

**THE UNIVERSITY OF MEDICINE AND PHARMACY
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THESIS

**THE DIAGNOSTIC VALUE OF SOME NEW GENETIC AND
EPIGENETIC MARKERS IN PROSTATE
ADENOMOCARCINOMA**

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Prostate cancer is known to be the most common neoplastic disease of men and the second leading cause of cancer death, with an estimated 1.7 million new cases diagnosed annually by 2030, currently the number of cases diagnosed annually is 899,000 .

We find the highest incidence of prostate cancer among African-Americans and Jamaicans of African origin

Worldwide it is the sixth leading cause of cancer death, with an estimated 258,000 deaths annually, reaching 499,000 deaths by 2030. In the US, 10% of all cancer deaths are prostate cancer. The mortality rate among African-Americans remains 2.4 times higher than for Caucasians, although it has been steadily declining since 1990. Between 1973 and 2008, the mortality rate for those diagnosed with Cp was 35%. Early diagnosis, PSA screening and curative therapy led to a significant decrease in mortality.

The introduction of PSA screening has had a major effect on the incidence and mortality of prostate cancer worldwide. In the United States, the risk of Cp doubled from 7.8% to 15.3%, while mortality decreased from 3% to 2.6%.

In 2012, after the publication of the results of the PLCO and ERSPC studies, the US Preventive Forces Service (USPSTF) does not recommend the routine use of PSA screening among healthy men, regardless of age, race or family history, so PSA screening receives a grade recommendation D, meaning there are major doubts about the net benefit or the side effects outweigh the benefits. Given the plurifactorial nature of neoplastic pathologies in general, part of the risk factor research efforts included genetic inheritance in prostate cancer.

The first reference to the familial component of prostate cancer was made in the mid-twentieth century and brought into question the increased risk of Cp in men with first-degree relatives who suffered the disease. Subsequent studies have demonstrated the theory, and twin studies have concluded that the risk of developing Cp, in which family inheritance is incriminated, is 40% higher than in any other cancer. The relative risk increases with the degree of kinship, the number of affected members and the age at which they were diagnosed. About 15% of prostate cancers are considered to be genetically inherited. Prostate adenocarcinoma is a hormone-dependent neoplastic pathology, more precisely, it depends on the interaction between testosterone and prostate tissue. The main androgen hormone, with trophic effects on the prostate, is dihydrotestosterone (DHT), the activated form of testosterone (T), the transformation being catalyzed by 5α -reductase. DHT interacts with intracitoplasmic androgen receptors (ARs), with a much higher affinity than T, and as a result of this interaction, the translocation process of the steroid-receptor complex at the nuclear level is amplified, with the activation of androgenic response elements (AREs). 5α -reductase type 1 is expressed predominantly in the skin and liver, less on the prostate level, whereas the type 2 enzyme is distributed exclusively in the prostate and other genital tissues. Phenotypes with inherited deficiency of 5α -reductase show minimal prostatic tissue, predominantly stromal, histopathological studies demonstrating the absence of the epithelial component. The deficiency of T, the hormonal substrate of the enzyme, seems to provide a protective effect, with the argument of prostatic atrophy consistent with surgical castration. However, there are documented cases of ADC-P in hypogonadal adults, and these cancers induce, etiopathogenic, other tumor proliferation mechanisms, intuitively independent of androgen activity. Currently, the diagnosis of prostate cancer is performed routinely by correlating PSA with the result of rectal examination. Most often, the diagnosis is obtained by PBP under transrectal ultrasound guidance, before the appearance of the clinical picture, and the post-diagnostic behavior has as central purpose the staging of the pathology, by the evaluation of the tumor extension, and the classification in a risk group, in order to establish the prognosis and management strategy. In addition to PSA and TR, histopathological features (SG and tumor volume) and imaging studies (assessment of loco-regional extent and presence of metastases) will facilitate informed management decisions. Unfortunately, evaluation by PSA and rectal touch cannot diagnose prostate adenocarcinoma in the early stages, one of the reasons being that PSA analysis is not specific for prostate cancer, the value of the specific prostate antigen may be influenced by non-

neoplastic prostatic insult. On the whole, the presence of a prostatic pathology (Cp, benign prostatic hyperplasia - HPB or prostatitis) is the main factor influencing the serum expression of PSA, the theory being postulated that the increases of serum PSA occur in the context of altering the glandular architecture, which facilitates the release in circulation. Although an increased level of PSA may indicate the presence of prostatic pathology, not all men with prostatic pathology report increased serum PSA levels, and PSA growth is not specific for Cp, being observed in the context of prostatic manipulation (TR , PBP, transurethral resection - TUR-P). A number of serum, urinary and genetic biomarkers have been proposed. Consolidated serum biomarkers are PSA and PSA derivatives, PSMA (prostate specific membrane antigen) Human Kalicrein 2 (hK2), a PSA-like biomarker.

The first urinary biomarker of prostate adenocarcinoma was PCA3 initially described by Bussemakers et al. (1999). Although the function of PCA3 remains unknown, multiple studies have shown that PCA3 is a species of lncRNA, which does not exhibit extraprostatic expression, and the expression level of PCA3 is generally much increased in malignant prostatic tissue compared to benign tissue. Unlike PSA, PCA3 expression values are independent of prostate volume. The concept of a unique prostate cancer biomarker, capable of answering all the important clinical questions related to pathology, is becoming increasingly unlikely, which is why multiple initiatives to develop integrative systems of Cp biomarkers have been launched, which represent added value to the diagnostic process.

Thanks to the revelation that most of the genomic DNA does not encode protein arrangements, the utility of these sequences in modulating gene expression has become a topical topic in the literature. Possibly the most important components of this type of gene modulation are microRNA (miRNA) species, short non-coding single-stranded RNA sequences (19-22 nucleotides) involved in messenger RNA (mRNA) modulation. These have been detected in a wide variety of biological fluids and are being investigated as potential biomarkers for a variety of malignancies. Given the potential etiopathogenic role of miRNA species, they could provide essential diagnostic and prognostic information.

At the tumor cell level, miRNA species act by two distinct mechanisms: overexpression of miRNA species that promotes carcinogenesis, by inhibiting the expression of tumor suppressor genes, called oncogenic miRNA sequences (OncomiRNA), or defective expression of miRNA species

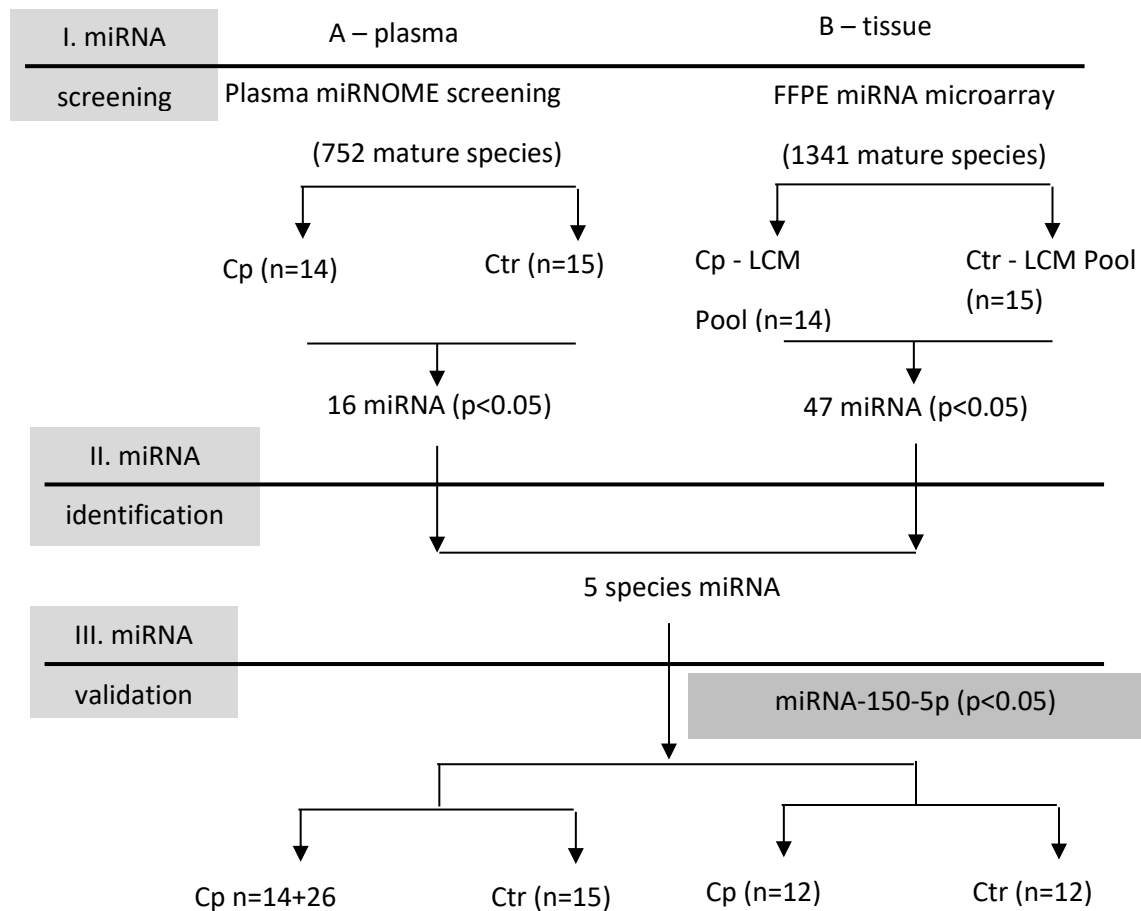
that, normally, suppresses neoplastic transformation, by inhibiting the expression of proto-oncogenes, called miRNA sequences of tumor suppression.

The doctoral thesis "The diagnostic value of new genetic and epigenetic markers in prostate adenocarcinoma" has as main objective the identification of circulating miRNA species, specific to prostate cancer, that can accurately discriminate cancer patients from healthy subjects, respectively, and serve as biomarkers for screening, diagnosis and clinical management of prostate cancer. The second objective is to validate the panel of circulating miRNAs as a biomarker, in a case-control study of prostate cancer and to investigate the association of miRNA species with clinical and pathological features. The third objective is the validation of circulating miRNA in paraffinized prostate tissue (FFPE). The fourth objective was an analysis of peripheral DNA methylation and hydroxymethylation as a biomarker in prostate cancer. The recruitment of the subjects included in the present study was approved by the Ethics Committee of the Scientific Research of the "Victor Babeș" University of Medicine and Pharmacy of Timisoara (CECS Opinion No. 09 / 13.05.2014), as well as by the Ethics Committee of the County Clinical Hospital of Emergency Timisoara (Opinion 71 / 05.08.2014).

To carry out the study, two groups of subjects were constituted: the group of patients - men with histopathologically confirmed prostate adenocarcinoma and the Gleason score greater than 5, over 50 years old, who did not suffer from other neoplastic pathology and the group control - men without prostate pathology with a PSA value of less than 4 ng / ml confirmed by chemiluminescent microparticle immunoassay (Abbott Diagnostics), over 50 years old and without symptoms associated with the lower urinary tract. All subjects included in this study signed the informed consent form. Subject data were centralized from the observation sheets, with the following aspects being observed: age, PSA value, Gleason score and the anatomopathological result of the biopsy piece where appropriate. Analysis of miRNA profile in plasma and tissue of patients with prostate cancer (Cp) identified 5 miRNA species whose expression is altered with respect to controls without prostate pathology (Ctr): miRNA-130a-3p, miRNA-145-5p, miRNA-148a -3p, miRNA-150-5p, miRNA-365a-3p. Of these, only 3 species showed consistent changes in tissue and plasma: miRNA130a-3p and miRNA-150-5p were overexpressed and miRNA-148a-3p was overexpressed. Of these, only miRNA-150-5p was validated to be statistically significant under-expressed in plasma and FFPE tumor samples with an expression change of -2.697 ($p < 0.001$) and -1.693 ($p = 0.035$). ROC analysis revealed an AUC value of 0.817 (95% CI: 0.680-0.995) for

plasma and 0.809 (0.616-1.001) for tissue. Our data suggest that the variation of miRNA-150 expression in plasma is consistent with that in tumor tissue.

A schematic presentation of the design that led to the identification of miRNA150 as a potential biomarker in prostate cancer is presented below.



The choice of miRNA-150-5p for individual validation was motivated by the small number of data in the literature. Following the analysis of the data obtained from the individual analysis, it was found that miRNA-150-5p is significantly sub-expressed in plasma and FFPE from Cp patients with an expression change of -2.697 ($p < 0.001$) in plasma and -1.693 ($p = 0.035$) in FFPE.

To confirm the literature data regarding miRNA-150-5p expression in prostate cancer cell lines, we investigated this in LnCAP cells (prostate cancer cells) compared to normal prostate epithelial cells (PPEC). The relative expression of miRNA-150-5p in LnCAP compared to PPEC confirms the significantly lower ($p < 0.005$) expression of this microRNA in cancer cells described in the

literature, as well as our observations in tissue and periphery. Regarding the level of methylation and hydroxymethylation, this is the first study that measures both the global level of DNA methylation and the level of hydroxymethylation in peripheral nucleated blood cells in prostate cancer patients compared to the control group. Although this is a pilot study with a limited number of samples, we confirmed the data presented in the only available study showing that the level of DNA hydroxymethylation in blood cells is not different in prostate cancer patients compared to the control group. The mentioned study used the same method of analyzing the level of hydroxymethylation in blood cells, obtaining similar values for the prostate and control cancer groups. However, subjects with benign prostatic hyperplasia (BPH) and atypical small acinar proliferation (ASAP) had significantly higher levels of hydroxymethylation compared with the control group. Unfortunately, we did not include subjects with BPH and ASAP in this study. Compared to normal prostate tissue, cancerous tissue has lower levels of 5hmC, which is regulated at least in part by enzymes of the ten-eleven translocation (TET) proteins, which oxidize 5-methyl cytosine to 5-hydroxymethyl cytosine and then to other derivatives. . It appears that this pattern is localized in prostate tissue and is not reflected by peripheral nucleated blood cells of prostate cancer patients. Of the more than 700 microRNAs analyzed in plasma and plasma exosomes from prostate cancer patients, 127 differentially expressed miRNAs were identified between exosomes and plasma in prostate cancer patients. A clear separation of the two groups (plasma and exosomes) is observed based on the different expression of miRNA species, suggesting the existence of specific miRNA species for the two blood fractions.

Bioinformatics analysis revealed that the main pathological process to which these miRNA species belong, is cancer and the main cellular and molecular processes involved are related to the development, proliferation, motility, cell cycle and cell survival, all of which are representative processes for the carcinogenetic process.

Regarding the comparative analysis of total plasma miRNA expression between patients and control subjects, a total of 262 miRNAs were detected in plasma samples, of which 147 were detected in both patients and plasma. controls, 113 miRNAs were detected only in controls, and 2 miRNA species only in cases. Of the 147 common miRNAs, 70 are differentially expressed between cases and controls, at a significance level of $p < 0.05$ adjusted for multiple comparisons. And in this case the bioinformatics analysis revealed that the main pathological process to which

these miRNA species belong is represented by cancer. Also, the only signaling network identified includes cancer, suggesting a clear involvement in these pathologies of these miRNAs.

Following the application of stringent criteria for statistical analysis and data normalization, a panel consisting of 16 miRNAs with a relatively different relative plasma expression between cases and controls was finally retained, all with a relatively high biomarker potential, expressed by area. AUC of more than 0.70. Moreover, the potential of the biomarker was also reinforced by the fact that some of them returned to an expression level similar to controls in patients with radical prostatectomy. Similarly, miRNAs from whole plasma, in the case of those present in exosomes, were finally retained a panel consisting of 7 miRNAs with significantly different relative plasma expression between cases and controls, the majority (> 70%) having relatively high biomarker potential, expressed by an AUC area of over 0.70. Moreover, the potential of the biomarker was also reinforced by the fact that some of them returned to an expression level similar to controls in patients with radical prostatectomy. Comprehensive analysis of the entire miRnome was performed in prostate tissue samples stored in paraffin blocks more than 10 years old, remarkable that this was achieved in samples processed by laser microdissection, this being one of the few studies of this kind. published so far in the case of prostate cancer.

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tumor cells among cancer cells were greatly reduced, reducing the sources of errors when measuring miRNA expression in tumor tissue.

Regarding the analysis of methylation and hydroxymethylation, it was observed that the average level of global DNA methylation was 1.15% for cancer patients and 2.29% for controls. The difference of methylation almost 2 times was statistically significant ($p < 0.0001$), the patients with prostate cancer having a pronounced hypomethylation compared to the control group. The mean level of global DNA hydroxymethylation was similar in prostate cancer patients and in the control group (0.022% and 0.028%, respectively), without statistical significance. To our knowledge, this is the first study to measure both the global level of DNA methylation and the level of hydroxymethylation in peripheral nucleated blood cells in prostate cancer patients compared to the control group.