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ACTIVITY OF ENZYMES OF THE PURINERGIC SYSTEM AND ITS ASSOCIATION WITH PSA LEVELS, GLEASON SCORE AND TUMOR CLASSIFICATION IN PATIENTS WITH PROSTATE CANCER

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Dissertation presented to the Graduate Program in Biomedical Sciences, Federal University of Fronteira Sul, *Campus* Chapecó, as a final requirement for obtaining the Master's degree in Biomedical Sciences.

Supervisor: Prof. Dr. Daniela Zanini Co-supervisor: Prof. Dr. Sarah FVO Maciel

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Dissertation presented to the Graduate Program in Biomedical Sciences, at the Federal University of Fronteira Sul-UFFS, to obtain the title of Master in Biomedical Sciences, defended by the Examining Board on 08/10/2023

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SUMMARY

Changes in the morbidity and mortality profile of prostate cancer (PC) in recent decades have made tornadothis pathology a public health problem in several countries, including Brazil. Inflammatory processes and purinergic signaling can directly influence prostate carcinogenesis, as well as tumor progression. The enzymatic action of ectonucleotidases and tissue response to adenine nucleotides and nucleosides play a fundamentally important role ,and should be increasingly explored. Considering this interrelationship with PC, the objective of the present study was to evaluate the activity of E-NTPDase and Adenosine deaminase (ADA) in patients with PC and its association with serum levels of PSA (Prostate Specific Antigen), the of Gleason, tumor staging and ISUP scale (International Society of Urological Pathology) of these patients. This is a cross-sectional observational quantitative study associated with a qualitative approach . For the statistical analyses, firstly, the data were tested for normality using aplicando-sethe Shapiro-Wilk test. Afterwards, Student's t test and One-way ANOVA were used. The significance level used was 0.05 and the statistical program used was GraphPad Prism 8.0.1. Lymphocyte samples were collected by venipuncture (30 mL) from 39 patients diagnosed with PC, prior to surgical treatment of the tumor, and from 37 control individuals, of similar age range. After the invitation and acceptance to participate in the study, the participants signed the Term of Free and Informed Consent to participate in it. From the analysis of the results, it was possible to observe that in relation to age, the mean was 66 ± 6.72 years in patients, and 65.81 ± 10.30 in controls (p=0.8503). Of the group of patients, 21 (54%) had serum levels of PSA ≥4 to ≤10 ng/mL before the therapeutic intervention. Regarding the Gleason score, 27 (69.2%) pacientes had a score of 7. Regarding the clinical staging, 17 (43.6%) pacientes had a T2 classification ,and 14 (35.9%) had a T3 classification. Regarding the ISUP score, 19 (48.8%) pacienteshad ISUP classification 2. E-NTPDase activity, for the hydrolysis of ATP in lymphocytes, is significantly increased in the group of patients with CP when compared to the control group (p=0.0001). The activity of E-NTPDase, for the hydrolysis of ADP in lymphocytes, is significantly reduced in the group of patients with CP when compared to the control group (p=0.0001). ADA activity in lymphocytes is reduced in patients with CP when compared to the control group (p=0.0003). Regarding serum PSA levels, ATP hydrolysis was statistically higher in patients with CP compared to the control group, in both analyses, serum PSA levels < 10 (p=0.0037) and PSA ≥ 10 (p=0.0001) . ADP hydrolysis was statistically lower in patients with CP compared to the control group when PSA ≥ 10 e PSA< 10, ambos vp-value=0.0001 . ADA activity significantly decreased with increasing serum PSA levels compared to the control group, both PSA < 10 (p=0.0104) and PSA ≥ 10 (p=0.0086) . Regarding the Gleason score, ATP hydrolysis was higher in the patient group when compared to the control group in both analyses, Gleason <7 (p=0.5664), Gleason 7 (p=0.0001) and Gleason > 7 (0.0056). ADP hydrolysis was lower in the patient group when compared to the control group in both analyses, Gleason <7 (p=0.0034), Gleason 7 (p=0.0001) and Gleason > 7 (0.0012). ADA activity was lower in the patient group when compared to the control group in both analyses, Gleason <7 (p=0.3647), Gleason 7 (p=0.0171) and Gleason > 7 (0.3955). Regarding clinical staging, ATP hydrolysis was increased in the patient group when compared to the control group in both analyses, T1 (p=0.1601), T2 (p=0.0004), T3 (p=0.0003). ADP hydrolysis was reduced in the patient group when compared to the control group in both analyses, T1 (p=03557), T2 and T3 (p=0.0001). ADA activity was reduced in the patient group when compared to the control group in both analyses, T1 (p=0.1877), T2 (p=0.0303), T3 (p=0.0397). Regarding the ISUP scale, ATP hydrolysis was increased in the patient group when compared to the control group in all analyses, ISUP 1 (p=0.2200), ISUP 2 (p=0.0024), ISUP 3 (p=0.0192), ISUP 4 and 5 (p=0.0108). ADP hydrolysis was decreased in the patient group when compared to the control group in all analyses, ISUP 1 (p=0.0005), ISUP 2 and ISUP 3 both

(p=0.0001), ISUP 4 and 5 (p =0.0007). ADA activity was reduced in the patient group when compared to the control group in all analyses, ISUP 1 (p=0.0832), ISUP 2 (p=0.0068), ISUP 3 (p=0.2625), ISUP 4 and 5 (p=0.2530). For the first time, it was possible to relate E-NTPDase and ADA activity to serum PSA levels, Gleason score, tumor staging, and ISUP values in patients with CP. In time, additional experiments on the expression of receptors and analyzes in tumor tissue are necessary to corroborate the results found in the present study, so that the modulation of purinergic signaling can form the basis of new drugs and efficient therapeutic protocols in controlling the development and progression of CP.

Keywords : Prostate Cancer; Ectonucleotidases; PSA; Tumor Progression.

ABSTRACT

Changes in the morbidity and mortality profile of prostate cancer (PC) in recent decades have made this pathology a public health problem in several countries, including Brazil. Inflammatory processes and purinergic signaling can directly influence prostate carcinogenesis, as well as tumor progression. The enzymatic action of ectonucleotidases and the response of tissues to nucleotides and adenine nucleoside play a fundamentally important role, and should be increasingly explored. Considering this interrelation with PC, the objective of the present study was to evaluate the activity of E-NTPDase and Adenosine deaminase (ADA) in patients with PC and its association with serum levels of PSA (Prostate Specific Antigen), the score of Gleason, tumor staging and ISUP scale (International Society of Urological Pathology) of these patients. This is a cross-sectional observational quantitative study associated with a qualitative approach. For the statistical analyses, firstly, the data were tested for normality by applying the Shapiro-Wilk test. Afterwards, Student's t test and One-way ANOVA were used. The significance level used was 0.05 and the statistical program used was GraphPad Prism 8.0.1. Lymphocyte samples were collected by venipuncture (30 mL) from 39 patients diagnosed with PC, prior to surgical treatment of the tumor, and from 37 control individuals, of similar age range. After the invitation and acceptance to participate in the study, the participants signed the Term of Free and Informed Consent to participate in it. From the analysis of the results, it was possible to observe that in relation to age, the mean was 66 ± 6.72 years in patients, and 65.81 ± 10.30 in controls (p=0.8503). Of the group of patients, 21 (54%) had serum PSA levels ≥4 to ≤10 ng/mL before the therapeutic intervention. Regarding the Gleason score, 27 (69.2%) patients had a score of 7. Regarding clinical staging, 17 (43.6%) patients had a T2 classification, and 14 (35.9%) had a T3 classification. Regarding the ISUP score, 19 (48.8%) patients had ISUP classification 2. E-NTPDase activity, for the hydrolysis of ATP in lymphocytes, is significantly increased in the group of patients with CP when compared to the control group (p=0.0001). The activity of E-NTPDase, for the hydrolysis of ADP in lymphocytes, is significantly reduced in the group of patients with CP when compared to the control group (p=0.0001). ADA activity in lymphocytes is reduced in patients with CP when compared to the control group (p=0.0003). Regarding serum PSA levels, ATP hydrolysis was statistically higher in patients with CP compared to the control group, in both analyses, serum PSA levels < 10 (p=0.0037) and PSA ≥ 10 (p= 0.0001). ADP hydrolysis was statistically lower in patients with CP compared to the control group when PSA ≥ 10 and PSA < 10, both p-value=0.0001. ADA activity significantly decreased with increasing serum PSA levels compared to the control group, both PSA < 10 (p=0.0104) and PSA ≥ 10 (p=0.0086). Regarding the Gleason score, ATP hydrolysis was higher in the patient group when compared to the control group in both analyses, Gleason <7 (p=0.5664), Gleason 7 (p=0.0001) and Gleason > 7 (0.0056). ADP hydrolysis was lower in the patient group when compared to the control group in both analyses, Gleason <7 (p=0.0034), Gleason 7 (p=0.0001) and Gleason > 7 (0.0012). ADA activity was lower in the patient group when compared to the control group in both analyses, Gleason <7 (p=0.3647), Gleason 7 (p=0.0171) and Gleason > 7 (0.3955). Regarding clinical staging, ATP hydrolysis was increased in the patient group when compared to the control group in both analyses, T1 (p=0.1601), T2 (p=0.0004), T3 (p=0.0003). ADP hydrolysis was reduced in the patient group when compared to the control group in both analyses, T1 (p=03557), T2 and T3 (p=0.0001). ADA activity was reduced in the patient group when compared to the control group in both analyses, T1 (p=0.1877), T2 (p=0.0303), T3 (p=0.0397). Regarding the ISUP scale, ATP hydrolysis was increased in the patient group when compared to the control group in all analyses, ISUP 1 (p=0.2200), ISUP 2 (p=0.0024), ISUP 3 (p=0.0192), ISUP 4 and 5 (p=0.0108). ADP hydrolysis was decreased in the patient group when compared to the control group in all analyses, ISUP 1 (p=0.0005), ISUP 2 and ISUP 3 both (p=0.0001), ISUP 4 and 5

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Keywords: prostate cancer; Ectonucleotidases; PSA; Tumor Progression.

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LIST OF ACRONYMS AND ABBREVIATIONS

ADA - Adenosine deaminase

Ado – Adenosine

ADP - Adenosine Diphosphate

ADT - Androgen Deprivation Therapy

AMP - Adenosine Monophosphate

ATP - Adenosine Triphosphate

CEP - Research Ethics Committee

Cl^- - Chloride anion

COVID-19 - Coronavirus disease 2019

PC - prostate cancer

CSF - Family Health Center

DAMPs - Molecular patterns associated with damage

e5NT/CD73 - Ecto-5'-nucleotidase

E-NTPDase - Ecto-Nucleoside Triphosphate Diphosphohydrolase

ESF - Family Health Team

FA - alkaline phosphatase

HClO - Hypochlorous acid

HRO - West Regional Hospital

ISUP - International Society of Urological Pathology

O_2^- - Superoxide anion

O_2 - Oxygen

WHO - World Health Organization

PCA3 - Prostate antigen 3

PSA - Prostate Specific Antigen

PSMA - Prostate membrane specific antigen

SBU - Brazilian Society of Urology

TCLE - Free and informed consent form

TNM - Classification of malignant tumors

UFFS - Federal University of Fronteira Sul

SUMMARY

1. INTRODUCTION

Prostate cancer (PC) is recognized as one of the most important health problems faced by the male population, being one of the most prevalent cancers worldwide and the cause of death of thousands of men every year (IARC, 2023) . Although radical prostatectomy (the most common treatment for o PC) achieves good overall results, its performance develops some unpleasant side effects , such as urinary incontinence and erectile dysfunction, contributing to the morbidity associated with this neoplasm (COSTELLO, 2020).

Tumor classification and the degree of cell differentiation are defined by performing a prostate biopsy, which allows the pathologist to distinguish tumors. Currently, the Gleason system, which emphasizes the glandular architecture, is the most used to predict the evolution and predict the pathological stage of PC. Using this system, lesions with a Gleason score of 2 to 4 (well differentiated) have a milder behavior, while tumores com scores of 7 to 10 (undifferentiated) are more aggressive, responding poorly to different treatment options. The most common site of PC dissemination is the bone tissue, and the presence or absence of bone metastasis is fundamental data that directs the therapeutic protocol (SHARMA; MIYAMOTO, 2018).

CP generally affects glandular cells and, in this context, the literature points out that among the main mechanisms associated with the development of this type of tumor, inflammatory processes and the expression of androgen receptors should be highlighted - which potentiate carcinogenesis due to the intense production of radicals free (RL) and reactive oxygen species (ROS). Oxidative processes have the ability to alter the DNA, promoting significant cellular alterations - such as apoptotic events and mutations - which are presented as the original basis of oncological diseases (GANDAGLIA *et al* ., 2021).

Allied to these events, as well as in other cancers, age is an important risk factor for the occurrence of tumors, since the development of PC increases dramatically after the age of 50 (PERNAR et al . , *2018*) . The study of the mechanisms involved in the increase in the incidence of PC with age is of great interest, being important for the understanding of this disease. A possible explanation may be associated with the imbalance between oxidizing agents and antioxidant defense mechanisms in various tissues, including the prostate, which occurs concomitantly with aging and leads to an oxidative state and damage to the organism (SHUKLA et al., *2020*) .

Although some evidence has already been outlined regarding the pathophysiology of PC, it is essential that other mechanisms related to the pathology are better understood, so that an early diagnosis can be made and it is possible to expand the less aggressive treatment options for this disease (OCHOA- CORTES *et al* ., 2014).

It is known that in tumor cells, significant changes occur in the activity of signaling pathways - affecting a wide range of cellular functions, from growth and proliferation to apoptosis, invasion and metastasis (VAGHARI-TABARI et al., 2021 *)* . In this light, the action that extracellular nucleotides and nucleosides (ATP, ADP, AMP and its metabolite adenosine) produce in response to various pathological processes, such as apoptosis, control of cell proliferation and differentiation (BURNSTOCK; DI VIRGILIO , 2013). Along these lines, several studies have suggested the involvement of purinergic signaling in the pathophysiology of tumors, including PC (ARAÚJO et al., 2005; MALDONADO et al., 2008; ZANINI et al., 2012; DI VIRGILIO; ADINOLFI, 2017; PFAFFENZELLER *et al* ., 2020).

Lymphocytes play an important role in antitumor immune responses, are direct targets of some antineoplastic therapies, and the composition and spatial organization of intratumor T cell populations are prognostic in some types of cancer (COWELL., 2020). It is also known that the tumor microenvironment is rich in nucleosides and nucleotides, with extracellular ATP being an important pro-inflammatory molecule and adenosine a key component - highly immunosuppressive (DI VIRGILIO *et al* ., 2016) . Despite the understanding of many actions linked to these molecules, their effects on the systemic tumor-host interaction are not completely understood (DI VIRGILIO *et al* ., 2016).

The levels of circulating adenine and adenosine nucleotides, as well as their concentrations in the tumor microenvironment, are directly associated with the activity of enzymes capable of hydrolyzing these molecules. Among these enzymes we can mention: i) E-NTPDase (CD39) (Ecto-Nucleoside triphosphate diphosphohydrolase) which degrades ATP to ADP and ADP to AMP; ii) ecto-5'-nucleotidase (CD73) which degrades AMP to adenosine; and iii) adenosine deaminase (ADA) which degrades adenosine to inosine. Equally important, associated with the complex enzymatic system of purinergic signaling, are the purinergic receptors - which constitute the P1 and P2 families (P2X and P2Y) (DI VIRGILIO; ADINOLFI, 2017).

In this scenario, despite some studies involving ectoenzimas do sistema purinérgicohaving been developed in patients with CP, there is still a lack of studies to elucidate how the joint action of the purinergic signaling components takes place, paying attention to PSA levels, Gleason score and tumor staging in these patients. patients.

Thus, considering that: 1) PC is a worldwide public health problem related to high incidence, morbidity and mortality rates; 2) the components of purinergic signaling may play a fundamental role in the development and progression of CP; 3) alterations in the activity of enzymes in some types of cancer are already recognized do sistema purinérgico , but few scientific studies have been carried out seeking to relate the activity of ectonucleotidases and ADA with diagnostic and prognostic markers of

PC, it is of fundamental importance to deepen the knowledge about this of the joint behavior of these factors in individuals with CP, in order to promote improvements in the diagnosis and treatment of patients affected by this pathology, promoting a decrease in morbidity and mortality rates.

2 OBJECTIVES

2.1 GENERAL OBJECTIVE

Evaluate the activity of E-NTPDase and ADA enzymes, associating them with different clinicopathological parameters in patients with CP.

2.2 SPECIFIC OBJECTIVES

- Carry out the clinical-pathological characterization of the sample group;
- To analyze the activity of E-NTPDase in lymphocytes from patients diagnosed with PC and in control individuals;
- Evaluate ADA activity in lymphocytes from patients diagnosed with PC and in control individuals;
- Associate the activity of E-NTPDase and ADA enzymes with serum PSA levels, Gleason score, tumor staging and the ISUP scale.

3 THEORETICAL BACKGROUND

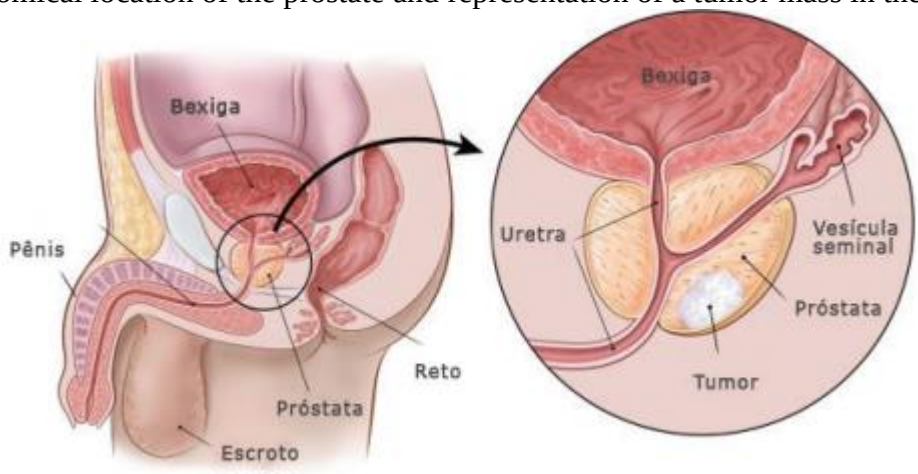
3.1 PROSTATE CANCER - BIOMARKERS AND TUMOR CLASSIFICATIONS

Worldwide, PC is considered the second cancer that most affects men. The World Health Organization (WHO) points out that in 2020 it corresponded to about 13.5% of the most recurrent cancers in the world (BRASIL, 2020).

The estimated number of new cases of PC in Brazil, for the three-year period from 2023 to 2025, is 71,730, corresponding to an estimated risk of 67.86 new cases per 100,000 men. Regionally, the data show that the most affected population will be the Southeast region; followed by the Northeast, Midwest, South and North, respectively (BRASIL, 2022).

Given the epidemiological data presented, it is important to emphasize the pathophysiology of PC. The prostate is a gland that is part of the male genitourinary system and is responsible for producing the fluid that protects and nourishes the sperm in the semen, and may have different sizes, according to the structure and physiology of each individual (ACS, 2019). It is located in front of the rectum and below the urinary bladder, normally of small volume and frequently subject to the risk of injuries and tumor involvement. In Figure 1, it is possible to verify the anatomical position of the prostate and the representation of a tumor mass in the gland.

Figure 1 - Anatomical location of the prostate and representation of a tumor mass in the gland.



Source: BRAZIL (2021) .

Tumors are abnormal cell growths that may be related to several factors. PC does not have an exact scientifically proven cause, however there are related factors that may favor the appearance of

such a tumor. The lesion develops more commonly in individuals over 50 years of age, with more than half of diagnosed cases occurring in the age group of 65 years or older (BRASIL, 2020).

In addition, hereditary and/or acquired genetic mutations are the preponderant factors for the development of PC. Family history, DNA mutations, activation of oncogenes and metabolic syndrome are related to a higher risk of developing o CP. As if that were not enough, diabetes and exposure to ultraviolet rays are associated with a higher incidence, while smoking and obesity are capable of increasing specific mortality, while regular physical activity can decrease the progression of the disease (GANDAGLIA *et al* ., 2021).

The diagnosis of PC usually occurs after screening, given that the disease has an asymptomatic course. When symptomatic, it may indicate a poor prognosis, since in 6% of cases the cancer is already metastatic at the time of diagnosis and one of the main symptoms is bone pain, since bone metastases are the most common in PC (COLLIN *et al* ., 2009).

As stated above, most of the time CP has a silent evolution, and may not show signs and symptoms in its initial phase. However, the most common clinical manifestations are: - difficulty urinating, - delay in starting or completing the urinary stream, - reduced urinary stream, - need to urinate several times during the day or night and, -presence of blood in the urine . These signs and symptoms are nonspecific and medical evaluation is essential for the purpose of differential diagnosis (BRASIL, 2021).

According to the Brazilian Society of Urology (SBU), screening for PC is indicated by measuring the Prostate Specific Antigen (PSA) and digital rectal examination for all men, from 50 years of age, and individuals at higher risk of disease development - black men or with first-degree relatives who have a history of PC - should start screening at age 45 (SBU, 2020).

PSA is among the most clinically useful tumor markers known so far. It is a glycoprotein, secreted in the lumen of the prostatic ducts through the epithelial cells of the prostate, whose function is to solubilize the sperm after ejaculation, as well as to liquefy the seminal clot. The cutoff point for the test is 4.0 ng/mL, with higher values requiring additional evaluation. The predictive value of the test is 20% in patients with slightly elevated plasma levels (between 4.0 ng/mL and 10.0 ng/mL) and reaches up to 60% when plasma levels exceed 10.0 ng/mL . Furthermore, the negative predictive value of the test reaches 85% for invasive tumors (COLEY *et al* ., 1997; THOMPSON *et al* ., 2004).

PSA has been recommended as a molecular target for antineoplastic therapy, since it plays a key role in PC signaling pathways, including proliferation, invasion, metastasis, angiogenesis, apoptosis, immune response, and regulation of the tumor microenvironment . *al* ., 2019).Currently, the PSA cannot be considered only as a guide for the presence or absence of CP. Its determination can also

help the urologist to decide on the most convenient treatment for a patient with benign prostatic hypertrophy, as a criterion for disease progression (COZAR et al., *2020*).

In addition, PSA is a key factor for the follow-up of patients with prostate adenocarcinoma at any stage who have received treatment (surgery, radiotherapy or focal therapies and/or hormone therapy). It also serves as a guide for the identification of biochemical recurrences and suspected local or distant recurrences, as well as being used to propose or rule out adjuvant treatments. The role of PSA as a screening tool has recently been reinforced by the observation of increased mortality rates and the existence of more aggressive cases of PC in those countries where the use of this tool has declined (COZAR et al., 2020 *)* .

Together, for the screening of PC, the digital rectal examination is performed, in which the examiner looks for abnormalities in the prostate that can be palpated, such as hardened nodules. The use of digital rectal examination doubles the chances of detecting a clinically important cancer (COLEY *et al* ., 1997; THOMPSON *et al* ., 2004). Unfortunately, many men are still very resistant to performing this test, so much so that Pereira et al. (2021) report that the rectal examination is linked to the transgression of masculinity, which causes fear of doing it.

Other tests have also been gaining notoriety for use as biomarkers that help in the screening of PC, in the sense of submitting patients with inconclusive tests to biopsy or not. Among them we can mention Prostate Antigen 3 (PCA3) , discovered in 1999 and which is overexpressed in most tumor samples of PC, but not in healthy tissues or only hypertrophied ones. It is usually collected through a urine sample obtained after performing the digital rectal examination. Its indication is for men with PSA tests in an undetermined range (2.5 to 10.0 ng/mL) and men with negative previous biopsies, and who continue to have elevated PSA. In addition, another recent marker is Prostatic Membrane Specific Antigen (PSMA), whose indication is based on helping to determine the risk stratification of PC and, consequently, the bases of treatment, given that a lower expression was observed of it in samples with a Gleason 3 pattern, than in those with a Gleason 4 pattern (BRAVACCINI *et al* ., 2018).

Given the above , it should be noted that PSA monitoring and histopathological examination of tumor biopsies remain gold standards in the diagnosis of PC (BOERRIGTER *et al* ., 2020).

In this context, the Gleason scoring system is a reliable method to quantify PC aggressiveness, as it provides an important reference value for clinical evaluation of therapeutic strategies (LU et al., *2022*) . To obtain the total Gleason classification score, which varies from 2 to 10, the pathologist grades the two most frequent areas of the tumor from 1 to 5 and adds the results. The lower the Gleason score, the better the patient's prognosis. Scores between 2 and 4 represent that the cancer is likely to be slow growing. Intermediate scores, between 5 and 7, can mean a slow or fast growing cancer - and the

speed of growth will depend on a number of other factors, including how long the patient has had the cancer. Scores between 8 and 10 represent a very fast growing cancer (BRASIL, 2016).

The Gleason score also predicts the chance of CP spreading to other organs. For scores from 2 to 4, there is about a 25% chance that the cancer will spread outside the prostate within 10 years, with damage to other organs, affecting survival. Accordingly, a Gleason score of 5 to 7 presents approximately a 50% chance of this event occurring, while for a Gleason score of 8 to 10 the probability increases to 75% (BRASIL, 2016).

According to Samarutanga et al. (2016) ,the ISUP classification system consists of five grades: grade 1 (Gleason score 3 + 3), grade 2 (Gleason score 3 + 4), grade 3 (Gleason score 4 + 3) , grade 4 (Gleason score 4 + 4, 3 + 5, 5 + 3) and grade 5 (Gleason score 9 or 10).

In this sense, the Gleason classification is based on the cellular and histological characteristics that constitute the tumor. Cells that, although tumorous and malignant, are more similar to those of normal prostate, more differentiated and less aggressive, are classified as grade 1. Those that present cellular characteristics already very different from normal ones, and therefore much more aggressive, are classified as grade 5. The final classification of the Gleason score is attributed according to the two predominant cell types in the tumor. The final result results from the sum of the degrees of the two most frequent degrees (SAMARUTANGA *et al* ., 2016).

In addition to the Gleason scale, the accurate staging of the primary PC is important for the selection of the therapeutic protocol (local *versus* systemic) (POMYKALA *et al* ., 2019) . The TNM staging of CP is shown in Chart 1, which is shown below (SOCIEDADE BRASILEIRA DE PATOLOGIA, 2019).

Chart 1 – TNM classification of prostate cancer

pT - PRIMARY TUMOR:
p TX- Primary tumor cannot be evaluated
pTO– No evidence of primary tumor
T1- The tumor is an incidental histological finding, it is not palpable on rectal examination or visualized by imaging techniques
T1a – Tumor in 5% or less of resected tissue
T1b – Tumor in more than 5% of resected tissue
T1c– Tumor identified on needle biopsy (elevated PSA, but tumor not palpable to touch and not visualized by imaging techniques)
pT2 – Tumor limited to the prostate
pT2a– Tumor affects up to half of a lobe, or less
pT2b– Tumor affects more than half of one lobe, but not both lobes
pT2c– Tumor affects both lobes
pT3– Tumor extends beyond the prostate
pT3a–Extraprostatic extension (unilateral or bilateral); includes microscopic involvement of the bladder neck

pT3b– Tumor invades the seminal vesicle(s) *pT4– Tumor is fixed or invades adjacent structures other than the seminal vesicle: external sphincter, rectum, levator muscles, or pelvic wall*
pN - REGIONAL LYMPHO NODES:
pNX– Regional lymph nodes cannot be evaluated *pNO– Absence of metastases in regional lymph nodes* *pN1– Metastasis(ies) in regional lymph node(s)*
pM- DISTANCE METASTASIS (M):
pMO– Absence of distant metastases *pM1– Distant metastases* *M1a– Non-regional lymph node(s)* *M1b- Bone(s)* *M1c- Other location(s)*

Source: BRAZILIAN SOCIETY OF PATHOLOGY (2019).

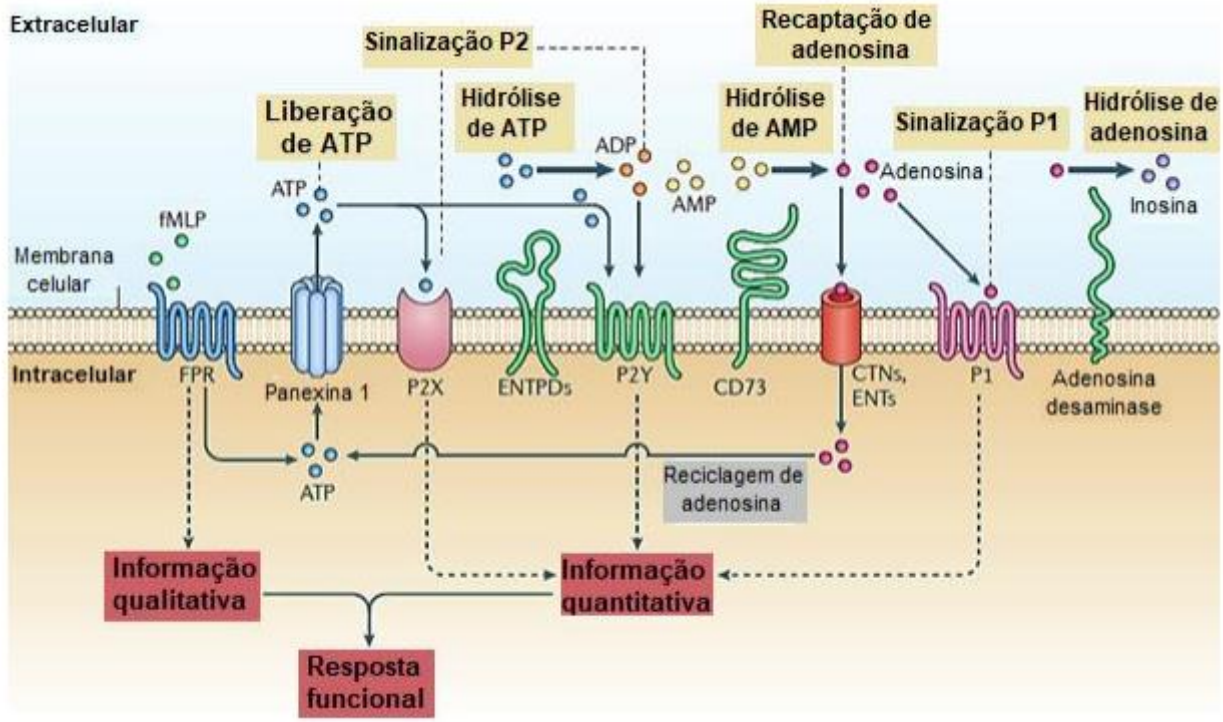
Patients with CP can also be stratified into groups of: i) low, ii) intermediate and, iii) high risk for the occurrence of disease recurrence. For low-risk patients, an option for therapeutic management is active PC surveillance. Still, brachytherapy is also an option for patients at low or intermediate risk of recurrence - it can be used as a booster, after external radiotherapy, for high-risk patients. For intermediate and high-risk patients, radical prostatectomy and radiotherapy should be considered. Furthermore, in addition to radiotherapy, concomitant Androgen Deprivation Therapy (ADT) may be required . Finally, after radical prostatectomy and depending on pathological, biological and clinical factors, radiotherapy associated with ADT may be proposed as adjuvant or rescue treatment (ACHARD *et al* ., 2021). ADT uniquely can be offered to patients with no indication for definitive therapy, such as patients with limited life expectancy (BEKELMAN *et al* ., 2018).

3.2 PURINERGIC SYSTEM AND PROSTATE CANCER

The purinergic system is composed of signaling molecules (ATP, ADP, AMP and adenosine (Ado)), enzymes that degrade these nucleotides and adenine nucleoside, in addition to purinergic receptors composed of: a) P1 family: (A2, A2A, A2B and A3); and b) P2 family (P2X1-7 and P2Y 1, 2, 4, 6, 12, 14) (DI VIRGÍLIO, 2012).

In order to exemplify the joint action of the purinergic signaling components, we present Figure 2, which highlights the enzymes, receptors and signaling molecules.

Figure 2 - Joint action of purinergic signaling components .



Source: Adapted from Junger (2011).

Extracellular signals (especially ATP, ADP and Ado) participate in different pathophysiological processes such as: - stimulation or inhibition of apoptosis; - cell proliferation, migration and differentiation; - secretion of growth factors and inflammatory mediators. Therefore, pathological processes such as cancer can be modulated by the purinergic system (DI VIRGILIO; ADINOLFI, 2017).

In this context, many studies demonstrate the effect of purinergic signaling on cell growth, and ATP is strongly involved in both cancer cell metabolism and antitumor immunity (DI VIRGÍLIO, 2012).

ATP can be released from cells by specific and non-specific pathways. An unregulated release occurs from dead and damaged cells, while active release involves exocytic granules, plasma membrane-derived microvesicles, specific ATP-binding cassette transporters and membrane channels, modulators of calcium homeostasis 1, regulated anion channels by volume and maxi-anion channels (VULTAGGIO-POMA; SARTI; DI VIRGILIO, 2020).

The events associated with the stages of tumor cell proliferation generate an inflammatory microenvironment, favorable for the release of Molecular Patterns Associated with Damage (DAMPs), since in tumorigenesis there is a dynamic process between cell proliferation and injury/destruction (DI VIRGILIO; SARTI ; COUTINHO, 2020) . One of these DAMPs is ATP, and thus, in neoplastic

diseases, this nucleotide plays a crucial role in activating inflammatory cells, becoming an important component of purinergic signaling involved in the development and progression of neoplasms (DI VIRGÍLIO, 2012).

During the inflammatory process, ATP exerts a series of effects. It is involved in the development of inflammation through a combination of actions such as: i) the release of histamines from mast cells; ii) the increase in the synthesis of prostaglandins and; iii) the production and release of cytokines from cells of the immune system (DI VIRGILIO *et al* ., 1998). The action of ATP, during the inflammatory process, occurs mainly via activation of the P2X7 purinergic receptor, causing cell apoptosis and secretion of pro-inflammatory cytokines, such as IL-1β and IL-18 (BURNSTOCK, 2002; BULANOVA et *al* . , 2005, PELEGRIN AND SURPRENANT, 2009).

Thus, ATP is one of the main biochemical components of the tumor microenvironment, and may favor both tumor progression and tumor suppression - depending on its concentration and the action of ecto-nucleotidases and specific receptors expressed by immune and cancer cells.

Regarding Ado, it is a biomarker of cell damage that mediates anti-inflammatory actions, in addition to being a potent immunosuppressive molecule. Ado is normally present at high levels during tumor development, and is able to induce the growth and development of cancer cells through mechanisms such as induction of angiogenesis and immunosuppressed state (MALDONADO et al., 2012 *)* . Studies show that Ado accumulation contributes to tumor progression and represents a promising immunotherapeutic target, since this nucleoside has already been shown to impair the effector function of T cells (MASTELIC-GAVILLET et al., 2019 *)* . Controversially, Ado is also known to induce apoptosis and, in this sense, ADA inhibition would be considered an antitumor strategy (CAMICI *et al* ., 2019).

The signaling molecules mentioned above are metabolized by purinergic enzymes, such as ectonucleotidases. Here we can mention family members (E-NTPDase1 (CD39), 2, 3 and 8), CD73 and ecto-nucleotide pyrophosphatase/phosphodiesterase, which are (E-NPP)located on the cell surface and exhibit a catalytic site facing the extracellular space. The CD39 enzyme catalyses a hidrólise de ATP and ADP into AMP, which is subsequently converted to Ado by CD73. Both CD39 and CD73 are expressed in different cells and tissues, highlighting the abundant expression of CD39 in endothelial cells and smooth muscle cells, dendritic cells and lymphocytes (ROBSON; SÉVIGNY; ZIMMERMANN, 2006), and the CD73 being expressed in colon, kidney, brain, liver, heart, lung and prostate (MORIWAKI *et al* ., 1999; ZIMMERMANN, 1992, 2006).

CD39 activity on lymphocytes is a relevant mediator of tumor progression, since it can play an indirect immunosuppressive role by hydrolyzing ATP and ADP, which will later be converted into Ado - a molecule that induces tumor progression (KÜNZLI et al . , 2011; MASSÉ *et al* ., 2015).

an analysis *in silico* showed that several tumor types have a significant correlation between an epithelial-mesenchymal transition score and expressions of CD73 and CD39, with the strongest correlations being observed in prostate adenocarcinoma (ISER *et al* ., 2022).

In turn, the ADA ectoenzyme has the function of promoting the hydrolytic deamination, on the cell surface, of Ado into inosine (ROBSON *et al* ., 2006; YEGUTKIN, 2008). Its deficiency contributes to the development of pathologies, due to the abnormal increase in extracellular Ado concentrations (MARTINS *et al* ., 2016).

ADA plays an important role in immune regulation, especially in lymphocyte proliferation, maturation and differentiation. Its role has been studied in inflammatory processes and malignant diseases (CHEN *et al* ., 2015), since changes in ADA activity have already been detected in patients with various types of cancer (ZHULAI *et al* ., 2022). Low ADA activity in lymphocytes may be responsible for the decrease in cellular immune function in cancer patients (CAMICI *et al* ., 2019).

On the contrary, some studies show that the high activity of ADA can be advantageous for cancer cells, and may be a compensatory mechanism against the toxic accumulation of their substrates (CAMICI et al., *2019*).

Additionally, exploring the common pathways that associate P2-purinergic receptor dysregulation with urologic disease may ultimately help to gain new mechanistic insights into disease processes and therapeutic direction. Here, we highlight the convincing evidence for the exploration of purinergic P2 receptors as biomarkers and therapeutic targets in urological cancers and other diseases of this system. Likewise, there is currently optimism for P2 sreceptor- targeted therapies for the treatment of inflammation and pain related to urologic diseases.

In this sense, studies have shown that the P2X7 receptor is abnormally expressed in PC and is related to PSA levels , and may be an early biomarker of this disease. It is essential in the occurrence and development of this pathology, mainly influencing the invasion and metastasis of cancer cells through genes related to epithelial mesenchymal transition/invasion and the PI3K/AKT and ERK1/2 signaling pathways. For all these reasons, the e s e receptor can be a promising therapeutic target for CP (QIAO *et al* ., 2022).

In addition to P2X7, studies suggest that the P2X4 receptor plays a key role in the onset of PC and is a clinically useful therapeutic candidate, since inhibitors of this receptor are already available and have the potential to suppress disease progression (HE et *al.* ., 2020). On the other hand, the activation of the P2Y1 purinergic receptor induced apoptosis through the Caspase 3/7 pathway and through the ROS signaling pathway. In this way, it is evidenced that the newly synthesized ligands, as agonists of this receptor, could potentially act as antitumor molecules in the treatment of CP (LE *et al* ., 2019).

Given the above, research is of fundamental importance to understand the involvement of purinergic signaling in PC .

4 METHODOLOGY

4.1 STUDY DESIGN

This a research was approved aby the Ethics Committee for Research with Human Beings (CEP) of the Federal University of Fronteira Sul (UFFS), under protocol number: 87508918.4.0000.5564.

The present study is characterized as a cross-sectional observational quantitative study associated with a qualitative approach.

With regard to sample selection, it was carried out for convenience and material was collected from 39 patients from September 2021 to March 2023. The study analyzed the activity of NTPDase, for the hydrolysis of ATP and ADP, and the ADA activity in men newly diagnosed with PC, before starting surgical and/or pharmacological treatment. The control group consisted of 37 men, aged similar to the investigated group and who did not have a diagnosis of CP. The patients were selected based on previous contact between the researchers and the surgeon responsible for the evaluation of patients at the Hospital Regional do Oeste (HRO) ,and the selected patients were informed by the researchers about the objectives of the research and about the interventions that would be performed if they accepted. participate, signing the Term of Free and Informed Consent (TCLE).

After selection, patients and controls were contacted by the researchers to perform a single blood collection, in a volume of 30 mL, by venipuncture. Biochemical and molecular analyzes were carried out in the research laboratories of the Federal University of Fronteira Sul (UFFS) - *Campus* Chapecó (SC). Medical record information was obtained from patient files filed at the HRO, and was used to complement the biological analyses. This collected information is contained in Appendix II. Based on these analyses, the results obtained between the groups were compared and changes in the evaluated biological systems due to the presence of CP were verified.

4.2 EXPERIMENTAL PROTOCOL

4.2.1 Collection of biological samples

With regard to sample selection, sampling was done for convenience, and thirty-nine (n= 39) patients diagnosed with CP were collected, and thirty-seven (n= 37) control individuals, matched by age, in the city from Chapecó (SC), following the inclusion and exclusion criteria. Soon after the diagnosis, the individuals were informed about the objectives of the study, and were invited to

participate in it, consenting, by signing the TCLE, to provide information from the examination reports and biopsies performed from the medical records a. Afterwards, blood was collected in *vacutainer tubes* with EDTA as an anticoagulant (for the separation of lymphocytes). Blood collection from patients and controls was performed in a single moment, after the result of the biopsy performed, and before the start of surgical and/or pharmacological treatment of PC in the case of patients. The lymphocytes were placed in an *eppendorf -type microtube* , stored in specific boxes, identified and frozen in a freezer at -80 ºC for subsequent analysis. The information obtained from the medical records was related to age, histology, TNM, histological grade (Gleason score), serum PSA levels, ISUP score, family history of cancer and chronic diseases.

4.2.2 Processing and separation of lymphocytes

For lymphocyte isolation, blood samples collected with EDTA were diluted in an equal volume of saline solution. Subsequently, the diluted sample was transferred to a conical tube containing Lymphoprep (Ficoll-Histopaque) and centrifugation was performed at 1800 rotations per minute (rpm), at room temperature, for 30 minutes. After centrifugation, through the formation of a density gradient, it is possible to visualize an intermediate layer composed of mononuclear cells (lymphocytes and monocytes) between the layers of plasma and Ficoll. This cloud of cells was carefully removed and transferred to a clean conical tube. To the cells, saline was added and the sample was centrifuged for 5 minutes at 1800 rpm. Next, the supernatant was discarded and 5 ml of hemolytic buffer were added to the lymphocytes, followed by a new centrifugation. Again, the supernatant was discarded and 5 mL of saline solution was added, homogenizing the contents and performing a new centrifugation for 5 minutes at 1800 rpm. After the end of this process , the lymphocytes were stored in *eppendorfs* containing 600 μL of saline solution. The samples were stored in a freezer at -80ºC until the experiments were carried out (Leal, 2005).

4.2.3 Protein dosage

The quantification of protein levels in lymphocyte samples from control and patient individuals was measured by the *Comassie Blue* method according to Bradford (BRADFORD, 1976), using bovine albumin as standard.

4.2.4 Determination of E-NTPDase activity

The activity of E-NTPDase, in lymphocytes, was performed according to Leal e colaboradores(2005). Enzyme activity was determined by measuring the amount of inorganic phosphate (Pi) released using a colorimetric assay. Um volume de 20 uL of lymphocyte sample was added to 160 uL of NTPDase incubation system and pre-incubated for 10 minutes at 37°C. For NTPDase activity in lymphocytes, the incubation system contains 1200 mM NaCl, 600 mM Glucose, 50 mM KCl, 500 mM Tris-HCl buffer pH 7.4, 50 mM $CaCl_2$ and *Milli Q* H_2O . The reaction was started by adding 20 uL of ATP or ADP as substrate. The NTPDase reaction was stopped by the addition of 150 µL of 15% trichloroacetic acid (TCA). The Pi released by the hydrolysis of ATP and ADP was measured by the CHAN methode colaboradores (1986). Readings were taken in a spectrophotometer at comprimento de onda de 630 nm. Patient and control samples were analyzed in triplicates. The non-enzymatic hydrolysis control was performed as described for the samples, but without the addition of enzyme. Enzyme activity was expressed in nmol Pi released/min/mg protein.

4.2.5 Determination of ADA activity

ADA activity, in lymphocytes, was determined according to the protocol already described by Giusti and Galanti (1974). The colored reaction, which reveals the amount of ammonia released by the action of the enzyme, is carried out according to the reaction by Chaney and Marbach (1962), which produces a blue color. This technique is based on the dosage of ammonia released by the transformation of Ado inosine, catalyzed by ADA. Ammonia forms, in the presence of phenol, in an alkaline solution, an indophenol derivative that is blue in color and can be read in a no comprimento de onda de 620 nm spectrophotometer. Enzymatic activity was expressed in U/mg of protein.

4.3 INCLUSION CRITERIA

- Volunteer patients, over forty-five (45) years of age, diagnosed by an oncologist or urologist according to ICD 10 of CP;
- Have not performed surgical removal of the tumor or initiated pharmacological treatment at the time of participation in the study;
- The selected control individuals were volunteers, aged over forty-five (45) complete years, who did not have a diagnosis of CP.

4.4 EXCLUSION CRITERIA

- Patients who had another diagnosis of cancer prévio ou atual;
- Control subjects who had any type of cancer .

4.5 STATISTICAL ANALYSIS

Statistical analysis was performed using *GraphPad Prism* 8.0.1 software (GraphPad Software, San Diego, California, USA). Data normality was analyzed using the Shapiro-Wilk test, which showed normal distribution. Outliers were analyzed by the *software* itself and removed only for the analysis of variables that differed from the other data . Regarding the study variables, the differences between patients with CP and control individuals were evaluated using Student's t test and the Mann-Whitney test for non-parametric data. Differences between subgroups for enzymatic activities were assessed using one-way analysis of variance (One-way ANOVA). Results are presented as mean ± standard deviation. Differences in which the probability of rejecting the null hypothesis was less than 5% ($p<0.05$) were considered statistically significant.

4.6 ETHICAL CONSIDERATIONS

All procedures performed were submitted to evaluation by the UFFS CEP, in accordance with the norms of Resolution 466/12 of the National Health Council on Research involving human beings.

To participate in the study, the selected individuals agreed with the TCLE (APPENDIX I) provided by the researchers. All research participants were warned about the risks and benefits brought by their participation and by the collection procedure, and personal data will be kept confidential and each individual will be identified by a different number. The material collected will remain in the possession of the responsible researcher and will be kept in a lockable cabinet, in the researcher's room, on the UFFS premises, or in a freezer in the research laboratory, which only the researchers involved have access to. After the five-year period, the files (physical or digital) will be destroyed.

5RESULTS

5.1 CHARACTERIZATION OF THE SAMPLE

By analyzing Table 1, we can observe general characteristics related to the sample population, such as age, marital status, smoking, diagnosis of Covid-19, comorbidities and history of CPfamily members with 1st degree kinship.

Regarding the patients' age, the average is 66 years, 74.3% are married and more than 82% of patients with CP were non-smokers. Of this group of patients, 87.2% were not diagnosed with Covid-19 and 61.5% did not have DM (Diabetes Mellitus) or SAH (Systemic Arterial Hypertension). Another relevant data is that the vast majority (84.6%) of patients did not have a history of PC in 1st degree relatives.

Table 1 - General characteristics of patients and controls

Characteristics	**Patients**	**Controls**	**p value**
sample number	39	37	-
Age			
Average	66	65.81	0.8503
Standard deviation	±6.72	±10.30	
marital status			
Singles	6 (15.4%)	4 (10.8%)	
Married	29 (74.3%)	29 (78.4%)	
Divorced	2 (5.1%)	1 (2.7%)	
widowers	2 (5.1%)	3 (8.1%)	
smoking			
Yes	7 (17.9%)	2 (5.4%)	
No	32 (82.1%)	35 (94.6%)	
Diagnosis of COVID-19			
Yes	5 (12.8%)	4 (10.8%)	
No	34 (87.2%)	33 (89.2%)	
Comorbidities			
They do not have DM and	24 (61.5%)	22 (59.5%)	
SAH	0 (0%)	0 (0%)	
DM	11 (28.2%)	13 (35.1%)	
SAH	4 (10.3%)	2 (5.4%)	
DM and SAH			
1st degree de kinship historyCP			
Yes	6 (15.4%)	1 (2.7%)	
No	33 (84.6%)	36 (97.3%)	

Source: elaborated by the authors (2023).

The Table 2presents information regarding serum PSA levels before cancer treatment, Gleason score, tumor histology, tumor staging and ISUP score of the patients involved in the study.

Table2 - Tumor classification of patients withCP

	N (%)
PSA pretreatment	-
<4 ng/mL	2 (5.1%)
≥4 a ≤10 ng/mL	21 (54%)
>10 a ≤20 ng/mL	14 (35.9%)
> 20 ng/mL	1 (2.5%)
Not in the record	1 (2.5%)
Histological grading (Gleason)	-
gleason 6	5 (12.9%)
Gleason 7	27 (69.2%)
Gleason 8	4 (10.2%)
Gleason 9	3 (7.7%)
Histology	-
prostate adenocarcinoma	39 (100%)
***TNM**	-
T1c	3 (7.7%)
pT2	4 (10.3%)
pT2b	4 (10.3%)
pT2c	9 (23%)
pT3	1 (2.5%)
pT3a	7 (18%)
pT3b	6 (15.4%)
Not in the record	5 (12.8%)
***Clinical staging** -	
T1c 3 (7.7%)	
T2 17 (43.6%)	
T3 14 (35.9%)	
Does not appear in medical records 5 (12.8%)	
ISUP score -	
ISUP 1 5 (12.8%)	
ISUP 2 19 (48.8%)	
ISUP 3 8 (20.5%)	
ISUP 4 4 (10.3%)	
ISUP 5 3 (7.6%)	

*Data related to the involvement of regional lymph nodes “N” and the presence of distant metastases “M” were not included in the medical records, for this reason only the staging from “T” was described.

Source: elaborated by the authors (2023).

Pela análise da Tabela 2 observamos que a maioria dos pacientes participantes do estudo (54%) apresentava níveis séricos de PSA entre ≥4 a ≤10 ng/mL, antes da intervenção terapêutica. Além disso, a maioria (69,2%) dos pacientes apresentava escore de Gleason 7 e que, em relação ao estadiamento clínico, 43,6% dos pacientes apresentaram classificação T2. Já em relação ao escore da ISUP a maioria (48,8%) apresentava classificação ISUP 2.

5.2 E-NTPDase AND ADA ACTIVITY

We can observe, by analyzing Figure 3, the activity of the E-NTPDase and ADA enzymes in lymphocytes. Figure 3A depicts E-NTPDase activity for ATP hydrolysis. We can observe that ATP hydrolysis was significantly higher in the group of patients with CP than in the group of control individuals (136.2 ± 51.27 *vs.* 229.2 ± 105.97 nmol Pi/min/mg of protein, in controls and patients, respectively), p-value=0.0001. Figure 3B depicts E-NTPDase activity for ADP hydrolysis. It is possible to observe that ADP hydrolysis was statistically lower in the group of patients with CP than in the group of control individuals (124.8 ± 50.46 *vs.* 60.81 ± 75.85 nmol Pi/min/mg of protein, in controls and patients, respectively), p-value=0.0001. Figure 3C depicts ADA activity. By analyzing this figure, we observed that ADA activity was significantly lower in the group with CP when compared to the control group (190.8 ± 104.5 *vs.* 105.5 ± 35.70 U/mg of protein, in controls and patients , respectively), p-value=0.0001.

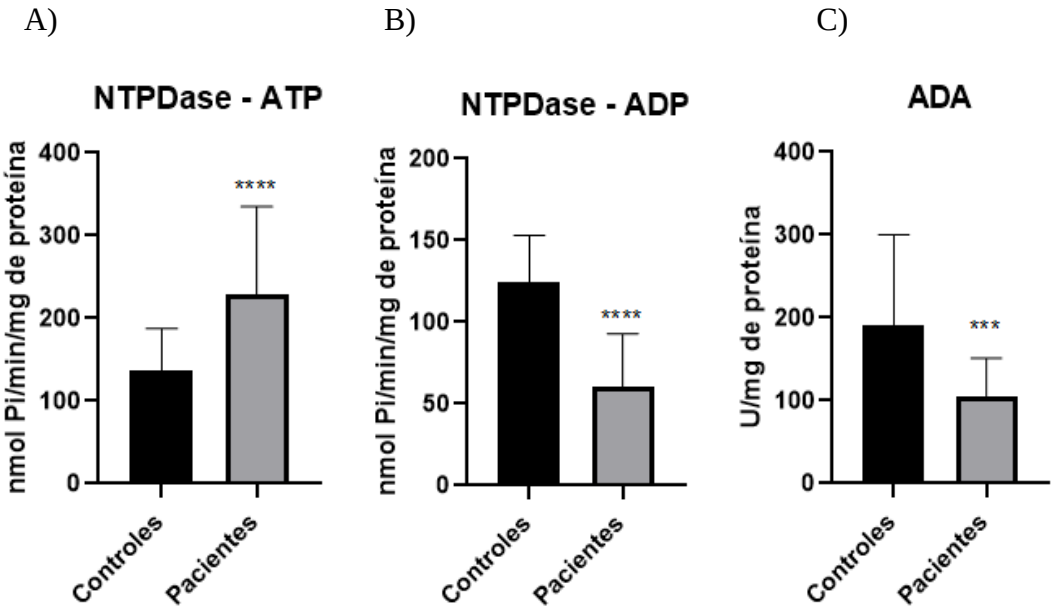


Figure 3 - Atividade das enzimas E-NTPDase e ADA em linfócitos de pacientes com CP. A) Atividade da E-NTPDase para a hidrólise de ATP. B) Atividade da E-NTPDase para a hidrólise de ADP. C) A atividade da ADA. Os resultados foram expressos como média ± desvio padrão (n= 37) para controles e (n= 39) para pacientes. ***p ≤ 0.0005 ****p ≤ 0.00005.

Figure 4 presents the values of E-NTPDase and ADA activity, in lymphocytes, according to serum PSA levels. It is noteworthy that the control group, based on the reference values in the literature, was considered to have serum PSA levels < 4.0 ng/mL.By analyzing Figure 4A, we can see that ATP hydrolysis increased significantly with the increase in serum levels of (control PSA: 20.7.6±99.28 nmol Pi/min/mg of protein; PSA < 10: 136 ±47.27 nmol Pi/min/mg protein, p-value=0.0037; PSA ≥ 10: 251.5±6.07 nmol Pi/min/mg protein), p-value=0.0001 . Analyzing Figure 4B, we observed that ADP hydrolysis was statistically lower in patients with CP compared to the control group (: 124.8± 50.46 nmol Pi/min/mg of protein; PSA < 10: 61.14±75.85 nmol Pi/min/mg protein; PSA ≥ 10: 61.01±70.18 nmol Pi/min/mg protein), p-value=0.0001 . By analyzing Figure 4C, it is possible to observe that ADA activity decreased significantly with the increase in serum levels of (control PSA: 190.8 ± 104.5 U/mg of protein; PSA < 10: 109.1 ± 83.42 U/mg protein, p-value=0.0104; PSA ≥ 10: 105.1 ±15.59 U/mg protein), p-value=0.0086 .

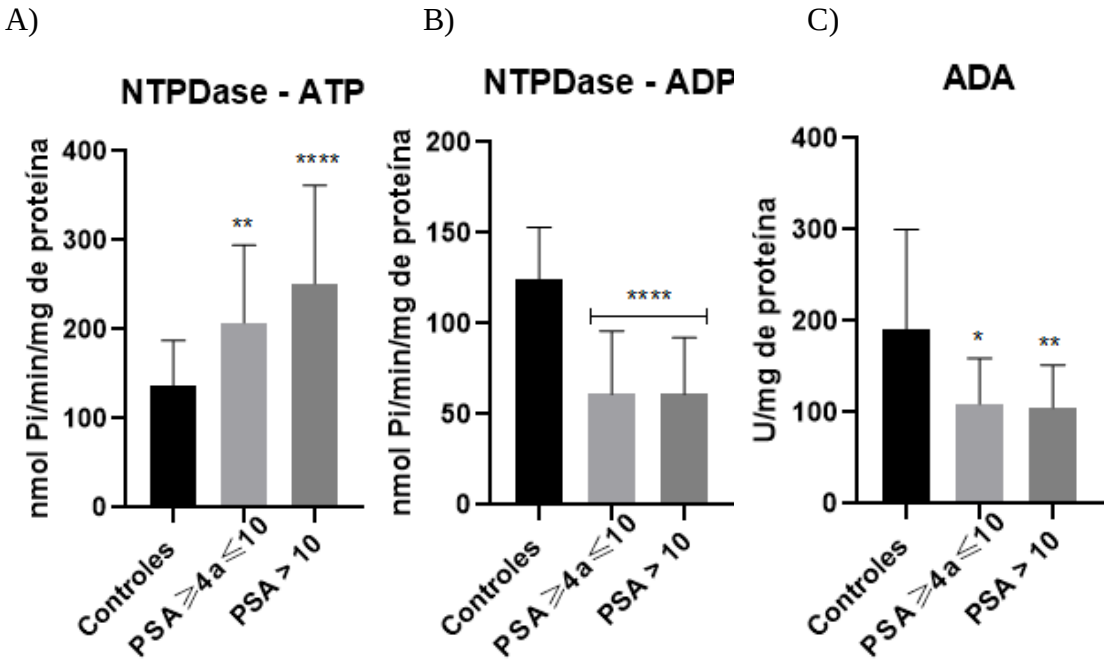


Figure 4 - Atividade das enzimas E-NTPDase e ADA em linfócitos de pacientes com CP e em controles de acordo com os níveis séricos de PSA. A) Atividade da E-NTPDase para a hidrólise de ATP. B) Atividade da E-NTPDase para a hidrólise de ADP. C) A atividade da ADA. Os resultados foram expressos como média ± desvio padrão (n= 37) para controles e (n= 39) para pacientes. * p < 0,05) **p < 0,005 ****p ≤ 0.00005

Figure 5 shows the activity values of E-NTPDase and ADA, in lymphocytes, according to the Gleason score. Observing Figure 5A, it is possible to notice that ATP hydrolysis was significantly higher in patients with a Gleason score greater than or equal to 7. Between the control group and the Gleason score < 7, there was no statistically significant difference 136.2±47 (, 27 *vs.* 186.7±41.65 nmol Pi/min/mg of protein, in controls and Gleason score < 7, respectively), p-value=0.5664 . There was a statistically significant difference between the control group and Gleason score 7 and Gleason score > 7 (control: 136.2±47.27 nmol Pi/min/mg of protein; Gleason score 7: 234.1±107, 32 nmol Pi/min/mg of protein; Gleason score > 7: 247±91.81 nmol Pi/min/mg of protein), p value=0.0001 . By analyzing Figure 5B, it is possible to observe that the hydrolysis of ADP was significantly lower in patients with CP when compared to the control group. We observed that there was a statistically significant difference between all groups with CP, regardless of the Gleason score value. Gleason score 7: 64.55 ± 42.80 nmol Pi /min/mg protein, p-value=0.0034; Gleason score 7: 52.94±33.91 nmol Pi/min/mg protein, p-value=0.00001; Gleason score > 7: 63.60±30.76 nmol Pi/min/mg of protein), p value=0.0012 Analyzing Figure 5C, we observed that ADA activity was statistically lower only in the group of patients with Gleason score 7 (189 ±138.04 *vs.* 102.9±42.25 U/mg of protein, in controls and Gleason score 7, respectively), p value=0.0171 . However, between the control group and the Gleason score < 7 and Gleason score > 7, there was no statistically significant difference between (the control

group: 189±138.04 U/mg of protein; Gleason < 7: 73.70±15.36 U/mg of protein, p-value= 0.3647; Gleason score > 7: 108.1±16.56 U/mg of protein), p value= 0.3955 .

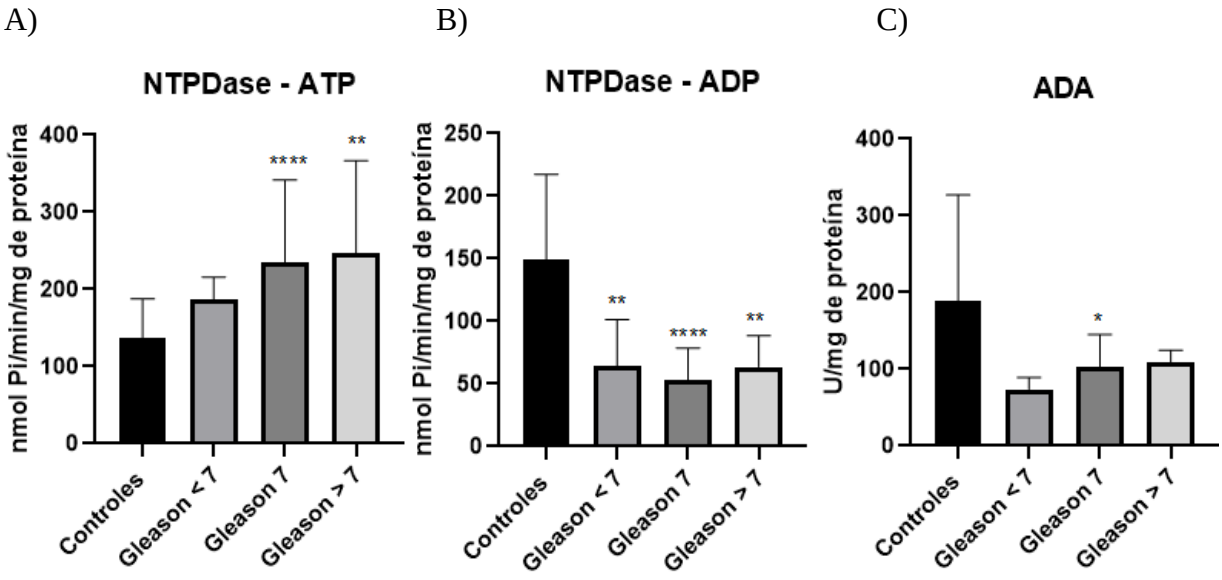


Figure 5 - Atividade das enzimas E-NTPDase e ADA em linfócitos de pacientes com CP de acordo com o escore de Gleason e em controles. A) Atividade da E-NTPDase para a hidrólise de ATP. B) Atividade da E-NTPDase para a hidrólise de ADP. C) A atividade da ADA. Os resultados foram expressos como média ± desvio padrão (n= 37) para controles e (n= 39) para pacientes. * p < 0,05 **p <0,005 ***p < 0,0005 ****p ≤ 0.00005

Figure 6 shows the activity of E-NTPDase and ADA, in lymphocytes, according to the clinical staging of the patients. By analyzing Figure 6A, we observed that ATP hydrolysis foi was significantly higher in patients with CP with clinical stages T2 and T3 compared to the control group (: 136.2±47.27 nmol Pi/min/mg of protein; T2: 228.2±99.28 nmol Pi/min/mg protein; p-value=0.0004. T3: 238.6±18.59 nmol Pi/min/mg protein, p-value=0.0003). Between the control group and clinical staging T1, there was no statistically significant difference (136.2±47.27 vs. 225.1± 94.74 nmol Pi/min/mg of protein, in controls and T1 group, respectively), p value=0.1601 . By evaluating Figure 6B, it is possible to observe that ADP hydrolysis was significantly reduced in patients with CP with clinical stages T2 and T3 compared to the control group control (: 124.8±50.46 nmol Pi/min/mg of protein; T2: 52.02±33.91 nmol Pi/min/mg protein, p-value=0.0001; T3: 60.53±34.91 nmol Pi/min/mg protein, p-value=0.0001). Between the control group and clinical staging T1, there was no statistically significant difference for hydrolysis of ADP (124.8±50.46 vs. 95.12±7.36 nmol Pi/min/mg of protein, in controls and T1 group, respectively), p value=0.3557 . By analyzing Figure 6C, we can observe that ADA activity was statistically lower in patients with CP with clinical stages T2 and T3 compared to the control group (control: 190.8±104.50 U/mg of protein; T2: 111.2±20.50 U/mg protein, p-value=0.0303; T3: 109.3±52.99 U/mg protein, p-value=0.0397). There was no statistically significant

difference between the control group and clinical staging T1 (190.8±104.50 *vs* . 73.70±15.36 U/mg of protein, in controls and T1 group, respectively), p value=0.1877 .

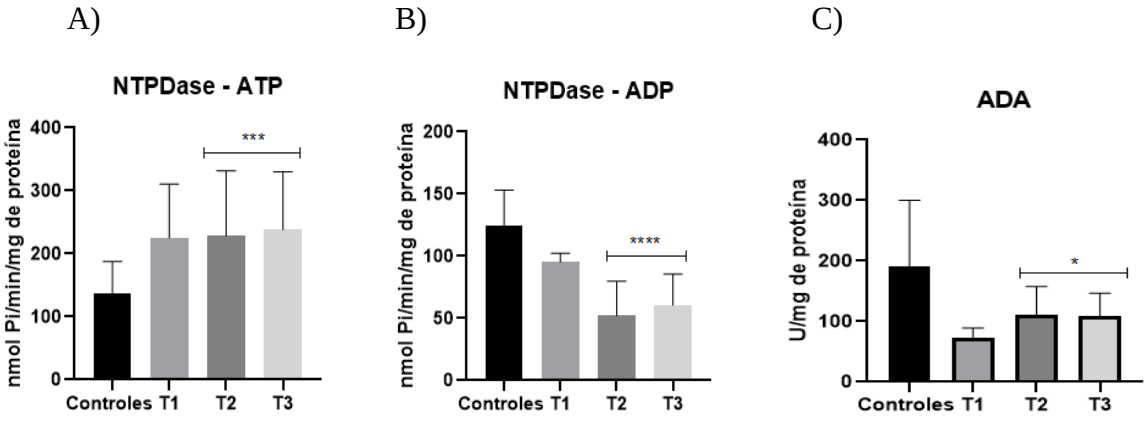


Figure 6 - Atividade das enzimas E-NTPDase e ADA em linfócitos de pacientes com CP de acordo com o estadiamento clínico e em controles. A) Atividade da E-NTPDase para a hidrólise de ATP. B) Atividade da E-NTPDase para a hidrólise de ADP. C) A atividade da ADA. Os resultados foram expressos como média ± desvio padrão (n= 37) para controles e (n= 39) para pacientes. * $p < 0,05$ ***$p < 0,0005$ ****$p \leq 0.00005$

Figure 7 shows the activity of E-NTPDase and ADA, in lymphocytes, according to the ISUP of the patients e em controles. By analyzing Figure 7A, we can observe that between the control group and the group of patients with ISUP 1 there was no statistically significant difference for the hydrolysis of ATP (136.2±47.27 *vs.* 214±41.65 nmol Pi/min/mg of protein, in controls and ISUP 1 group, respectively, p-value=0.2200). Between the control group and ISUP 2, ISUP 3 and ISUP 3 and 4, there was a significant increase for the control ATP hydrolysis (: 136.2±47.27 Pi/min/mg of protein; ISUP 2: 224.5±99.28 Pi/min/mg protein, p-value=0.0024; ISUP 3: 234.3±155.74 Pi/min/mg protein, p-value=0.0192; ISUP 4 and 5: 247±119.7 Pi/min/mg protein, p-value=0.0108). By observing Figure 7B, it is possible to see a significant decrease in ADP hydrolysis for all ISUPs when compared to the control group (: 124.8±50.46 nmol Pi/min/mg of protein; ISUP 1: 64.55± 42.80 nmol Pi/min/mg protein, p-value=0.0005; ISUP 2: 51.26±75.85 nmol Pi/min/mg protein, p-value=0.0001; ISUP 3: 67.14±5.74 nmol Pi/min/mg protein, p-value=0.0001; ISUP 4 and 5: 74.07±35.85 nmol Pi/min/mg protein, p-value=0.0007). By analyzing Figure 7C, we observed a statistically significant decrease only in patients with ISUP 2 compared with the control group (: 190.8±104.5 U/mg of protein; ISUP 2: 102.6± 35.70 U/mg protein, p-value=0.0068).

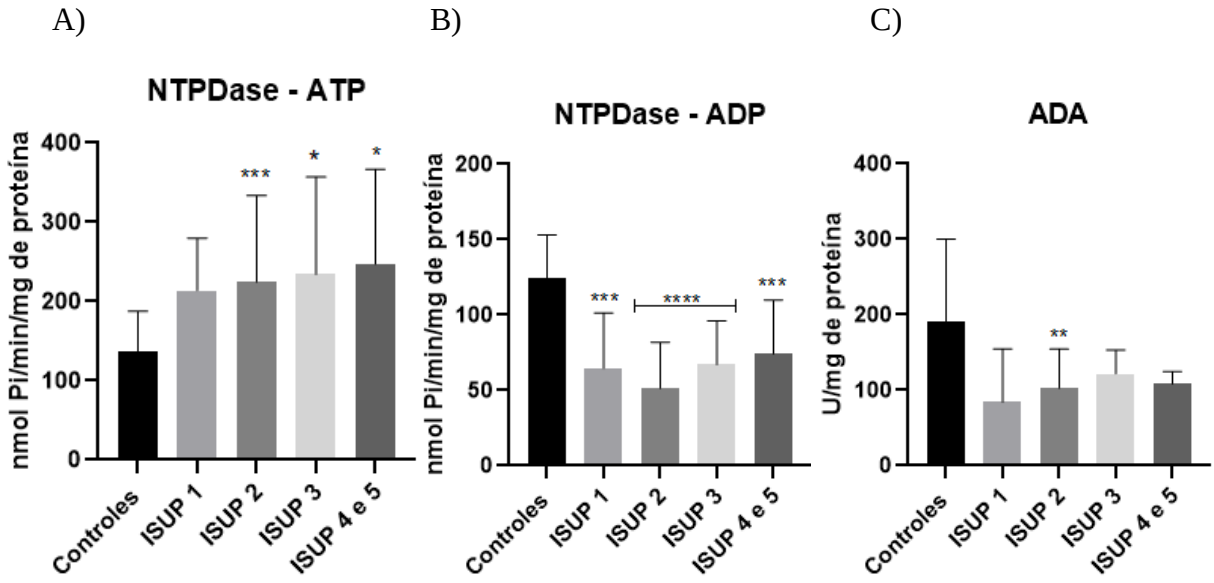


Figure 7 - Atividade das enzimas E-NTPDase e ADA em linfócitos de pacientes com CP de acordo com a escala da ISUP e em controles. A) Atividade da E-NTPDase para a hidrólise de ATP. B) Atividade da E-NTPDase para a hidrólise de ADP. C) A atividade da ADA. Os resultados foram expressos como média ± desvio padrão (n= 37) para controles e (n= 39) para pacientes. * $p < 0,05$ **$p < 0,005$ ***$p < 0,0005$ ****$p \leq 0.00005$

6 DISCUSSION

PC is the second most diagnosed type of cancer worldwide and the most frequent cause of cancer-related deaths in men. Hereditary CP has the highest heritability of all types of serious cancer in men, as the proportion of CP attributable to hereditary factors has been estimated to range from 5-15% (VIETRI et al., 2021 *)* . Twenty percent of all patients with CP have a positive family history (at least 1 first-degree relative with CP) and a portion of these patients have a genetic predisposition (MEISSNER ; JAHNEN ; HERKOMMER, 2021). In addition to family history and hereditary syndromes, ethnic aspects are also strong risk factors for PC. In our study, we found that only 15.4% of the patients had a history of CP1st degree dekinship.

As in our study, in which the mean age of patients was 66 years, other studies such as the one carried out by Gardani et al. (2019), also observed that individuals in their sixth decade of life are the most affected by PC. In addition, WHO data show that this is the age group with the highest incidence of this type of tumor.

In the meta-analysis by Guo et al. (2020) carried out with a total of 11 observational studies comprising 1,457,799 patients diagnosed with CP, the results indicated that single marital status (separated, divorced, widowed or never married) was associated with a risk higher all-cause mortality compared to the married state, particularly for divorced and never-married patients. In our research, we identified that most patients with PC, 29 (74.3%) are married, 6 (15.4%) are single, 2 (5.1%) are separated and also for widows.

Furthermore, we found in our study that 17.9% of the patients were smokers. In this sense, the literature has described that smoking and obesity can increase mortality, while regular physical activity is capable of reducing the progression of the disease (GANDAGLIA *et al* ., 2021). O consumo de álcool, o tabagismo, a ingestão de carne vermelha e índice de massa corpórea (IMC) ≥ 25-30 kg/m² revelaram uma tendência de aumento do risco do desenvolvimento do CP (CIRNE *et al* ., 2022). In parallel, metabolic syndrome has been associated with the risk of developing high-grade CP (GANDAGLIA *et al* ., 2021).

The authors Macke and Petrosyan (2022) report that excessive alcohol consumption is related to increased risk of PC and is also directly linked to the lethality of this pathology, as it can accelerate the growth of prostate tumors and significantly reduce the time of progression. for metastatic CP. In this sense, Oczkowski e colaboradores (2021) state that consumption of certain nutrients (saturated and trans fatty acids) and food products (e.g., processed meat products) leads to disruption of prostate hormone regulation, induction of oxidative stress and inflammation, and alteration of factor signaling.

of growth and lipid metabolism, which contribute to prostate carcinogenesis. These conditions may be associated with the development of CP in our studied population.

Brito-Dellan, Tsoukalas, and Font (2022) report that cancer and coronavirus disease 2019 (COVID-19) have unusual similarities: both result in a markedly elevated risk of thrombosis, exceptionally high levels of D-dimer, and therapy failure. anticoagulation in some cases. Cancer patients are more vulnerable to COVID-19 infection and have a higher mortality rate. In our study, only 5 (12.8%) of the patients were diagnosed with COVID-19.

With regard to E-NTPDase and ADA activity in lymphocytes, our results demonstrated an increase in E-NTPDase activity for ATP hydrolysis, a decrease in ADP hydrolysis, and a decrease in ADA activity in patients with CP.

It is extensively reported in the literature that nucleotides, especially ATP, act as endogenous signaling molecules of injuries, triggering an immune system response (ZHANG; MOSSER, 2008). Studies reveal that ATP is involved in several functions in the immune system: in T cells, ATP is able to mediate the immune response acting as a pro-inflammatory agent, because it stimulates the proliferation of lymphocytes and potentiates the release of cytokines, such as IL-2 and IFN-g (LANGSTON et al., 2003; BOURS et al., 2006); in circulating monocytes, ATP is involved in their recruitment to target tissues (VENTURA; THOMOPOULOS, 1995); in dendritic cells, ATP induces migration and differentiation (LA SALA et al., 2003); in macrophages, it stimulates the production of IL-1β (ELSSNER et al., 2004) and tumor necrosis factor-α (TNF-α) (GUERRA et al., 2003).

In the process of development and tumor progression of PC, there is notably a favoring of injury and injury to prostatic cells, collaborating for the release of large amounts of ATP into the extracellular environment - which will propitiate the development of a pro-inflammatory microenvironment, which is amplified as tumor progression is established. It is also evident that the greater availability of extracellular ATP favors the maximum activity of E-NTPDase, given the presence of substrate in high concentrations. Similar results were observed in studies carried out in patients with breast cancer and in patients with uterine cervix neoplasia (ARAÚJO *et al* ., 2005; MALDONADO *et al* ., 2010), reinforcing the relationship between the development of neoplastic diseases and alterations in activities of the enzymes of the purinergic system.

E-NTPDase is an essential enzyme in the vasculature and on the surface of platelets, as it limits thrombotic events and preserves the antithrombotic properties of the endothelium (MORELLO *et al* ., 2021). In our research, we verified that the activity of E-NTPDase in lymphocytes, for ADP hydrolysis, is significantly reduced in the group of patients with CP in relation to the control group.

The increase in ATP hydrolysis by E-NTPDase promotes the formation of ADP, which has prominent pro-aggregant activity. Stimulation of platelets by ADP leads to changes in format,

aggregation and generation of TxA_2, and the co-stimulation of P2Y1 and P2Y12 receptors is required for the induction of platelet aggregation by this nucleotide (KAHNER *et al* ., 2006). Additionally, stimulation of the P2X1 receptor by ATP is involved in changing the shape of platelets and helps to amplify responses mediated by other agonists (KAHNER et al . , 2006). Binding of ADP to P2Y1 receptors triggers the activation of phospholipase A2, which activates the generation of TxA_2. Both the activation of P2Y1 receptors and the binding of ATP to P2X receptors (mainly P2X1) generate mobilization and influx of Ca^{2+}, culminating in a change in the shape of platelets. Together, all these factors lead to platelet activation and the stabilization of existing platelet aggregates and, given the above, we can suggest that the patients with PC involved in this study may be more susceptible to the development of thrombotic processes.

Considering the pro-carcinogenic properties exerted by Ado - among which stand out the functions that promote tumor growth, stimulate angiogenesis and reduce tissue hypoxia, through its vasodilator activity - (RATHBONE, 1992; SPYCHALA, 2000 ; GESSI; VARANI; MERIGUI, 2007), this molecule has become an important therapeutic target for cancer (LOSENKOVA *et al* ., 2020).

In our study, we observed a decrease in ADA activity in lymphocytes from patients with CP, a phenomenon that promotes an increase in Ado levels. Other studies, such as the one developed by Zanini *et al* . (2019) in patients with lung cancer, also showed that ADA activity was reduced in erythrocytes and lymphocytes, proposing that the accumulation of Ado in the extracellular environment could favor tumor progression. In the work by Mânica et al. (2018) in patients with melanoma, they report knowing that immunosuppression is closely related to the development of lymphatic metastases in patients with melanoma, the increase in ADA activity may be associated with malignant processes even after removal tumor surgery. Also, the authors Wang, Du and Chen (2022) refer that, in general, in the context of cancer, the accumulation of extracellular Ado inhibits the normal function of the effector immune cells and facilitates the effect of the immunosuppressive cells to promote the proliferation and migration of malignant cells.

Regarding PC biomarkers, PSA is the first filter in the diagnosis of this neoplasm, according to Maestroni *et al* . (2022). Kohaar , Petrovich and Srivastava (2019) state that early detection of CP is largely determined by PSA, which is widely used.

With regard to the hydrolysis of ATP, ADP and ADA activity, when patients with CP were allocated into subgroups according to serum PSA levels, we identified that PSA values seem to cause a significant change in this signaling pathway - hydrolysis of ATP, ADP and ADA activity. Apparently, when PSA levels are strongly altered (>10 ng/mL) it is possible to associate these values with greater cell destruction/damage and significant immunosuppression in patients with CP.

Since PSA is released by prostatic cells and, in tumoral conditions, there is a clonal expansion

of these cells, an increase in serum levels of PSA is assumed according to the size of the tumor mass in the PC. As mentioned in the literature, solid tumor cells are exposed to a hypoxic environment, which favors injury and cell lysis processes. These events contribute to the externalization of molecules to the extracellular environment, such as ATP, contributing to the increase in serum levels of this nucleotide. Thus, the increase in E-NTPDase activity for ATP hydrolysis observed in our work may be directly associated with the greater availability of its substrate in patients with CP. Thus, ATP hydrolysis appears to be directly associated with PSA levels.

As stated by the author Egevad e colaboradores(2019), the histological grade of PC is one of the most important tissue parameters to predict the outcome and response to treatment. And, in this sense, the Gleason score continues to be the basis of the CP classification. Tagai et al. (2019) report that this classification is a key component of the diagnosis of PC, as it indicates the aggressiveness of the disease. In addition, GEYBELS e colaboradores(2016) state that this score is the best prognostic predictor of prostatic tumors.

In our study, more than 69% of the patients had a Gleason score of 7. We observed that the higher the Gleason score, the greater the ATP hydrolysis and the lower the ADP hydrolysis and ADA activity. As this score is associated with the aggressiveness of the disease, we can suggest that individuals with higher scores have a greater availability of extracellular ATP - from the processes of destruction of tumor cells in hypoxia, a greater availability of ADP - which favors the formation of thrombi veins, and an immunodeficiency caused by the increase in serum levels of Ado.

Corroborating our results, the work carried out by Battisti e colaboradores(2013), showed a decrease in ADA activity in platelets and serum in the group of patients with CP with a Gleason score greater than 7.

It is clear from our work that NTPDase and ADA activities are also influenced by clinical staging, especially in T2 and T3 classifications. In our work, we identified that there is a direct association between the increase in tumor size, the greater ATP hydrolysis and the decrease in ADA activity. In the same sense, Mandapathil e colaboradores(2018) identified that CD39 expression in head and neck squamous cell carcinoma positively correlated with tumor stage and seems to predict poor prognosis.

Additionally, corroborating our findings, Battisti e colaboradores (2013) state that, when patients were subdivided considering the presence or absence of metastasis, a reduction in ADA activity was observed only in patients with metastasis. These results are interesting and support the idea that Ado may be involved in tumor progression and metastasis. In our study, it should be noted that data relating to the involvement of regional lymph nodes and distant metastases are not included in the medical records and the patients had not undergone surgical removal of the tumor or initiated

pharmacological treatment at the time of participation in the study.

Regarding the ISUP scale, it was possible to observe that there seems to be an increase in ATP hydrolysis and a decrease in ADP hydrolysis the higher the scale value. We can suggest that higher values for the ISUP scale are associated with a worse prognosis, observing the actions of ATP, ADP and Ado in this protumorigenic signaling pathway.

In view of the above, the analysis of these results, together, is of great relevance for the medical and public health context, since preventive and palliative measures can be adopted in more efficient ways, so that patients diagnosed with PC can present a better quality of life and longer survival.

7 CONCLUSION

For the first time, it was possible to relate E-NTPDase and ADA activity to serum PSA levels, Gleason score, tumor staging, and ISUP values in patients with CP. Notably, increased serum PSA levels, Gleason score, tumor staging, and ISUP scale appear to be directly associated with increased E-NTPDase activity for ATP hydrolysis, possibly because all are correlated with size. , tumor progression and invasiveness. Thus, knowing that the carcinogenic process involves events related to cell injury and apoptosis, greater amounts of ATP are released into the extracellular environment, increasing the availability of this substrate for NTPDase and, consequently, its activity. On the other hand, the decrease in NTPDase activity for ADP hydrolysis favors the biological availability of this molecule, which has potent procoagulant activity, a fact that would facilitate the occurrence of thrombotic processes in patients with more advanced CP.

In the same sense, the lower activity of ADA in patients with more advanced CP promotes the accumulation of Ado in the body, which can compromise immune surveillance against tumor development, since the immunosuppressive action of this molecule is known, collaborating with the progression of CP.

In time, additional experiments on the expression of receptors and analyzes in tumor tissue are necessary to corroborate the results found in the present study, so that the modulation of purinergic signaling can form the basis of new drugs and efficient therapeutic protocols in controlling the development and progression of CP.

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APPENDIX I - TERMS OF FREE AND INFORMED CONSENT (TCLE)

TERMS OF FREE AND INFORMED CONSENT (TCLE)
Research Ethics Committee - CEP/UFFS

Dear participant,

You are being invited to participate in the research: **Biorepository of tumor biopsies for evaluation of gene and protein expression of receptors and enzymes of the purinergic system with emphasis on breast, prostate and colorectal tumors, developed** by students and professors of the Medicine course from the Federal University of Fronteira Sul (UFFS), Campus Chapecó – SC, under the coordination of Professor Dr. Sarah FVO Maciel.

1. Central Objective

Construction and maintenance of the Biorepository of tumor samples, in order to research molecular markers involved in the development of cancer, and with possibilities of acting in the treatment of breast cancer, prostate cancer, colorectal cancer and bladder cancer .

2. Inclusion criteria

Patients: both sexes, diagnosed by a specialist with breast cancer (invasive ductal carcinoma), prostate cancer (adenocarcinoma), colorectal cancer (adenocarcinoma) and bladder cancer, over 18 years of age, who so far have not undergone surgery to remove the tumor or any type of treatment (chemotherapy, radiotherapy, hormone therapy, immunotherapy, precision therapy). Patients with a previous diagnosis of cancer, or with chronic inflammatory diseases (diabetes, hypertension, Crohn's disease, ulcerative colitis, benign prostatic hyperplasia, mastitis) will be excluded. **Controls:** based on age (plus or minus 3 years) and gender matching of patients who do not have a past or current diagnosis of cancer of any type or chronic inflammatory disease. Your participation is not mandatory and you have full autonomy to decide whether or not to participate , in addition to being able to withdraw from collaborating in this study at any time, without the need for an explanation. You will not receive remuneration or any type of reward for this research.

3. Mechanisms to ensure secrecy and privacy

The confidentiality and privacy of the information provided by you will be guaranteed. The biological samples and clinicopathological data of the participants will be identified by sequential numbering, having no link with the patient's identification. At any time, you may request information from the researchers about your participation and/or about the research, which can be done through the contact means explained in this Term. In case of withdrawal, biological materials and clinicopathological information of participants will be discarded.

4. Participant identification throughout the work

Your name will not be mentioned during any stage of this research, as well as in any publications, courses, reports and the like. Only the name of the Institution will be mentioned. To maintain your anonymity, a sequential numerical coding will be used. Each participant will have a distinct number on all materials and data related to him.

5. Collection/procedure/experiment duration time

Your participation in the research consists of: 1. Patients - answering the **questionnaires about style and quality of life** ; use of **tumor and blood material (30 ml)** collected during the tumor removal surgery, materials that would normally be discarded after pathological analysis; availability of **information from medical records** (age, gender, histological tumor subtype, staging, etc.). The research will not generate any prejudice in the diagnosis and treatment of the disease in question; 2. Controls - respond to the **style and quality of life questionnaires** ; use of **blood material (30 ml)** collected during the interview with the researchers. Collections will be carried out by the responsible researchers and/or specialist physicians, in a suitable environment at the HRO itself. The duration of collections will be a maximum of 30 minutes.

6. Storage of data and materials collected in the research

All biological materials will be stored in a freezer, duly identified with sequential numbering, project name and responsible researcher. The other materials from the research will be stored in a locked cabinet, to which only the responsible researcher will have access. The tables with information on research participants will be stored on the computers of the researchers involved, with access only with a password. All materials will be kept for the duration of the research (5 years). Upon completion of the research, biological materials and clinicopathological data will be discarded.

7. Direct benefits (individual or collective) to research participants

Meeting groups with research participants and other patients from the Oncology sector of the HRO, voluntarily by both parties, in a reserved room at the HRO itself, where the researchers involved will provide guidance and clarification on the pathologies included in research and sharing experiences, in order to improve the quality of life of the participants. Later, these groups will also allow the sharing of the results obtained in the research, in an adequate way

for the participants to understand.

8. Prediction of risks or discomforts

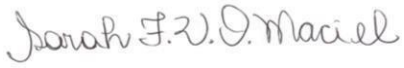
Participation in research may carry risks. A predictable risk is discomfort at the time of blood collection, as a result of the needle stick. To minimize it, the collection will be carried out by trained professionals, aiming at the safety of the participants. Researchers will explain the research content in detail and advise participants that their participation is not necessary if they do not feel comfortable doing so. If the anticipated risks occur, you will receive treatment and follow-up until these discomforts disappear. Other damages that may result from the research are psychological, since the pathologies in question can cause psychosocial changes. To minimize them, the objective and purpose of their contribution to the research will be explained to participants. However, if any psychological disorders are noticed in the participant resulting from the research, he will be referred to the psychological support service of the reference Family Health Center (CSF), with the Family Health Team (ESF).

9. Disclosure of research results

The feedback on the results obtained in the research will be carried out through scientific publications and participation in scientific events in the area, with lectures and with the use of *posters* and *banners* or online newsletters. The personal data of participants will not be disclosed at any time.

If you agree to participate, one copy of this term will remain in your possession and the other will be delivered to the researcher. We thank you in advance for your participation!

Chapecó – SC, _____from _____from ____.



Responsible Researcher Signature

Professional contact with the responsible researcher: Tel: 49-30254508/ e-mail: sarah.maciel@uffs.edu.br. In case of doubt regarding the ethical conduct of the study, contact the UFFS Research Ethics Committee: Tel and Fax - 49-2049-3745 / e-mail: cep.uffs@uffs.edu.br .

Mailing Address: UFFS Research Ethics Committee, Federal University of Fronteira Sul, Library Block, Room 310, 3rd floor, Rodovia SC 484 Km 02, Fronteira Sul, CEP 89815-899, Chapecó, Santa Catarina, Brazil.

I declare that I understand the objectives and conditions of my participation in the research and I agree to participate.

Participant's full name and contact:

Signature:

APPENDIX II - CLINICAL PATHOLOGICAL INFORMATION

CLINICAL PATHOLOGICAL INFORMATION

Information was obtained from research participants based on their reports and consultation of medical records.

1) Age

2) Marital status

3) Smoking

4) Diagnosis of Covid-19

5) History of comorbidities (systemic arterial hypertension, diabetes mellitus, among others)

6) History of prostate cancer in individuals with 1st degree kinship

7) Pre-treatment PSA value

8) Histological grading (Gleason)

9) Histology

10) TNM (classification of malignant tumors)

11) Clinical staging

12) ISUP score