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DOCTORAL THESIS

CELLULAR AND MOLECULAR STUDIES IN ADVANCED PROSTATE CANCER

THESIS ABSTRACT

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TABLE OF CONTENTS

The doctoral thesis includes: ‣229 pages, of which 52 pages Current state of knowledge ‣ 65 figures, of which 22 in Current state of knowledge ‣ 28 tables, of which 2 in Current state of knowledge ‣ 235 bibliographical references

Research motivation

Despite advances in medical care, advanced cancer is still a very common problem. For some patients, having advanced cancer means having a disease that is no longer treatable. For others, it means having the uncertainty of not knowing whether the disease will be treated. Prostate cancer (PC) is a major public health problem worldwide due to its high prevalence, mortality, and morbidity. Although it is currently the second most common cancer overall, it is estimated that by 2035 there will be over 75,000 new cases of prostate cancer each year, making it the most common neoplasm overall. (1) According to statistics, prostate cancer is rarely detected before the age of 40; it is estimated that 6 out of 10 cases are diagnosed in men over the age of 65. Studies have shown that the average age at diagnosis is approximately 66 years. (2)

Currently, there is an increase in the incidence of this pathology due to the aging of the population and as a result of widespread screening. It is important to diagnose and intervene therapeutically as early as possible for aggressive forms and not to radically treat tumors without clinical impact. It is essential to develop and use diagnostic and predictive biomarkers to diagnose early prostate cancer, at the earliest possible stage and at the same time to facilitate the choice of optimal, personalized therapeutic regimens. (2, 3) Despite its high prevalence, the molecular mechanism involved in the occurrence of PC and its progression has not been deciphered to date. Histopathological and genetic data show that tumors originate from a single cell but, as tumors grow, they become increasingly heterogeneous, due to genetic and phenotypic changes that occur during tumorigenesis. (4)

Thus, due to the heterogeneous phenotype of prostate cancer, diagnosis and prognosis are often difficult: it is often a multi-focal tumor, presenting more than one histological grade and is frequently juxtaposed/combined with benign prostatic hyperplasia (5,6). It is absolutely necessary in the case of patients with advanced PC, who by definition have a poor prognosis, to identify biomarkers of response to treatment, which ultimately translates into the choice of personalized, patient-centered therapies and the reduction of costs by avoiding the use of medication on the “One Size Fits All” concept.

Despite the progress made in identifying the pathogenic mechanisms involved in the neoplastic process, currently, studies aimed at identifying biomarkers useful in the optimal choice of treatment for patients with advanced PC are of great relevance (6), and they aim to

identify cellular and molecular mechanisms, with increased specificity and sensitivity compared to standard biomarkers (PSA - prostate-specific antigen, TR - rectal swab and prostate tissue biopsy for HP - histopathological examination), in order to obtain early diagnosis and a highly accurate prognostic factor in prostate cancer. (7)

Recent genomic research has transformed the understanding of prostate cancer, providing new tools for diagnosing, monitoring, and treating this disease. (8) Although challenges and limitations remain, progress in this field promises to significantly improve patient care and pave the way for personalized medicine in oncology.

Therefore, it is imperative to improve diagnostic methods by exploring biomarkers with increased potential for detecting treatment-resistant forms of prostate cancer that may benefit from targeted curative therapy or for improving the overall prognosis of the disease.

Assessing the molecular profile of this pathology will allow the establishment of a management plan appropriate to the clinical and biological particularities so that each patient can benefit from personalized treatments for the molecular subtype of prostate cancer. By understanding the detailed genomic profile of each patient, precision medicine becomes a reality, allowing treatments specifically tailored to each individual and thus improving the prognosis and quality of life of patients.

In the general part of the paper, we presented the current state of knowledge in the literature on the pathology addressed, reviewing the existing knowledge on the epidemiology and biology of prostate cancer, including the incidence and prevalence of the disease at global and national levels, as well as the risk factors and etiology of the disease. Emphasis is placed on the anatomical and physiological aspects of the prostate, as well as on the molecular and cellular mechanisms involved in the carcinogenesis process.

The section aimed at personal research describes the research methodology and the results obtained from clinical studies conducted on patients diagnosed with advanced prostate cancer. The study was conducted in collaboration with the Constanta County Emergency Clinical Hospital and the CEDMOG research center, using modern molecular biology equipment to evaluate the expression of biomarkers involved in carcinogenesis.

The thesis includes three main studies. The first study clinically and morpho-histochemical characterizes patients with prostate cancer. The second study explores the tumor microenvironment and the role of biological processes in disease progression, and the third focuses on the analysis of genetic and signaling interactions involved in advanced prostate cancer. These studies aim to correlate clinical and molecular data to obtain a detailed understanding of the mechanisms of tumor progression and to improve therapeutic approaches.

The results of each study are discussed with data from the specialized literature, and at the end of each study, the most relevant conclusions are drawn, supported by the observations presented.

The thesis is notable for its rich iconography, graphs, images and conclusive tables to present the results obtained as clearly as possible and ends with the "General Bibliography", in which the literature consulted is presented in alphabetical order, at the end of each chapter of the thesis there is a selective bibliography, in which the authors are presented in the order of citation in the text.

The value of the results obtained allowed me to translate the results of the studies carried out within this doctoral research into scientific articles that were published in WoS-rated specialized journals, all with a high impact factor above 3 (such as: Biomedicine, FI = 4.7; International Journal of Molecular Sciences and Biomedicines, FI = 4.9) or to present them at important international conferences, reflecting the impact and relevance of the research for the scientific community.

Thus, the thesis offers a deep and complex perspective on advanced prostate cancer, with the potential to influence future research directions and improve existing therapeutic approaches.

I. CURRENT STATE OF KNOWLEDGE

1.1. Epidemiology of prostate cancer

Prostate cancer is the most common cancer diagnosed in men in many developed countries. According to Globocan 2022 (1), prostate cancer ranks first among cancers diagnosed in men globally, with approximately 1.46 million new cases diagnosed annually, being the third leading cause of death from cancer in men globally. In 2022, approximately 397,430 men died from prostate cancer worldwide. (1, 2). Approximately 8,000 new cases of prostate cancer were diagnosed annually in Romania and 3,000 deaths were reported annually due to prostate cancer. The incidence of PC increases with age, tripling after the age of 50 according to statistics. Although its incidence is increasing, mortality from prostate cancer is decreasing, mainly due to overdiagnosis of diseases that would not have become clinically evident during the patient's lifetime. (2, 3)

1.2. Biology of prostate cancer

The biology of prostate cancer involves complex processes at the molecular and cellular levels. The prostate is made up of two main types of cells: epithelial cells, which form the glands and ducts of the prostate, and stromal cells, which form the supporting tissue. Prostate cancer usually begins in the epithelial cells of the prostate glands (adenocarcinoma). This process involves the transformation of normal cells into cancer cells, which lose control of their growth and division.(8)

Molecular and Cellular Mechanisms Involved in Carcinogenesis

Prostate carcinogenesis is a complex process involving a number of molecular and cellular mechanisms. These mechanisms contribute to the development and progression of prostate cancer and include genetic, epigenetic, and cell signaling alterations.

Growth Factors and Major Cell Signaling Pathways

• Androgen Hormones: Testosterone and other androgens are essential for the development and maintenance of the prostate. Prostate cancer cells are usually sensitive to these hormones. The prostate is stimulated by androgens, and cancer cell proliferation is often dependent on them.(9)

• ***Androgen receptors (AR):*** Androgen receptors play a crucial role in the development of prostate cancer. These receptors bind to androgens and activate genes that promote cell growth. In many cases of prostate cancer, these receptors are overexpressed or mutated, leading to uncontrolled cell proliferation. (10)

• ***Signaling pathways:*** Other signaling pathways, such as the PI3K/Akt pathway and the MAPK pathway, are also involved in prostate cancer progression. These pathways are involved in processes such as cell proliferation, cell survival, and metastasis. (11,12)

The tumor microenvironment

The tumor microenvironment (or tumor microenvironment) plays a crucial role in prostatic carcinogenesis, influencing the development, progression, and metastasis of prostate cancer. (13) This complex environment includes not only tumor cells but also a variety of non-tumor components, such as stromal cells, blood vessels, extracellular matrix, and immune cells. (14,15) Important in tumorigenesis are the cellular immune components of the tumor microenvironment (TME), including intact, highly activated T helper cells, cytotoxic T lymphocytes (CTLs), M1 tumor-associated macrophages (TAMs), and natural killer (NK) subsets. After the initiation of oncogenesis, immunological rejection of tumors is largely mediated by tumor-infiltrating T cells. (49) Chronic activation results in the upregulation of molecules associated with exhaustion, including programmed death ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), and T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3). (16, 17, 18) The tumor microenvironment in prostate cancer plays a critical role in carcinogenesis, influencing tumor development, progression, and metastasis through complex interactions between tumor cells and non-tumor components of the microenvironment. (19,20) Understanding these interactions may provide new insights into tumor mechanisms and guide the development of more effective therapeutic strategies. (20-22)

1.3. Classification and staging of prostate cancer

Staging and classification of prostate cancer are essential to determine appropriate treatment and prognosis. (22) These processes involve assessing tumor extension, lymph node involvement, and the presence of metastases. (23, 24) There are several systems used to classify and stage prostate cancer, the most important of which are the TNM system, the Gleason score system, and PSA staging. (25) Staging of prostate cancer is crucial for (26,27):

- treatment planning: determines the appropriate type of treatment (surgery, radiotherapy, hormonal therapy, chemotherapy, etc.).
- prognosis: provides information about the prognosis of the disease and the chances of survival.
- assessment of response to treatment: helps monitor the effectiveness of treatment and adjust it according to the patient's response.

Early diagnosis and correct staging are essential for the effective management of prostate cancer. Regular medical check-ups and PSA testing are important methods for the early detection of prostate cancer.

1.4. Diagnosing advanced prostate cancer

The diagnosis of advanced prostate cancer involves a series of methods and techniques to confirm the presence of the disease, determine its extent and plan appropriate treatment. (28)

Diagnostic and prognostic biomarkers in prostate cancer

Biomarkers are extremely important in the context of prostate cancer, having multiple applications in diagnosis, prognosis, treatment monitoring and personalization of therapy (29 – 38). They can be serum, urinary or tissue and provide valuable information about the presence, extent and aggressiveness of the disease. (29)

- **Diagnosis**: biomarkers can help identify prostate cancer in its early stages, when clinical symptoms are not obvious. For example, prostate-specific antigen (PSA) is the most widely used biomarker in prostate cancer screening. However, PSA is not perfect, as it can be influenced by other conditions, such as benign prostatic hyperplasia.

Specificity and ***sensitivity***: serum and urine biomarkers provide a more accurate diagnosis, reducing the need for unnecessary biopsies.

Non-invasiveness: urine and blood tests are less invasive than tissue biopsies.

- **Prognosis**: biomarkers can also help assess the aggressiveness of prostate cancer, helping doctors predict the course of the disease and can help estimate the risk of recurrence after treatment.

Prediction of tumor aggressiveness: tissue biomarkers, such as Gleason score and Ki-67, help assess the aggressiveness of the cancer.

Disease monitoring: PSA and other serum markers are used to monitor treatment response and disease progression.

- **Treatment planning:**

Therapy selection: molecular and genetic markers, such as AR-V7 and PTEN, can guide the selection of personalized treatment.

Prediction of treatment resistance: in some cases, prostate cancer can become resistant to conventional treatments, and biomarkers can help identify these cases. For example, in the case of castration-resistant prostate cancer (CRPC), biomarkers that indicate specific genetic mutations or altered cell signaling may suggest the need for alternative therapies.

1.5. Cellular and molecular mechanisms in advanced prostate cancer

Advanced prostate cancer is characterized by a number of complex cellular and molecular mechanisms that contribute to disease progression, treatment resistance, and metastasis. (37)

Genomic studies

Several studies have identified mutations in key genes, recurrent mutations in genes such as TP53, PTEN, BRCA1/2, RB1, and PIK3CA. These mutations play a critical role in promoting genomic instability and cancer progression. Gene amplifications and deletions, for example, MYC gene amplifications and deletions in PTEN and RB1 genes, are frequently observed in advanced prostate cancer. The TMPRSS2-ERG fusion (40), is one of the most common gene fusions in prostate cancer, present in approximately 50% of prostate cancer cases. This fusion activates the ERG signaling pathway, contributing to oncogenesis.

Epigenetic studies

DNA methylation (41-45). One mechanism implicated is promoter hypermethylation. Tumor suppressor genes such as GSTP1 are frequently hypermethylated in prostate cancer, leading to decreased expression. Global DNA methylation studies have revealed specific methylation patterns associated with tumor progression and aggressiveness.

Histone modifications (41-45). Another mechanism implicated is histone acetylation and methylation. Post-translational modifications of histones, such as acetylation and methylation, play a role in regulating gene expression. For example, H3K27me3 methylation is associated with the repression of tumor suppressor genes. Other studies have highlighted the role of histone-modifying enzymes. Dysregulation of histone-modifying enzymes, such as HDAC and EZH2, contribute to oncogenesis and represent potential therapeutic targets.

Non-coding RNAs (41-45). Dysregulation of microRNAs (miRNAs), such as miR-21 and miR-221, is involved in the regulation of cell proliferation, apoptosis, and metastasis.

1.6. Innovative therapies for advanced prostate cancer

Treatment of prostate cancer often involves androgen deprivation therapy to reduce androgen levels or to block androgen receptors. However, many tumors develop resistance to this therapy over time, through mechanisms such as androgen receptor mutations or activation of alternative signaling pathways. (1)

Recent research includes the development of immunotherapy and gene therapy strategies for the treatment of prostate cancer, but these are still in the experimental and developmental phases. (45) Personalized medicine and the use of biomarkers represent an innovative approach in the treatment of advanced PC, allowing for the personalization of therapy based on the molecular and genetic characteristics of each patient.

Future prospects

Liquid biopsy and precision medicine will revolutionize the diagnosis and monitoring of prostate cancer, allowing early detection of recurrence and real-time adjustment of therapy. Future research focuses on combinations of treatments that overcome resistance mechanisms. Another solution is the use of artificial intelligence (AI) in diagnosis and treatment. AI algorithms can analyze molecular and imaging data to guide therapeutic decisions. An important direction is the development of new therapeutic targets. Studies of tumor metabolism and the microenvironment could lead to new and more effective treatments. Prostate cancer remains a major challenge, but technological advances and personalized medicine offer hope for more accurate diagnosis, more effective treatments, and more accessible care for all patients. Personalized medicine and the use of biomarkers represent a promising approach to improve the treatment of advanced prostate cancer. By identifying the specific molecular characteristics of each patient, it is possible to personalize therapy to achieve the best results. Continuing research and development of new biomarkers and targeted therapies will be essential for advancing this approach and improving patients' quality of life.

II. PERSONAL CONTRIBUTION

2.1. Research methodology

The present study aims to perform a detailed clinical-morphological analysis of advanced prostate cancers through studies at the cellular and molecular level, in order to obtain new and profound insights into their oncogenetic process. Through this research, I aimed to identify and correlate the clinical and morphological characteristics of advanced prostate tumors with the main molecular and genetic mechanisms that drive their development and progression. To achieve the established objectives, the cases were selected from the Constanta County Emergency Clinical Hospital "Sf. Apostol Andrei". I selected working methods and tools with a high degree of relevance for the phenomenon studied (Table 1).

Types of Studies:

Longitudinal, retrospective, indirect study – which allowed the formation of s***tudy group I.***

Cross-sectional, prospective, direct study – which allowed the formation of ***study groups II*** and ***III.***

Group I was obtained following a longitudinal, retrospective indirect study, being composed based on data extracted from the observation sheets of the Clinical Anatomy Pathology Service (SCAP) and information obtained from the statistics department of SCJU "Sf. Apostol Andrei" in Constanța, covering a period of 5 years (2019 - 2023).

Inclusion criteria:

- Patients have a definite diagnosis of prostate cancer, confirmed by biopsy and histopathological evaluation.
- Availability of complete clinical data.
    - Patients must have a detailed and accessible medical history, including information about previous and current treatments (TPA, chemotherapy, radiotherapy, surgical interventions), as well as the response to them.
    - Data on PSA levels, staging imaging, and other clinical assessments are needed for correlation with morphological and molecular parameters.
- Informed consent of all participants, after they have been fully informed about the purpose, methods, risks, and benefits of the study.

***Table 1**. Working methods used in the study – research design*

N0.	Study	Cases	Methods
1.	**RETROSPECTIVE, INDIRECT STUDY (Cohort I)** **Clinical-morphological characterisation of prostate-cancer cases**	**1925 prostate-pathology cases (2019 – 2023)** ↓ **440 malignant prostate tumours** ↓ **96 cases analysed**	• **Retrieval and review of electronic medical records** (Hipocrat system) • **Extraction of clinical and demographic data:** age, medical history, PSA levels, disease stage, prior treatments, hormone assays, inflammatory markers, imaging, etc. • **Examination of SCAP registries** to identify prostate-cancer cases • **Statistical analyses** • **Comparison with national and international cancer registries**
2.	**PROSPECTIVE, DIRECT STUDY (Cohort II)** **Assessment of the tumour micro-environment and biological processes involved in prostatic carcinogenesis**	**80 cases (40 prostate cancer cases and 40 benign prostatic hyperplasia cases)**	• **Systematic collection and analysis of clinico-morphological data** (age, history, PSA, stage, prior therapies) for longitudinal evaluation • **Use of archived biological specimens** (paraffin-embedded tissues) • **Immunohistochemical analysis of investigated cases** • **Genomic and proteomic molecular studies** • **Statistical analyses**
3.	**PROSPECTIVE, DIRECT STUDY (Cohort III)** **Genetic-interaction and signalling-pathway analysis in advanced prostate cancer**	**43 advanced prostate cancer cases**	• **Collection and analysis of clinico-morphological data** (age, history, PSA, stage, Gleason score, etc.) • **Use of archived biological specimens** (paraffin-embedded tissues) • **Immunohistochemical analysis** • **Molecular cytogenetic studies by FISH** • **Statistical analyses**

Groups II/III were formed through a direct, prospective study of cases treated within the Urology Department of the "Sf. Apostol Andrei" County Emergency Hospital in Constanta.

Inclusion criteria:

- Patients have a confirmed diagnosis of advanced prostate cancer, documented by biopsy and histopathological evaluation.
    - Disease progression can be defined either by metastases (e.g., bone metastases or to other organs) or by local progression of the tumor that can no longer be effectively treated by surgery or localized radiotherapy.
- Participants are in advanced stages of the disease, such as stage III or IV (according to the TNM system), or have castration-resistant prostate cancer (CRPC).
    - Patients with signs of disease progression under androgen deprivation therapy (ADT) or chemotherapy would be prioritized for inclusion.
- Access to appropriate biological samples of tumor tissue for morphological and molecular analyses.
    - These may include recent biopsies or tumor tissue embedded in paraffin blocks stored under appropriate conditions for further studies.
    - Samples should be of sufficient quality to allow detailed histological evaluation and extraction of genetic and protein material for further analysis.
- Exclusion of other types of active malignancies to avoid interference with the study results.
    - This exclusion helps to focus exclusively on mechanisms specific to advanced prostate cancer.
- Signed informed consent of all participants, after they have been fully informed about the purpose, methods, risks and benefits of the study.

The histopathological diagnosis was performed by the pathologists from the SCAP of the SCJU Constanta. All images were digitized using the TISSUEscope 4000XT automatic scanner produced by Huron Technologies from the Virtual Microscopy Laboratory of the CEDMOG Center. Genomic and proteomic determinations were performed in the laboratories of the CEDMOG Center of the "Ovidius" University of Constanta.

2.2. Results and discussions

2.2.1. Study I: Clinical-morpho-histochemical characterization of prostate cancer cases of patients diagnosed at SCAP of SCJU "Sf. Apostol Andrei" of Constanta

Through this study, *I aimed to identify predictive factors that improve the diagnosis of prostate cancer and that may have a predictive role for the evolution towards aggressive forms, respectively for an appropriate therapeutic attitude, associated with the prognosis of each case. Also, another objective was to analyze the predictive role of the neutrophil/lymphocyte ratio (NLR) in the oncological prognosis of patients with localized and locally advanced prostate cancer, who underwent radical prostatectomy (RP).*

Based on information obtained from the registers within the SCAP of the SCJU "Sf. Apostol Andrei" in Constanța and the hospital's IT system, we identified a number of 96 cases of prostate cancer investigated and diagnosed during the period 2019 - 2023, which met the inclusion criteria in the study: confirmed diagnosis of prostate cancer (localized + advanced, in which radical prostatectomy was performed), documented by biopsy and histopathological evaluation; available clinical and laboratory data, suggestive PSA values (PSA above 10 ng/mL), staging imaging and informed consent signed by the patient. All clinical data necessary for correlation with morphological and molecular parameters were taken from the patients' electronic files. During this period, 440 malignant tumors were diagnosed, most of which were diagnosed in 2019 (115 cases), and the fewest in 2021 (64 cases). 266 benign pseudoneoplastic lesions of the prostate were diagnosed between 2019 and 2023. Of these, most were in 2020 (65 cases), and the fewest in 2023 (41 cases).

The results obtained confirm that inflammation plays a role in promoting metastasis in prostate cancer. This suggests that chronic inflammation may contribute to tumor dissemination by creating a microenvironment favorable to cell migration and invasion. However, inflammation does not appear to influence intrinsic tumor characteristics, such as Gleason score or tumor stage, highlighting the importance of the tumor microenvironment in disease progression. For patients with an elevated NLR and proven nodal metastases, overextended lymphadenectomy may have the potential to improve oncological outcomes. The simultaneous analysis of a panel of biomarkers to predict the pathological stage represents a strength of the present study, but, nevertheless, when analyzing the research results, we must take into account the characteristics of the group, the small number of cases included in the

evaluation, and the possibility of combining preoperative variables or including other risk factors that can make a highly accurate prediction regarding the final pathological stage.

2.2.2. Study II: Characterization of the tumor microenvironment and biological processes involved in prostatic tumorigenesis

Intratumoral heterogeneity of the prostate, resulting from epithelial-mesenchymal plasticity, contributes to the limited tumor response to conventional treatments, highlighting the importance of using biomarkers to detect and analyze this variability in an efficient and rapid manner. Identification and monitoring of these biomarkers are essential for improving patient prognosis and survival. Flow cytometric analysis of ploidy and cell cycle, together with the assessment of adhesion glycoproteins essential for cell proliferation and apoptosis, provides a rapid and efficient method for measuring characteristics of the tumor microenvironment (TME). Flow cytometry measures DNA content to determine the frequency of cells in different stages of the cell cycle (G0/G1, S, and G2/M, respectively) and assesses DNA ploidy. Evidence of aneuploidy serves as a marker of tumor presence, providing important clues regarding tumor progression and treatment efficacy. Integrating these techniques into clinical practice may facilitate a better understanding of tumor dynamics and contribute to the development of therapeutic strategies more tailored to the individual needs of patients.

Therefore, in the present study, *we aimed to characterize the tumor microenvironment and biological processes involved in prostatic tumorigenesis and to establish correlations between the studied parameters to highlight their role in prostatic carcinogenesis.*

In this study - **Study Group II** - 80 cases of prostate tumors from patients diagnosed with untreated prostate cancer (PCa) (n = 40) and patients with untreated benign prostatic hyperplasia (BPH) (n = 40) were included. Adjacent non-malignant tissue samples, recovered from patients with prostate cancer (PCa) or BPH, were used as controls. CaP, HBP and control tissues were used for the evaluation of T-cell infiltration, CD34, and the Ki-67 nuclear proliferation index by immunohistochemistry (IHC) methods within SCAP, respectively for flow cytometry analyses, including the evaluation of DNA content and cell cycle, cell apoptosis, autophagy and pyknosis, ROS and the expression of the biomarkers CD34, CD61 and CD42b, at the Cell Biology Platform, CEDMOG, "Ovidius" University of Constanta.

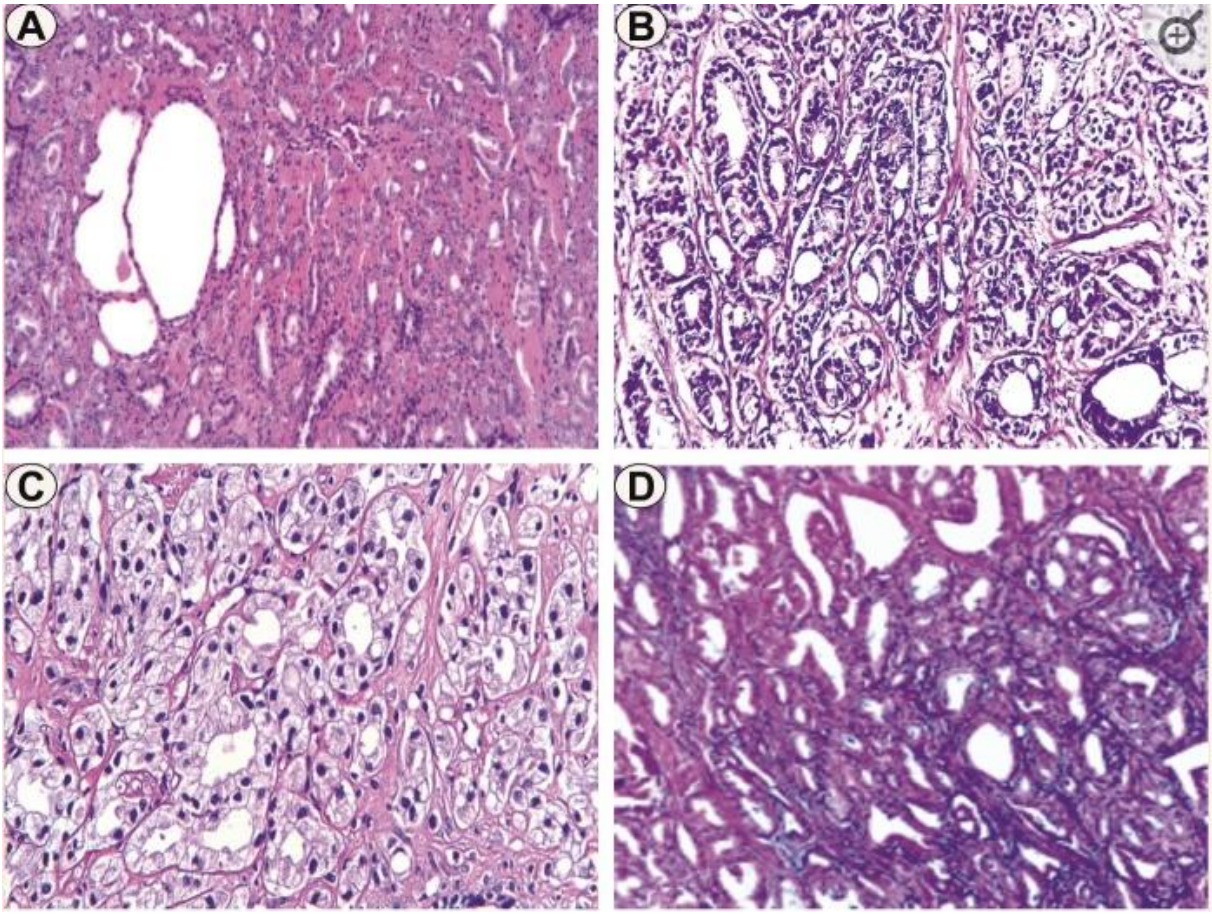

Fig.1 (A-D) - *Prostate adenocarcinoma. A. Conventional and atrophic patterns, GS 6 (3+3)/ISUP 1, HE stain, ×200; B. Conventional pattern, GS 7 (3+4)/ISUP 2, HE stain, ×400; C. Foamy gland patterns, GS 7 (3+4)/ISUP 2, HE stain, ×400; D. Conventional patterns, GS 7 (4+3)/ISUP 3, HE stain, ×200;*

The study confirmed the usefulness of flow cytometry for analysing the cell-cycle profile and DNA ploidy in prostate cancer (PCa) and benign prostatic hyperplasia (BPH). A substantial proportion of both PCa and BPH cases proved to be aneuploid, displaying a broad ploidy spectrum that included hypodiploidy, hyperdiploidy and tetraploidy. Tumours with a high S-phase fraction (≥ 12.0 %) were associated with an increased risk of recurrence and death, whereas tumours with a low S-phase fraction (< 7.0 %) carried a lower risk. These measurements may therefore aid in patient stratification and in guiding therapeutic decisions. A high prevalence of S-phase cells suggests uncontrolled proliferation, contributing to the emergence of aggressive disease; accordingly, an elevated S-phase fraction could act as an additional unfavourable biomarker, indicating a strong potential for tumour growth and dissemination.

In BPH, the apoptotic rate is relatively balanced but still lower than the proliferation rate, contributing to benign prostatic enlargement. In prostate adenocarcinoma, apoptosis is markedly suppressed, allowing cancer cells to survive and expand unchecked.

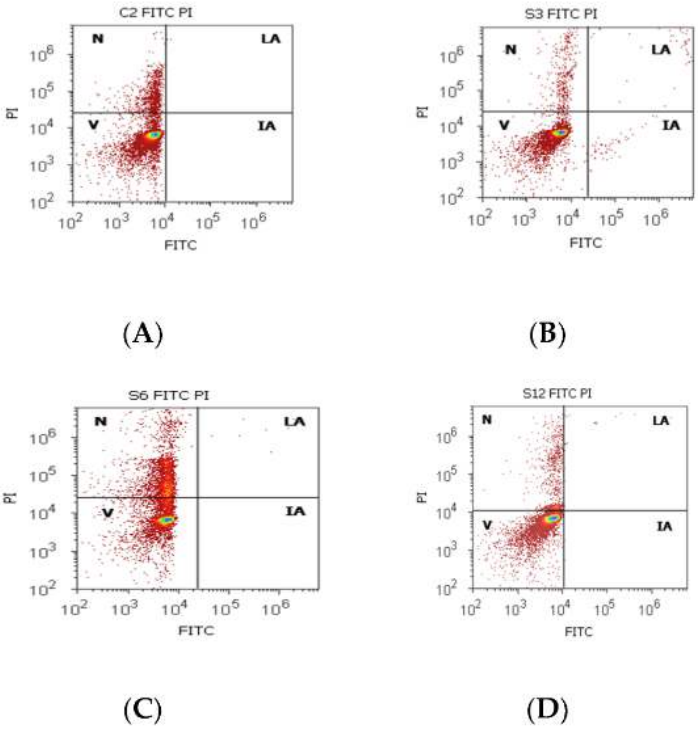

Fig.2 (A-D) - *Cell apoptosis assessed by dual Annexin V-FITC/propidium iodide (PI) staining. Viability: A – 83.50 % B – 94.72 % C – 68.79 % D – 87.90 %; Early apoptosis (EA): A – 0.00 % B – 0.54 % C – 0.005 % D – 0.025 %; Late apoptosis (LA): A – 0.00 % B – 0.49 % C – 0.07 % D – 0.22 %; Necrosis (N): A – 17.12 % B – 4.23 % C – 31.13 % D – 11.85 %. Legend: A – non-malignant prostatic tissue adjacent to nodular hyperplasia; B – prostatic adenoleiomyoma with chronic inflammatory hyperplasia; C – malignant prostatic tumour tissue; D – benign prostatic hyperplasia (BPH)*

As a prognostic biomarker, late apoptosis suggests a more unfavorable clinical outcome, which may help stratify patients according to the risk of disease progression.

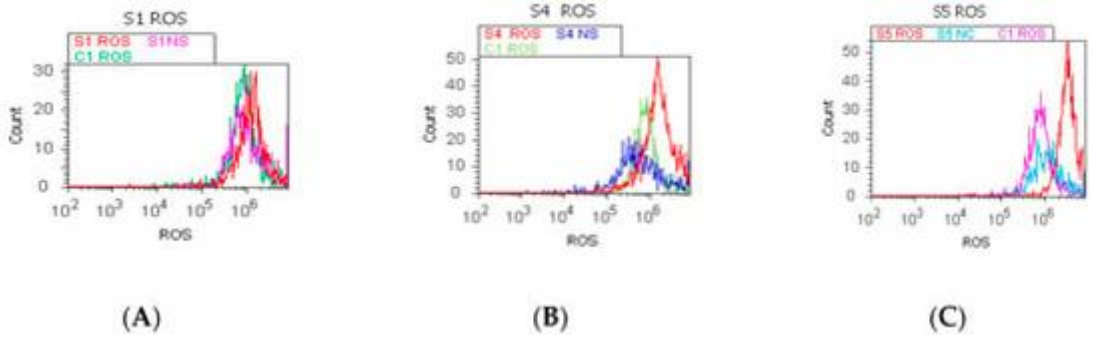

Fig. 3 - *Changes in oxidative stress in HBP (S5) and ADK (S4) samples versus control (S1). ROS: D-30 × 106; E-51 × 106; F-28 × 107; C1-35 × 106.*

The study highlighted the importance of assessing proliferation, apoptosis, and cell adhesion parameters in prostate cancer. It also highlighted the need for therapeutic strategies that include anti-angiogenic drugs and immunotherapy to improve prognosis and better respond to existing treatments.

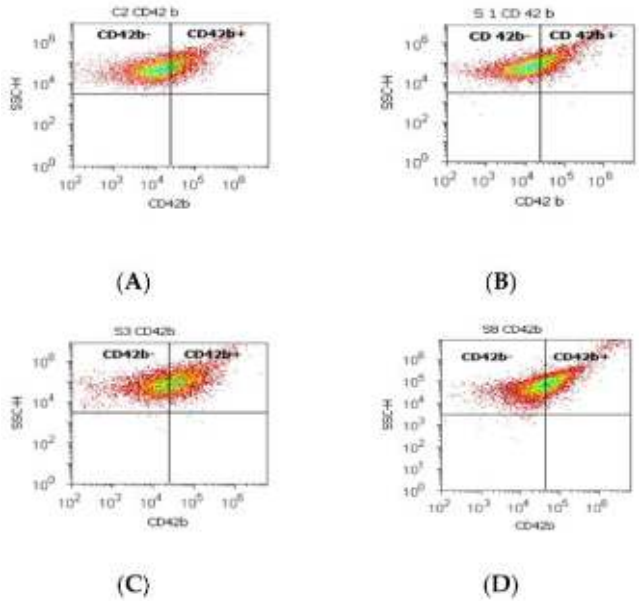

Fig.4 (A-D) - *Integral mediation of platelet aggregation to tumor and endothelial cells by highlighting positive and negative glycoprotein populations (CD42b-/CD42b+) with CD 42b-PE staining. CD42b+ population: (A) 25.80%; (B) 16.79%; (C) 41.10%; (D) 39.74%; CD42b- population: (A) 73.08%; (B) 82.88%; (C) 57.58%; (D) 57.23%. Legend: (A) non-malignant prostate tissue adjacent to nodular hyperplastic tissue; (B) adenoleiomyoma prostate tissue, chronic inflammatory hyperplasia; (C) malignant prostate tumor tissue; (D) benign prostatic hyperplasia.*

The pro-apoptotic signal revealed by the biochemical cascade shows a significant increase in early apoptosis (EA) in both BPH and PCa specimens compared with the control (BPH: 24.75 ± 16.50; PCa: 10.45 ± 1.12; vs. C1: 0.02 ± 0.01; $p < 0.05$; Figure 5 J–L).

- Early apoptosis (EA) refers to the initial stages of programmed cell death, in which the cell membrane begins to lose integrity while the nucleus remains intact.
- The data indicate a marked rise in EA in BPH samples (24.75 ± 16.50) and in prostate adenocarcinoma (PCa) samples (10.45 ± 1.12) relative to control tissue (0.02 ± 0.01). This finding underscores that cells from the pathological specimens are under heightened stress, predisposing them to programmed cell death and highlighting that pro-apoptotic processes are more pronounced under pathological conditions.
- The pro-apoptotic signal observed in both disease groups suggests that caspase activation and early apoptosis are common mechanisms in BPH and PCa, albeit with different magnitudes depending on the specific pathology.
- Caspase-3/7 activation indicates that, in both prostate disorders, a larger fraction of cells enter early apoptosis than in normal tissue—potentially a host strategy to eliminate dysfunctional or pre-malignant cells.

Taken together, these results emphasise the pivotal role of late-stage apoptosis in the chronic oxidative-stress response characteristic of both BPH and prostate adenocarcinoma.

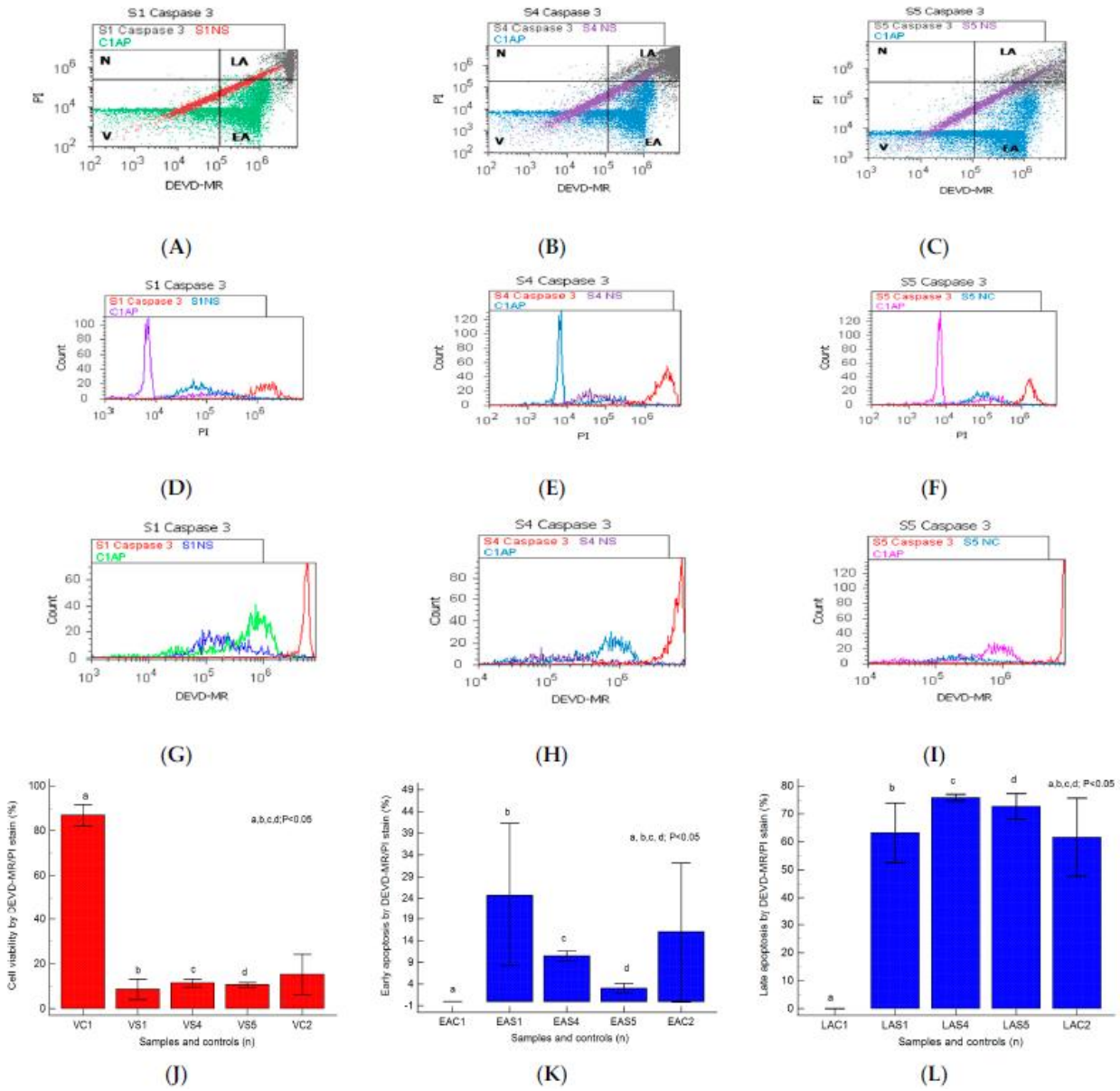

Fig.5 *- Caspase-3/7 activity, visualised by DEVD-MR/PI dual staining, is displayed graphically to illustrate cell viability (V), early and late apoptosis (EA, LA) and necrosis (N) in the BPH samples (B, E, H) and prostate adenocarcinoma (ADK) samples (C, F, I) compared with controls (A, D, G).(J–L) show the statistical analysis of caspase-3/7 activity: controls (J) versus BPH cases (K) and ADK cases (L). A *p*-value < 0.05 denotes a statistically significant difference between specimens and controls (Mann–Whitney test, MedCalc software).*
***Legend:** BPH – benign prostatic hyperplasia; ADK – prostate adenocarcinoma.*

The markedly higher levels of late apoptosis under these conditions, compared with normal tissue, point to a cellular adaptation to chronic stress and highlight the need to monitor this process during diagnosis and treatment. Such information can deepen our understanding of the pathophysiology of prostate disorders and support the development of more effective therapeutic strategies.

2.2.3. Study III: Analysis of genetic interactions and signaling pathways involved in advanced prostate cancer

The aim of the study is to determine and characterize gene mutations and fusion transcripts in advanced prostate cancer in order to discover biomolecular markers useful for early diagnosis, risk stratification and prognosis of patients, as well as to guide personalized therapeutic strategies. In this regard, the study aimed to analyze the frequency of common gene mutations (PTEN, AR-V7, TP53, ERBB2) in advanced prostate cancer by the FISH (Fluorescence In Situ Hybridization) technique, respectively, to investigate the incidence of TMPRSS2-ERG fusion in the evaluated advanced prostate cancer cases and the correlation with the aggressive phenotype and disease evolution. To carry out this study, we used formalin-fixed and paraffin-embedded tumor tissue samples from 43 patients with advanced or metastatic prostate cancer.

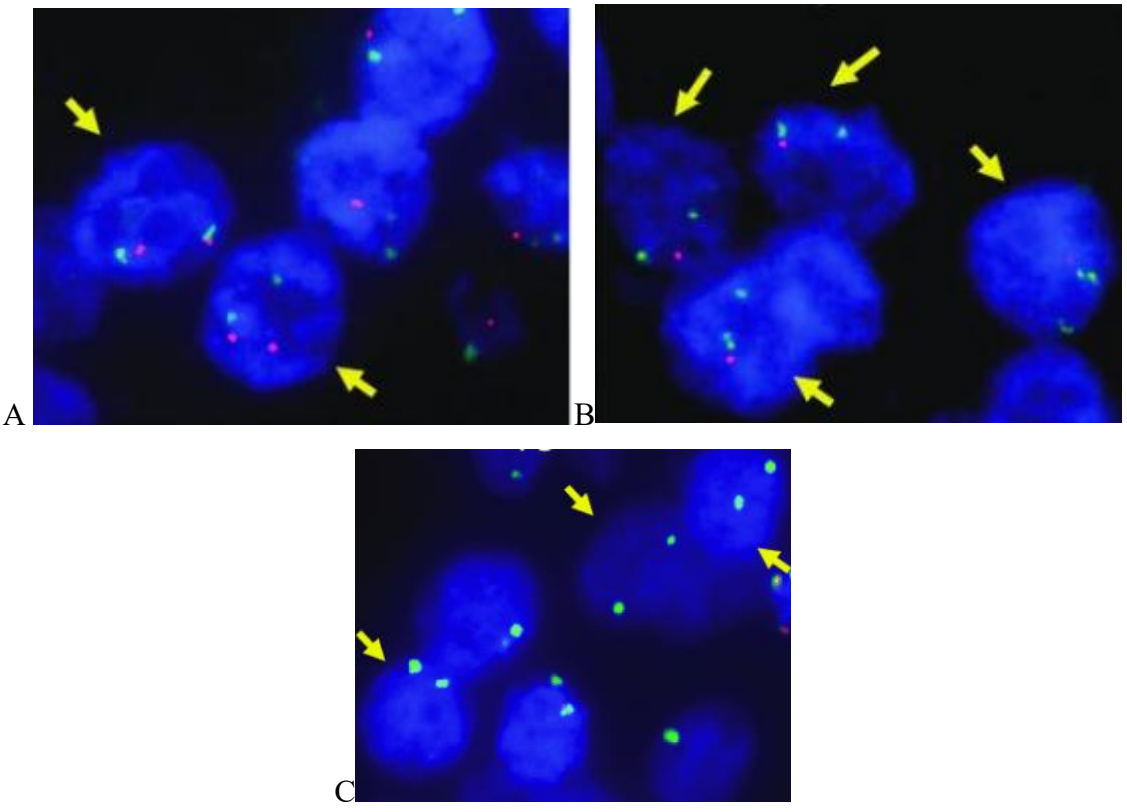

Fig. 6 - *FISH analysis of PTEN status in advanced prostate cancer.(A) The FISH image displays two red and two green signals per nucleus, indicating disomy.(B) The FISH image shows one red signal (locus 10q23.3) and two green centromere-10 signals per nucleus, consistent with a hemizygous PTEN deletion.(C) The FISH image shows no red signals (locus 10q23.3) and two green centromere-10 signals per nucleus, consistent with a homozygous PTEN deletion.*

The present study, which evaluated and identified the frequency of genetic alterations in the PTEN, AR-V7, TMPRSS2-ERG, TP53 and ERBB2 genes, revealed not only the distribution of these genetic alterations, but also important associations between molecular subtypes and disease aggressiveness. The results obtained highlight a significant molecular heterogeneity in prostate cancer, with PTEN deletion and TP53 mutations as the most frequent abnormalities. Combinations of markers such as PTEN-, TP53+ and AR-V7+ suggest aggressive tumors and potentially resistant to conventional treatments.

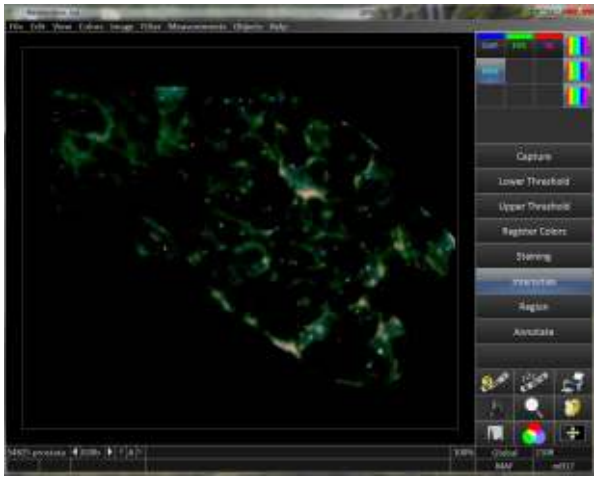

Fig. 7 – *Prostate adenocarcinoma. FISH image indicative of a TMPRSS2-ERG–positive status (gene fusion via interstitial deletion) — TMPRSS2 21q22.2-q22.3 (red), TMPRSS2 21q22.2-q22.3 (green), ERG 21q22.13-q22.2 (blue).*

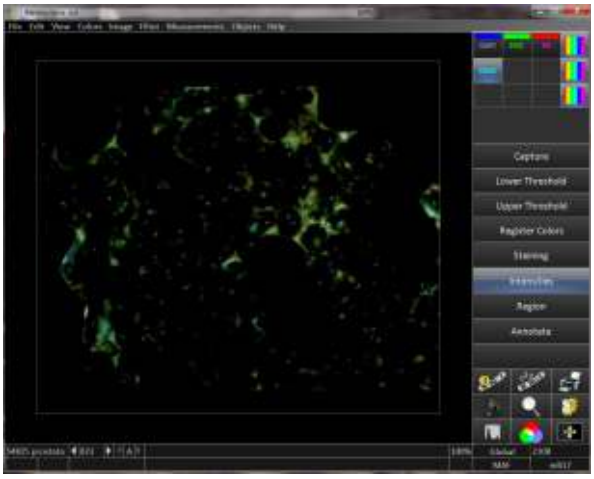

Fig. 8 - *Prostate adenocarcinoma. FISH image indicative of TMPRSS2-ERG positivity (gene fusion via insertion) — TMPRSS2 21q22.2-q22.3 (red), TMPRSS2 21q22.2-q22.3 (green), ERG 21q22.13-q22.2 (blue).*

The **AR-V7**+ biomarker is linked to a more aggressive tumour phenotype and a higher likelihood of seminal-vesicle invasion. Its presence signals resistance to androgen-deprivation therapy, emphasising the need for alternative treatment strategies in AR-V7+ patients. **TP53+** correlates with a pathological Gleason score > 7, indicating association with highly aggressive tumours and genomic instability. Consequently, TP53+ may predict poorer prognosis and should be factored into therapeutic decision-making.

These results highlight the importance of a case-by-case approach in the optimal management of prostate cancer. Most cases showed heterogeneous tumor foci with respect to PTEN gene alterations. This suggests that cells with different stages of gene inactivation can coexist within the same tumor, which may influence both disease progression and response to treatment. Intratumoral heterogeneity is an important factor contributing to the difficulty of cancer treatment, as different cells within the same tumor may have different responses to therapies.

3. General conclusions and future research directions

The doctoral thesis has made a significant contribution to the understanding of prostate cancer through three major research directions, each with relevant implications for diagnosis, prognosis and treatment. The synthesis of these results provides a comprehensive picture of the pathogenetic mechanisms, prognostic biomarkers and therapeutic strategies in prostate cancer.

The study of the biological mechanisms involved in the tumor microenvironment and the molecular pathways associated with prostate carcinogenesis provides essential information for accurate diagnosis and for the development of effective therapeutic strategies. Molecular biomarkers, such as AR-V7, ERBB2 and TP53, are key elements that can guide the selection of patients for targeted therapies. For example, patients with AR-V7+ expression may benefit from androgen inhibitors, and those with ERBB2+ from anti-HER2 treatments, thus optimizing treatments for better outcomes.

Although immunotherapy is not currently a first-line option in prostate cancer, the molecular markers identified could provide clues to which patients might benefit from novel immunotherapies or therapeutic combinations. For example, TP53 alterations and PTEN deletion could play an important role in selecting patients for immunotherapies targeting DNA repair mechanisms or immune pathways. In the long term, future research should further explore the interactions between biomarkers and cancer progression, as well as the potential for anti-inflammatory and personalized therapeutic interventions. These findings provide a solid basis for the development of more effective and targeted therapies with the aim of improving survival and quality of life for prostate cancer patients.

The research conducted in this thesis has clearly demonstrated the importance of a multidisciplinary approach in the diagnosis and treatment of prostate cancer. The integration of inflammatory, cytometric and genetic parameters offers a more complete and personalized approach, which can improve clinical outcomes and patient prognosis. The results emphasize that the treatment of prostate cancer can only be effective through a rigorous personalization of therapeutic strategies, depending on the characteristics of each tumor and the individual patient. This approach could significantly reduce relapses and contribute to an improvement in the quality of life of patients.

4. Originality of the doctoral thesis

- **First combined application of flow-cytometry, immunomarking, cytogenetic and molecular-biology techniques in a single cohort of prostate-cancer patients**: for the first time nationally, our study integrated cell-cycle analysis, DNA ploidy, adhesion glycoproteins (CD61 and CD42b), micro-vessel density (CD34) and T-cell infiltration in one multidimensional dataset. This comprehensive approach provides novel insights into the biology and management of prostate cancer.
- **First study to correlate the S-phase fraction with clinical risk:** we showed that the proportion of cells in S-phase is a powerful predictor of recurrence and mortality, offering more granular prognostic information and patient stratification than previous, broader assessments.
- **First national and among the earliest international reports to highlight the role of adhesion glycoproteins CD61/CD42b in prostatic tumour progression**, thereby proposing new anti-metastatic targets.
- **New evidence on multiparametric microvessel-density analysis:** unlike earlier work that merely quantified neovascularisation, our study correlated microvessel density with cellular proliferation and adhesion, clarifying its impact on disease course.
- **Additional data on T-cell infiltration and immune response:** we identified a distinctive T-cell infiltration pattern in prostate cancer versus BPH, with direct implications for selecting patients who may benefit from immune-checkpoint therapy.
- **Strengthened prognostic evidence for key biomarkers and personalised therapeutic recommendations**: in a well-characterised cohort we demonstrate that the molecular profile (PTEN, AR-V7, TP53, S-phase fraction, CD34) can guide systemic-therapy selection, translating concepts into actionable clinical algorithms.

The thesis therefore underscores the importance of inflammatory and genetic biomarkers in prostate-cancer progression. Its originality lies in the convergence of exhaustive biomolecular investigations with concrete clinical applications, advancing personalised management of advanced prostate cancer.

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