was critical to test the prognostic value of the microRNAs in a pre-therapeutic setting. In univariate analysis, most standard clinicopathological parameters associated with DSS and DFS, thus clinically validating this prostate biopsy dataset. The CAPRA score, however, only correlated with DSS but not DFS in this series. This was an unexpected result as CAPRA score determined at diagnosis, correlates with DFS in patients submitted to radical prostatectomy, i.e., those with clinically localized disease [30]. Notwithstanding, our prostate biopsy series included PCa at diverse clinical stages, submitted to different therapeutic modalities: radical prostatectomy, radiotherapy or androgen-deprivation therapy. This feature might explain the failure of CAPRA score to predict DFS. In multivariate analysis, only clinical stage, amongst all clinicopathological parameters tested, retained independent prognostic value, both for DSS and DFS. Remarkably, high miR-129-2 promoter methylation levels also predicted shorter DSS and DFS, suggesting that it might constitute a useful prognostic biomarker for PCa patients. It should be recalled that miR129-2 and miR-34b/c were selected for this analysis based on their association with pathological stage or Gleason score in the radical prostatectomy cohort. Considering the results of the multivariate analysis in the prostate biopsy cohort, we might assume that miR-129-2 promoter methylation levels are indicative of more clinically aggressive PCa, irrespective of disease extent at diagnosis.

Final conclusions

In this study we explored the biomarker potential of six candidate microRNA that were previously found to be hypermethylated in PCa by our group.

All candidates were validated in a large RP sample set. Moreover, we tested the PCa-specificity of methylation levels from each candidate, in comparison to other urinary tissues and neoplasms. Thus, the most promising diagnostic biomarkers (miR-34b/c, miR-193-b and miR-1258) were tested in an independent set consisting of voided urine samples, in which miR-193b was shown to be a very promising diagnostic biomarker with potential use as a non-invasive test for early detection of PCa.

Moreover, those microRNAs whose methylation levels were found to be associated with aggressive disease in the validation set (miR-34b/c and miR-129-2) were tested in a set of biopsy samples, seeking for prognostic value. MiR-129-2 surfaced as independent predictor of survival as higher promoter methylation levels associated with poorer DSS and DFS.

Although additional studies, preferably involving multiple institutions, are required to further validate these findings, we demonstrated herein that quantitative assays for specific microRNA promoter methylation (miR-193b and miR-129-2) might constitute important diagnostic and prognostic ancillary tools for clinical decision making.

Annex I

Table 11 - Current knowledge of the six putative candidates analyzed in this study.

MicroRNA	Genome location	Alterations in other tumors	Biomarker potential	Alterations in PCa
MiR–34b/c	chr11: 111512938-111513021 chr11: 111513439-111513515	Promoter polymorphisms increase risk of HCC and RCC, and decrease risk in gastric cancer [104-106]. Promoter hypermethylation is associated with late clinical stage in patients with soft tissue sarcomas [107].	Promoter methylation in stool samples for colorectal cancer [108].	Downregulated by promoter methylation [109, 110].
MiR–129–2	chr11: 43581394-43581483	Epigenetically repressed in gastric, esophageal, endometrial, lung, colorectal and hepatic tumors [90, 93, 111-113].	Expression in gastric juices [92]. Expression in plasma for HCC [93].	Not reported.
MiR–152	chr17: 48037161-48037247	Downregulated in lung, liver, ovarian, laryngeal and bladder cancer [114-118]. Proposed tumor-suppressor role in NSCLC [119, 120].	Expression differentiates high-risk from low-risk PCa [91].	Promoter is highly methylated. Has a tumor suppressor role [121, 122].
MiR–193b	chr16: 14303967-14304049	Downregulated in pancreatic tumors causing impaired cell growth [123]. Downregulated in ovarian carcinoma promoting metastasis [124, 125]. High expression correlates with poor survivability in melanoma and drives breast cancer progression (11).	Not reported.	Silenced by promoter methylation [126]. Has a tumor suppressor role [127].
MiR–663a	chr20: 26208186-26208278	Downregulated in chordomas and pancreatic tumors [128, 129].	Expression in serum samples for chordomas and pancreatic tumors [129].	Not reported.
MiR–1258	chr2: 179860836-179860908	Has a negative correlation with heparanase expression and is downregulated in NSCLC and breast tumors [130, 131].	Not reported.	Not reported.

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