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Artigo

Quantitative analysis of neutrophils, mast cells and eosinophils in human prostatic adenocarcinoma*

Análise quantitativa de neutrófilos, mastócitos e eosinófilos no adenocarcinoma prostático humano

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ABSTRACT: Objective: To evaluate the relationship between benign prostatic hyperplasia (BPH) and prostatic adenocarcinoma (AP) through the quantification of neutrophils, eosinophils and mast cells and to correlate with the histopathological grade of the neoplasm. **Methodology:** This is an observational cross-sectional study. Cataloged patient biopsies were sectioned into 5µm sections, stained and analyzed under a light microscope. In these sections, cells were quantified and compared between HPB and AP conditions by statistical analysis. **Results:** Biopsies of 47 patients were analyzed, 28 (59.6%) with BPH and 19 (40.4%) with AP. The median age of the BPH group was 69 years (range: 54 – 77 years) and the AP group was 66 years (range: 59 – 92 years). In the statistical analysis, a greater number of extravascular ($p < 0.001$) and total ($p < 0.05$) neutrophils was observed in the AP in relation to the BPH; however, there was no statistical difference between intravascular neutrophils, mast cells and eosinophils between the groups. By correlating the Gleason Score and the influx of inflammatory cells, it was observed that higher scores are associated with a lower influx of neutrophils and intact mast cells. In addition, it was observed that prostatic volume and weight with AP may be associated with inflammatory infiltrate. **Conclusion:** In this study, it was possible to suggest that prostate cancer is related to the innate immune response by the exacerbated influx of neutrophils in the tumor microenvironment and by the influence of these cells on the Gleason Score and on the values of weight and prostate volume. However, further studies are needed to better illustrate the role of neutrophils in tumorigenesis.

KEYWORDS: Prostate Cancer; Inflammation; Neutrophils; Mast Cells; Eosinophils.

RESUMO: Objetivo: Avaliar a relação entre a hiperplasia prostática benigna (HPB) e o adenocarcinoma prostático (AP) por meio da quantificação de neutrófilos, eosinófilos e mastócitos e correlacionar com o grau histopatológico da neoplasia. **Metodologia:** Este estudo é observacional transversal. Biópsias catalogadas de pacientes foram seccionadas em cortes de 5µm, coradas e analisadas no microscópio de luz. Nessas secções, as células foram quantificadas e comparadas entre as condições HPB e AP por meio de análises estatísticas. **Resultados:** Foram analisadas biópsias de 47 pacientes, sendo 28 (59,6%) com HPB e 19 (40,4%) com AP. A mediana da idade do grupo com HPB foi 69 anos (intervalo: 54 – 77 anos) e o com AP foi 66 anos (intervalo: 59 – 92 anos). Na análise estatística, foi observado um maior número de neutrófilos extravasculares ($p < 0{,}001$) e totais ($p < 0{,}05$) no AP em relação à HPB, porém não houve diferença estatística entre neutrófilos intravasculares, mastócitos e eosinófilos entre os grupos. Ao correlacionar o *Escore de Gleason* e o influxo de células inflamatórias, foi observado que maiores escores estão associados a menor influxo de neutrófilos e mastócitos intactos. Além disso, foi observado que o volume e o peso prostático com AP podem estar associados com o infiltrado inflamatório. **Conclusão:** Nesse estudo, foi possível sugerir que o câncer de próstata possui relação com a resposta imune inata pelo influxo exacerbado de neutrófilos no microambiente tumoral e pela influência dessas células no *Escore de Gleason* e nos valores de peso e volume prostático. Entretanto, novos estudos são necessários para melhor ilustrar o papel dos neutrófilos na tumorigênese.

PALAVRAS-CHAVE: Câncer de próstata; Inflamação; Neutrófilos; Mastócitos; Eosinófilos.

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INTRODUCTION

Prostate cancer is the second mostly diagnosed cancer among men and the second deadliest cancer in men worldwide, with an estimated amount of 1.4 million cases a year and mortality rate of 375,000 deaths a year[1]. In Brazil, prostate cancer is the mostly diagnosed malignant neoplasm and the second deadliest one among men[2]. It is estimated that the number of new cases of prostate cancer is going to be higher than 71,000 cases for each year in the 2023-2025 triennium[2]. The increase in life expectancy, the improvement and evolution of diagnostic methods and the quality of information systems in the country may explain the increase in the incidence rates. Age, ethnicity, and positive family history are well-stablished risk factors for the development of prostate cancer in the literature. The prostate cancer is rarely diagnosed before the 40 years old, and its incidence is high after 55 years old, with the United States presenting an average age of 66 years old[4].

As a man ages, the prostate undergoes hyperplasia, increasing its size, for this reason, it is common for men from the age of 50 to experience symptoms in the lower urinary tract[5]. A benign growth, called benign prostatic hyperplasia (BPH), and a malignant growth, called prostate cancer, may happen simultaneously in the prostate[6]. The BPH is a disease whose physiopathology is related to a chronic inflammatory process which starts between the ages of 25 and 40 with symptoms starting after 50 years old[6]. The most common histopathological type of prostate cancer is the adenocarcinoma, the neuroendocrine and squamous presentations among others are rare[7]. The cancer is diagnosed through a histopathological study of a prostatic fragment obtained through a biopsy. The histopathological report must provide the histological grading according to the Gleason score, whose objective is to inform the possible growth rate of the tumor and its tendency to spread according to the prostatic adenocarcinoma histological patterns[8]. In 2014, the International Society of Urological Pathology (ISUP) suggested the grading in groups from 1 to 5, according to the Gleason score, in which group 1 consists of Gleason 6, and successively group 2 (Gleason 3+4), group 3 (Gleason 4+3), group 4 (Gleason 8) and group 5 (Gleason 9 e 10)[8,9]. This grading system helps the therapeutic choice because it indicates the prognosis of each group and it improves the communication among clinicians, pathologists and patients[8,9].

The prostate consists of several alveoli lined by a columnar pseudostratified epithelium formed by basal cells and cylindrical cells which secrete a whitish serous fluid containing acid phosphatase, citric acid, zinc, prostate-specific antigen (PSA) and other proteases and fibrinolytic enzymes involved in liquefaction of semen. The alveoli are embedded in a highly vascularized stromal connective tissue in which immune system cells such as neutrophils; mast cells; eosinophils; natural killer cells; and B and T lymphocytes are present[10].

The immune system can be divided into innate immunity and adaptive immunity. The first one is comprised of macrophages; neutrophils; dendritic cells; natural killer cells; mast cells; basophils, and eosinophils; while B lymphocytes, T lymphocytes and antigen presenting cells (APC) are effector cells of the adaptive response[11]. Recent studies have shown that cancer development and progression, including prostate cancer, depend on interactions between tumor cells and immune system cells, in which the latter are the main regulators of neoplastic growth[12,13]. Among the immune cells associated with the tumor, macrophages, neutrophils, lymphocytes, and mast cells stand out[13].

The idea that inflammation is involved in the process of tumorigenesis is supported by the observation that the neoplasia often arises in areas of chronic inflammation, including prostate cancer[14]. At the beginning of the neoplastic process, inflammatory cells and their released mediators have regulatory capacity over neoplastic cells[15,16]. Immune system cells are attracted to the tumor for several factors, including hypoxia, cellular damage, tissue ischemia and tumor-derived chemoattractant chemoattractants[15]. Inflammation plays a very significant role in the development of several types of cancer, promoting carcinogenesis and cancer progression through a variety of mechanisms, including tissue remodeling; tumor promotion; changes in the extracellular matrix; loss of tissue architecture; DNA damage due to oxidative stress, and direct stimulation of tumor cells via cytokines[17].

Thus, this investigation aims to analyze the role of inflammation and specific immune cells in the mediation of prostate cancer progression to aid in targeting and optimizing immunotherapy goals.

METHODOLOGY

Location of the study

The study was carried out in two teaching and research institutions. At Centro Universitário Padre Albino (UNIFIPA), the following steps were carried out: search of pathology files with their respective paraffin blocks, microtomy of the paraffin-embedded blocks and data collection from the anatomopathological reports of the patients included in the study. At Faculdade Ceres (FACERES), staining, quantification, photomicrography, and statistical analyses were carried out.

Study design

This is an observational, cross-sectional, qualitative, and quantitative study.

Sample

The sample consisted of paraffin-embedded biopsies from 50 patients which had tumor tissue and benign prostatic hyperplasia obtained at the Pathology Service of Centro Universitário Padre Albino between 2011 and 2014. Throughout the study, 3 patients were excluded since the sample did not have prostate tissue. In the experiments, prostate fragments obtained in regions of benign prostatic hyperplasia in patients without neoplasia were used as control. Biopsies were obtained from patients with prostate cancer who had not undergone chemotherapy and/or radiation in surgeries or outpatient follow-up which happened at Padre Albino Hospital and Emílio Carlos Teaching Hospital, Padre Albino Foundation, SP. This project was submitted to the Ethics Committee for Research involving Human Beings (CEP/CONEP) (CAEE: 54898316.2.1001.5430).

Method

The paraffin-embedded blocks with fragments from fifty patients were catalogued in the pathological block bank of the Pathology Service and separated for microtomy, through which 10 sections of approximately 5 μm were obtained per patient using a microtome (DM 50, Leica, Germany). For the histopathological and morphological analyses of neutrophils and eosinophils under the light microscope, the sections were stained with Hematoxylin-Eosin (HE) and with the dye Toluidine Blue (AZT) for the mast cells analysis. Five sections were stained in HE and five sections in AZT for each patient. The number of neutrophils, mast cells and eosinophils were quantified under a light microscope (Axioskop Motplus II, Zeiss, GR). Inflammatory cells were quantified in 20 fields per slide using a 40X objective and the total number was divided by the total area of the fields in μm^2. After quantification, patient data were separated into two groups (BPH and PA), tabulated in an Excel spreadsheet and other variables were collected, such as age, Gleason score, Gleason score group grade (ISUP), weight and prostate volume using data from the anatomopathological reports.

Statistical analyses

Values were shown as mean ± S.E.M. of the number of cells per μm^2 of five 5μm sections (leaving a gap of 40μm between each section) per patient (n=47). The statistical difference between the groups was determined by the Mann-Whitney test and correlations by the Spearman rank correlation coefficient. P-values less than 0.05 were considered significant. Missing data were removed from the analysis by the statistical program. The statistical analysis was performed by using IBM SPSS Statistics 20 software and the graphs by using GraphPad Prism Software 9.0.

RESULTS

Altogether 47 patients were included in the analysis, 28 patients presented with benign prostatic hyperplasia (59.6%) and 19 patients presented with prostatic adenocarcinoma (40.4%). The median age was 67 years (54 – 92 years) considering the whole sample. Most of the patients were white (95%), and the remaining patients were black (5%). Patients who presented with benign prostatic hyperplasia (BPH) had a median age of 69 years (54 – 77 years) and were all white. The median age of the patients with prostatic adenocarcinoma (PA) was 66 years (59 – 92 years), 77.7% of them were white and 22.3% were black.

Regarding the method of sample collection from patients with BPH, 56.5% were obtained via transurethral resection of the prostate, 39.1% via prostatectomy, and 4.3% via prostate needle biopsy. In patients with PA, 50% of the samples were collected via prostatectomy, 28.6% via prostate needle biopsy and 21.4% via transurethral resection of the prostate.

In bivariate analysis, a greater influx of extravascular ($p < 0.001$) and total ($p < 0.05$) neutrophils was observed in prostatic adenocarcinoma compared to benign prostatic hyperplasia (Figure 1). On the other hand, there was no significant difference between the groups regarding the number of intravascular neutrophils (Figure 1); intravascular, extravascular, and total eosinophils (Figure 2), and intact, degranulated and total mast cells (Figure 3).

Patients who presented with prostatic adenocarcinoma had a Gleason Score of 6 (11%) - well differentiated or low grade; 7 (27.8%) - moderately differentiated or intermediate; 8 (50%) and 9 (11.1%) - poorly differentiated or highly differentiated. The prostate weight obtained through transvesical prostatectomy had a median of 56g (range: 40 - 80g) in patients who presented with BPH and 25g (range: 10 - 40g) in patients with PA undergoing radical prostatectomies. When comparing the Gleason Score with the prostate weight of patients with PA, a strong positive correlation was found (Table 1).

When correlating the Gleason Score with the influx of inflammatory cells, we obtained a weak negative correlation with intravascular, extravascular, and total neutrophils, while the influx of intact mast cells showed a weak positive correlation (Table 1), i.e., the higher the Score, the lower the influx of neutrophils and a greater number of intact mast cells. However, degranulated and total mast cells and intravascular, extravascular, and total eosinophils did not correlate with the Gleason Score (Table 1).

Regarding the Gleason Grade Group, the distribution of the patients was: group 1 (10.5%), group 2 (10.5%), group 3 (15.8%), group 4 (47.4%) and group 5 (10.5%). Only prostate weight had a strong positive correlation with Gleason Grade Group (Table 1).

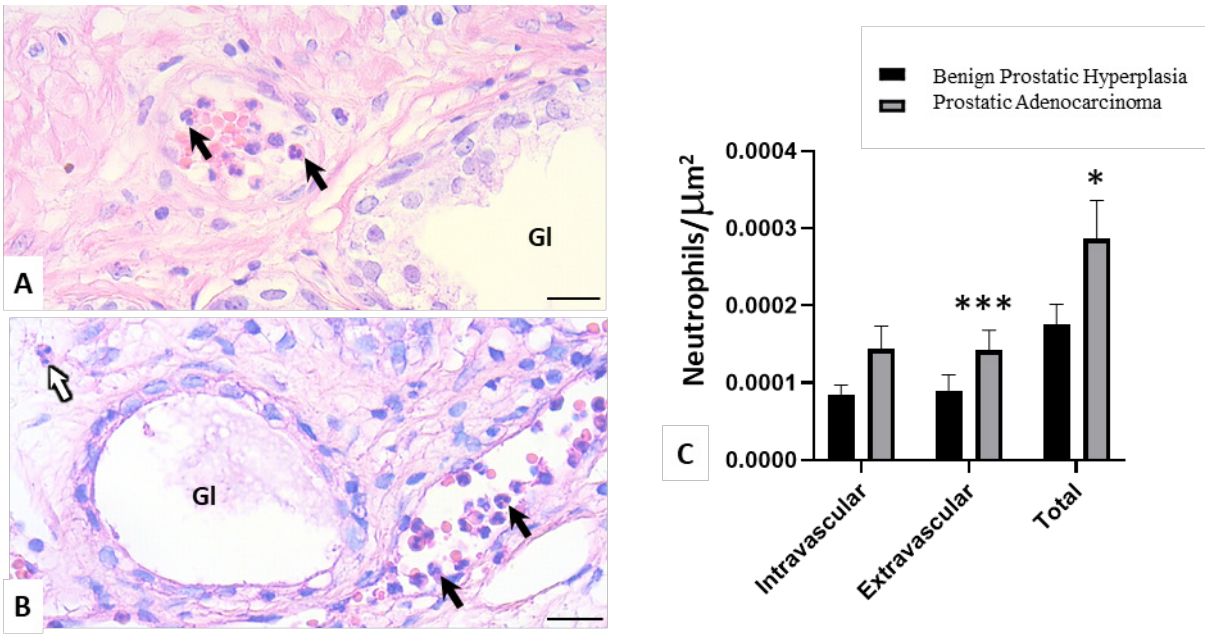

Figure 1 - Neutrophil infiltration in prostatic hyperplasia and adenocarcinoma. A) Photomicrograph of benign prostatic hyperplasia showing intravascular neutrophils (black arrows). Gl: gland. **B)** Section of prostatic adenocarcinoma indicating intravascular (black arrows) and extravascular (white arrow) neutrophils. Gl: gland. Staining: Hematoxylin-eosin. Bars: 10µm. Neutrophils were identified by the presence of a multi-lobed nucleus. **C)** Quantification of intravascular, extravascular, and total neutrophils. The values express the mean ±S.E.M. of cells per µm^2 (n=28 patients presenting with benign prostatic hyperplasia and 19 patients with prostatic adenocarcinoma). * p<0.05 versus benign prostatic hyperplasia (Mann-Witney U test). *** p<0.001 versus benign prostatic hyperplasia (Mann-Witney U test).

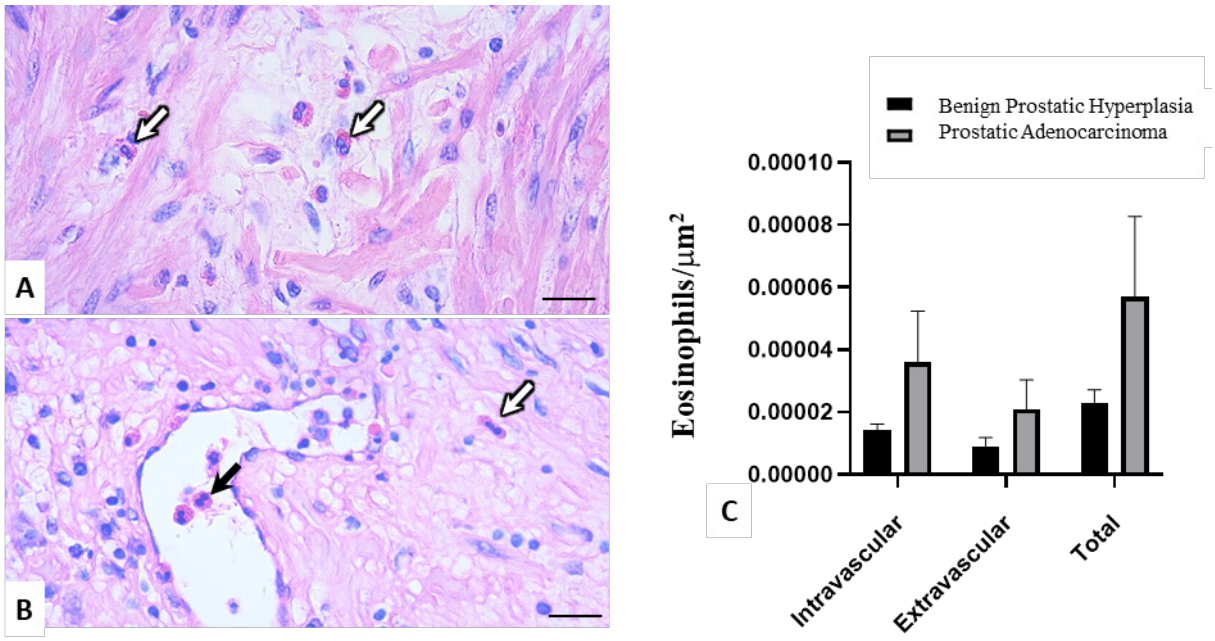

Figure 2 - Influx of eosinophils in prostatic hyperplasia and adenocarcinoma. A) Benign prostatic hyperplasia showing extravascular eosinophils (white arrows). **B)** Intravascular (black arrow) and extravascular (white arrow) eosinophils are observed in prostatic adenocarcinoma. Staining: Hematoxylin-eosin. Bars: 10µm. The eosinophils were identified by the presence of acidophilic cytoplasmic granules and a bilobed nucleus. **C)** Quantification of intravascular, extravascular and total eosinophils. The values express the mean ± S.E.M. of cells per µm^2 (n=28 patients presenting with benign prostatic hyperplasia and 19 patients with prostatic adenocarcinoma).

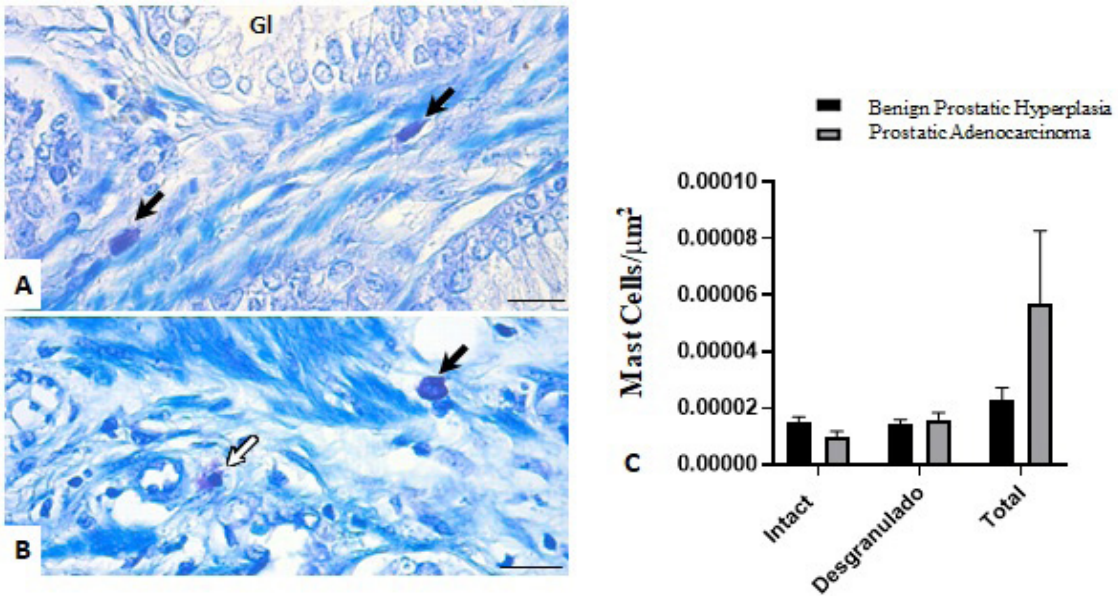

Figure 3 - Mast cell migration in hyperplasia and prostatic adenocarcinoma. A) Intact mast cells (black arrows) are visualized in benign prostatic hyperplasia. Gl: gland. B) Photomicrograph of prostatic adenocarcinoma showing intact (black arrow) and degranulated (white arrow) mast cells. Intact mast cells were identified by the presence of metachromatic cytoplasmic granules, while the degranulated ones were identified by the presence of granules with a purple color which underwent exocytosis. Color: Toluidine Blue. Bars: 10µm. C) Quantification of intact, degranulated and total mast cells. The values express the mean ± S.E.M. of cells per µm² (n=28 patients presenting with benign prostatic hyperplasia and 19 patients presenting with prostatic adenocarcinoma).

Table 1 - Relationship between Gleason Score and Gleason Grade Group (ISUP) with prostate weight, prostate volume, and inflammatory cells

	Gleason Score			***Gleason Grade Group***		
	r[1]	n	*p*	r[1]	n	*p*
Prostate Weight	0.891	7	< 0.0001*	0.891	7	< 0.0001*
Prostate Volume	0.188	8	0.38	0.188	8	0.38
Neutrophils						
Extravascular	-0.275	18	0.043*	-0.232	18	0.064
Intravascular	-0.292	18	0.032*	-0.232	18	0.092
Total	-0.334	18	0.014*	-0.254	18	0.064
Mast cells						
Degranulated	0.064	18	0.644	0.034	18	0.808
Intact	-0.309	18	0.023*	0.262	18	0.055
Total	0.176	18	0.203	0.139	18	0.315
Eosinophils						
Extravascular	-0.092	17	0.522	-0.117	17	0.412
Intravascular	-0.128	17	0.369	-0.117	17	0.412
Total	-0.214	17	0.131	-0.205	17	0.149

* Significant Values
[1] Spearman Correlation

When comparing inflammatory cells with the prostate weight of patients with PA, a moderate negative correlation was observed with the number of intravascular neutrophils, a strong positive correlation with intact

mast cells and a moderate positive correlation with intravascular eosinophils. Neutrophils (extravascular and total), mast cells (degranulated and total) and eosinophils (extravascular and total) did not correlate with prostate weight in patients with PA (Table 2).

Regarding prostate volume, patients with BPH had a median of 120.17 cm^3 (range: 63 – 400 cm^3) and patients with PA had a median of 86.62 cm^3 (range: 42 – 127.5 cm^3). When comparing inflammatory cells with the prostate volume of patients with PA, a strong negative correlation was found between intravascular, extravascular, and total neutrophils, a moderate negative correlation with degranulated mast cells and, finally, a moderate positive correlation with intravascular eosinophils. There was no association with the prostate volume of patients with PA and mast cells (intact and total) and eosinophils (extravascular and total) (Table 2).

Table 2 - Relationship between Weight, Prostate Volume, and Inflammatory Cells

	Prostate Volume			**Prostate Weight**		
	r[1]	n	*p*	r[1]	n	*p*
Prostate weight	-	-	-	0.4	7	0.072
Prostate Volume	0.4	7	0.072	-	-	-
Neutrophil						
Extravascular	-0.356	7	0.114	-0.795	8	< 0.0001*
Intravascular	-0.674	7	0.001*	-0.747	8	< 0.0001*
Total	-0.356	7	0.114	-0.88	8	< 0.0001*
Mast cell						
Desgranulado	0,038	7	0,871	-0,642	8	0,001*
Intacto	0,735	7	< 0,0001*	0,124	8	0,563
Total	0,359	7	0,11	-0,339	8	0,105
Eosinophil						
Extravascular	0,061	6	0,811	0,17	7	0,461
Intravascular	0,5	6	0,034*	0,453	7	0,039*
Total	0,219	6	0,383	0,337	7	0,136

* Significant Values

[1] Spearman Correlation

DISCUSSION

In this study, a quantitative analysis of inflammatory cells, neutrophils, mast cells and eosinophils were carried out in biopsies from patients with benign prostatic hyperplasia (BPH) and prostatic adenocarcinoma (PA).

Inflammatory cells in the prostate cancer microenvironment can interfere with the neoplasia initiation and progression through the secretion of cytokines and growth factors, and their role can change depending on the tumor stage[18].

In the present study, a greater influx of neutrophils was found in PA compared to BPH, corroborating data from the literature[19,20]. The neutrophil may have antitumor proprieties, which is exerted by the N1 phenotype, through the secretion of reactive oxygen species (ROS) and neutrophil elastase (NE) inducing cell death, inhibition of angiogenesis through the secretion of vascular endothelial growth factor A (VEGF-A) , release of the TNF-related apoptosis-inducing ligand (TRAIL) that induces apoptosis via caspase and stimulation of cytotoxic T lymphocytes[21,22]. However, the N2 phenotype, which is stimulated via transforming growth factor beta (TGF-β), has a pro-tumor action through the secretion of metalloproteinase-9 (MMP-9), inducing angiogenesis and remodeling of the extracellular matrix, predisposing to metastasis[22,23]. Other molecules are implicated in angiogenesis, such as VEGF-A and basic fibroblast growth factor (bFGF), which are directly linked to tumor progression[24]. In addition, the neutrophil can stimulate genetic instability producing mutations via ROS production and tumor growth through the release of growth factors and NE[25].

The acute inflammation, which is characterized by neutrophils, was associated with the lowest Gleason Score in the literature, as this study demonstrated an inverse relationship between them and, therefore, illustrating

the antitumor effect of neutrophils[26,27]. Recent studies have shown the neutrophil-lymphocyte ratio (NLR) as a biomarker in the prognosis of patients presenting with prostate cancer, an indicator of systemic inflammation[28]. A high NLR was associated with worse survival in patients who presented with castration-resistant prostate cancer with or without metastasis, treated with enzalutamide and abiratenone acetate, which is considered a marker of poor prognosis[29,30]. In addition, high Gleason Score was also associated with high values of NLR[31]. However, the role of neutrophils in the initiation and progression of prostate cancer remains unclear.

Mast cells are present mainly in tissues which have contact with the external environment, such as the respiratory tract, gastrointestinal tract, skin, and genitourinary tract, including the prostate[32]. These cells can be activated by the complement system (C3a, C5a), cytokines (IL-1, IL-12, TNF), chemokines, adenosine, growth factors (SCF), PAMPs (Pathogen-Associated Molecular Patterns), Fc receptors (IgE and IgG), neuropeptides and hormones[33,34]. After activation, mast cells release cytoplasmic granules containing histamine, serotonin, lysosomal enzymes, proteases (MMP-9, MMP-2, tryptase, chymase), cytokines (TNF, IL-15, IL-4), growth factors (VEGF, TGF-β, bFGF-2), heparin, among others[33,34]. The histamine is one of the main substances secreted by mast cells, VEGF and TNF-α are involved in angiogenesis, while proteases act on degradation in the extracellular matrix, affecting tumor progression[35,36]. Moreover, through the secretion of IL-10, histamine, adenosine and TNF-α, mast cells can induce immunosuppression in the tumor microenvironment[36]. In contrast, mast cells can present an antitumor response by activating the effector T lymphocytes by the inhibition of the regulatory T lymphocytes and they can also activate natural killer (NK) cells and dendritic cells[37].

Although our findings do not present significant differences in the influx of mast cells, other studies have implicated their influence on prostate cancer[19,38-40]. In localized prostate cancer, a reduced number of intratumoral mast cells was associated with an increased risk of tumor recurrence and low Gleason scores show higher mast cell densities[17]. However, a higher risk of recurrence and distant metastasis (liver and lung) after radical prostatectomy was associated with a higher number of extratumoral mast cells[41]. Therefore, previous work suggests that the location of mast cells and their degree of activation influence the prognosis of patients with prostate cancer.

Typically, eosinophils are involved in the immune response against parasites and in allergic reactions[42]. As in the literature, our study found no association between eosinophil quantification and prostate cancer[40,43]. An *in vitro* study showed that eosinophils inhibited the growth of prostate cancer cells[44]. Furthermore, another study related the increase in the expression of E-cadherin in human prostate cancer cell lines DU145 and PC3 by activated eosinophils, and thus playing an important role in the mechanism of metastasis[45]. In addition to prostate cancer, eosinophils have been implicated in other types of cancer, such as melanoma, colorectal, lymphoma, gastric, among others[46].

In our study, samples from patients with greater prostate weight had a higher Gleason Score, but published articles associate high-grade tumors with lower prostate weight[47,48]. When analyzing the prostate weight, a greater number of intact mast cells and intravascular eosinophils and a lower number of intravascular neutrophils were observed in PA with higher weight. On the other hand, when analyzing prostate volume, a greater number of neutrophils and degranulated mast cells were found in PA with smaller volumes and a greater number of intravascular eosinophils in PA with larger volume. There are controversies between studies that involve the variables of prostate weight and volume which imply clinical outcomes and, until the moment, it has not been possible to find data in the literature regarding the relationship between inflammatory cells and the variables weight and volume in the context of neoplasia.

This study presented the following limitations: as it was a retrospective study, it had a limited sample and the bias of only one institution for the data collection; in addition, it was not possible to collect clinical data from patients, such as clinical staging.

CONCLUSION

In this study, it was possible to suggest that the prostate cancer is related to the innate immune response due to the exacerbated influx of neutrophils in the tumor microenvironment and the influence of these cells on the Gleason Score and on prostate weight and volume values. However, information regarding the precise profiles of inflammatory cells in prostate cancer is still really limited. Inflammatory cells and the immune response present anti- or pro-tumorigenic activity, depending on cellular phenotypes, their combinations and location in the tumor microenvironment. Therefore, the identity, functional status, the distribution, and the interactions of inflammatory cells in the prostate must be fully characterized to improve and combine the current promising immunotherapies.

The effect of inflammation on cancer has been widely studied, including prostate cancer, but there are still conflicting data on the role of each inflammatory cell in this process, mainly because studies have different methodologies in which each tumor microenvironment has unique characteristics. Furthermore, efforts should be directed toward elucidating better indicators of potential response to facilitate the identification of optimal target populations as well as biomarkers to assess therapeutic efficacy. Progress in these areas reinforces existing optimism for immunotherapy to be part of a care regimen for future prostate cancer patients. Therefore,

it is necessary to carry out new, more in-depth studies, characterizing the phenotypes of innate response cells with the quantification of cytokines to better illustrate their function in tumorigenesis.

Contributions: Leonardo Cortez Guerra, Bruna Orbite Garcia, Thaís Santana Gastardelo Bizotto: Substantial contribution to the study outline, data interpretation and writing of the preliminary version. Ana Paula Girol: Participation in the review and approval of the final version. Thaís Santana Gastardelo Bizotto: Participation in the review and approval of the final version, compliance with being responsible for the accuracy and integrity of any part of the study.

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