Characterization of collagens fibers (I, III, IV) and elastin in the extracellular matrix of normal and neoplastic canine prostate

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Abstract: Collagen (Coll) is the most common protein in the extracellular matrix, responsible for providing tissue structure and support. In some types of cancer, including prostate cancer (PC) abundant collagen was identified and related to tumor progression and metastasis. This study aimed to investigate Coll-I, III, IV and elastin in normal canine prostatic tissue and PC, using the Picrosirius red (PSR) and Immunohistochemical (IHC) analysis. Eight normal prostates and 10 PC from formalin-fixed, paraffin-embedded samples were used. Collagen fibers area was analyzed with ImageJ software. The distribution of Coll-I and Col-III was approximately 80% around prostatic ducts and acini, 15% among smooth muscle and 5% around in blood vessels, in both normal prostate and PC. Immunostaining for type IV collagen was observed in the basal membrane of prostate acini, smooth muscle, blood vessels, and never fibers of normal and PC samples. Elastic fibers were found in the septa dividing the lobules and around the prostatic acini of normal samples. A high amount of elastic fibers was observed around the ducts and the urethra in normal and canine PC. The distribution and area percentage of staining for collagen are similar in normal and neoplastic canine prostate when analyzed with PSR and IHC.

Keywords: dog; prostatic tissue; extracellular matrix, picrosirius, immunohistochemistry.

1. Introduction

Cancer is of second leading cause of mortality worldwide. In men, prostate cancer (PC) is the third most common malignant neoplasia (after non-melanoma skin cancer and lung cancer) [1]. In advance stage, human PC often shows metastasis to bones and resistance to anti-androgen treatment [2]. Similar to the men, dogs spontaneously develops PC [3, 4]. In dogs, PC is very aggressive and highly metastatic [5]. Usually, bone metastasis is diagnosed at a late stage of highly aggressive tumor subtypes [3]. Due to similarities in the clinical and pathologic aspects of PC in both species, some authors suggest that the dog may be considered a good model for the study of human PC [4, 5, 6, 7].

Recently, has been demonstrated in the human PC that an interaction between the tumor cells and the proteins of extracellular matrix (ECM) plays an important role in PC development and progression [8, 9]. The ECM is a complex network of macromolecules [10]. The major constituents of ECMs are proteoglycans and fibrous proteins (collagen, elastin, fibronectin and laminin) [11]. Collagen is the most common protein of the ECM [10]. The main collagen function is to provide structure, support and tensile strength, as well as, regulation of cell adhesion, chemotaxis, migration and direct tissue development [12]. In some human cancers such as, breast, colon and prostate, occurs a formation of abundant collagenous stroma (reactive stroma) in their tumor microenvironment (TME), responsible for the tumor progression and metastasis [8, 13, 14]. High density of type I collagen and degradation of type IV collagen are frequently observed in solid cancer, associated with metastasis [9, 15-18]. In the veterinary medicine, few studies were conducted to understand the relationship between cancer and the collagens fibers, compared to human medicine [19].

Picrosirius red (PSR) is a staining method useful to visualize collagen fibers in different connective tissues [20, 21]. The combination of PSR and polarized light microscopy allows distinguish and analyze the type I and III collagens fibers, in according with their birefringence color [20]. The objective of this study was to characterize and compare the collagen fibers and elastin in the normal prostate and canine PC, using PSR and immunohistochemical test.

2. Materials and Methods

2.1 The Subjects

Eight canine normal prostates and 10 PC were retrieved from the archives of the Veterinary Pathology Service, FMVZ, UNESP, Botucatu, SP, Brazil (Supplementary Table 1). The prostates were collected from necropsies from animals that had an interval between death and necropsy less than 6 hours. Formalin-fixed paraffin-embedded (FFPE) samples from canine prostatic tissue were sectioned for histological diagnosis, which was performed by three pathologists (LGRC, CEFA, PEK), at the same time, in a multi-head microscopy. The histopathological classification was performed according to the human WHO from Tumors of the Urinary System and Male Genital Organs [22], which was recently adapted to canine PC [4]. (Supplementary Table 2).

2.2 Picrosirius (PSR)

The slides were deparaffinized in xylene and rehydrated in alcohol. After, PSR staining was performed using a commercial kit (HistokitTM, Easypath, SP, Brazil), according to manufacturer's instructions. The slides were examined in an optical microscopy with polarized light (Axio Imager A1, Zeiss®, Germany). The collagen fibers that presented red-orange birefringence were considered type I, while the collagen fibers with green birefringence were interpreted as type III [23].

2.3 Immunohistochemistry (IHC)

The slides were subject to immunohistochemical test using the peroxidase method . The antibodies, antigen retrieval, dilutions and incubation period are described in the Table 1. Endogenous peroxidase activity was inhibited with 4% hydrogen peroxide in methanol for 10 min at room temperature (RT). Then, the slides were treated with protein block serum-free for 15 min RT (Dako, Carpinteria, CA, USA). In each step of the immunohistochemical process, the slides were washed with Tris-buffered saline (pH 7.4). A LSAB system was used as secondary antibody; applied for 1 hour at RT, according to manufacturer's instructions (Dako, Carpinteria, CA, USA). Peroxidase activity was reveled with 3',3'-Diaminobenzidinechromogen (DAB, Substrate System, CA, Dako). For the counterstained, Harris's hematoxylin was used. As negative control, primary antibodies were replaced by Tris-buffered saline. Canine skin was used as positive control tissue from collagen and elastic fibers, according to the Human Protein Atlas (https://www.proteinatlas.org/).

Table 1. Primary antibodies, retrieval antigen, dilution and incubation period used in the IHC test.

Primary antibody	antigen Retrieval	Dilution	Incubation Period
Collagen I, rabbit, Novotec	Citrate buffer pH 6,0, microwave, twice for 5 min	1:100 0	Overnight at 4°C
Collagen III, rabbit, Novotec	Pepsin 2%, pH 1,4 in oven for 10 min at 60°C after for 30 min at 37°C.	1:100 0	Overnight at 4°C
Collagen IV, rabbit, Biorbyt	Pepsin 2%, pH 1,4, in oven for 10 min at 60°C after for 30 min at 37°C.	1:100 0	Overnight at 4°C
Elastin (BA-4), mouse, Santa Cruz.	Citrate buffer pH 6,0, pressure cooker (Pascal®, Dako, Carpinteria, CA, USA)	1:100	Overnight at 4°C

2.4 Interpretation of PSR and IHC staining

Five fields (20x magnification) were selected from each HE slide according to the percentage of neoplastic cells. The same areas in each slide, were captured with a digital camera (Axioncam MRc, Zeiss® Vision, Germany) for each antibody and PSR. The stained areas were analyzed with Image J 1.49v software (National Institutes of Health, USA) and were assessed by setting a threshold, using the Image J threshold tool in according to the procedure described by Bauman et al., [24]. The staining distribution and intensity of the collagens and elastic fibers were evaluated In both normal canine prostate and PC.

2.5 Data analysis

Descriptive statistics was used to define the median and percentile of Coll-I, Coll-III and elastin in normal and canine PC. After establishing the median in the normal group, the PC samples were considered as under or over expressed compared to normal median. Mann-Whitney U test was applied to compare the area percentage among normal and canine PC. Statistical significance was set at $p < 0.05$. All statistical analysis was done using GraphPad Prism 6 (GraphPad Software Inc. La Jolla, CA).

3. Results

In the normal prostates and PC stained with PSR, the distribution of Coll-I and Col-III was approximately 80% around prostatic ducts and acini, 15% among smooth muscle and 5% around blood vessels (Figure 1). A similar proportion of collagen distribution was observed for IHC test in both groups.

No statistic differences in the percentage area of PSR and IHC staining for type I and type III collagens fibers were observed between canine PC and normal samples ($p > 0.05$). Table 2 shows the median, 25% percentile and 75% percentile values of Coll-I and Coll-III according to the diagnosis group and test applied. No statistic differences in the area percentage of immunohistochemical for Coll-IV and elastin was observed in normal prostates and canine PC ($p > 0.05$, Table 2 and 3).
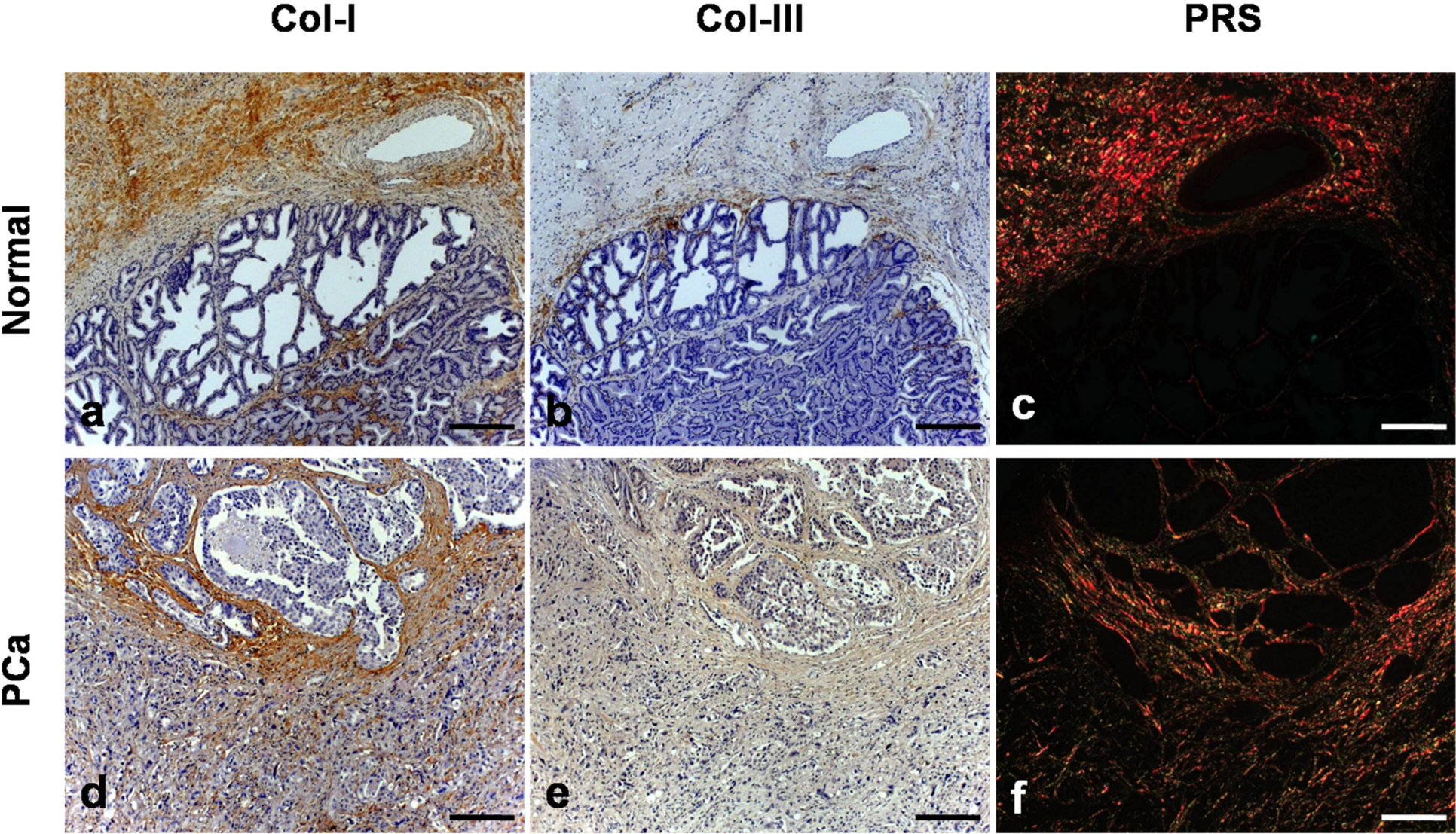
Figure 1. The immunohistochemistry and PSR stain in normal tissue and canine PC. a: immunostaining of Coll-I in the stroma of normal prostate (case No. 3). b: immunostaining of Coll-III in the stroma of the normal prostatic tissue (case No. 3). c: PSR staining observed in an optical microscopy with polarized light, the collagens fibers present red-orange birefringence (Coll-I) and green birefringence (Coll-III) in a smaller amount(case No. 3). d: immunostaining of Coll-I in the stroma of prostatic neoplastic tissue (case No. 11). e: immunostaining of Coll-III in the stroma of canine PC (case No. 11). f: PSR staining in the canine PC with similar amounts of Coll-I and Coll-III (case No. 11).

Table 2. Median, 25% percentile and 75% percentile values of area percentage staining for Coll-I and Coll-I in the normal and canine PC, according to the method used.

		PSR test				IHC test			
		25%	Median	75%	p	25%	Median	75%	p
	N	1.25	1.89	2.27		2.85	4.73	8.03	
Coll-I	PC	2.09	2.24	2.43	0.099	3.31	6.18	8.56	0.447
	N	1.33	1.64	2.06		1.81	3.22	5.03	
Coll-III	PC	1.68	2.25	3.11	0.120	3.72	5.07	6.44	0.082

Coll-I: Collagen I, Coll-III: Collagen III, N: Normal, PC: Prostatic carcinoma

Table 3. Median, 25% percentile and 75% percentile values of area percentage staining for Coll-IV and elastin in the normal prostate and canine PC samples.

	Group	IHC test			
		25%	Median	75%	p
	Normal	1.11	1.41	1.72	0.2722
	PC **Coll-IV**	0.58	1.14	1.61	
	Normal	0.25	0.26	0.42	0.0671
Elastin	PC	0.28	0.43	0.51	

Immunostaining for collagen type IV (Coll-IV) was observed in the basal membrane (BM) of prostate acini, smooth muscle, blood vessels, and never fibers of normal and PC samples. Acinar BM, showed weak immunostaining for Coll-IV in more than of 70% normal samples and PC (Figure 2). Only the blood vessels showed strong immunostaining in the two groups. The distribution of Coll-IV was approximately 70% in acinar BM, 15% in smooth muscle, 10% in blood vessels BM and 5% in nerve fibers in both groups. Absence of Coll-IV immunostaining was observed in the tumors with solid pattern due to loss of acinar BM (Figure 2). Immunostaining for elastin was observed with similar intensity and distribution than Coll-IV around of blood vessels innormal prostate and canine PC. Elastic fibers were found in the septa dividing the lobules and around the prostatic acini of normal samples. A high amount of elastic fibers was observed around the ducts and the urethra in normal and canine PC (Figure 2).
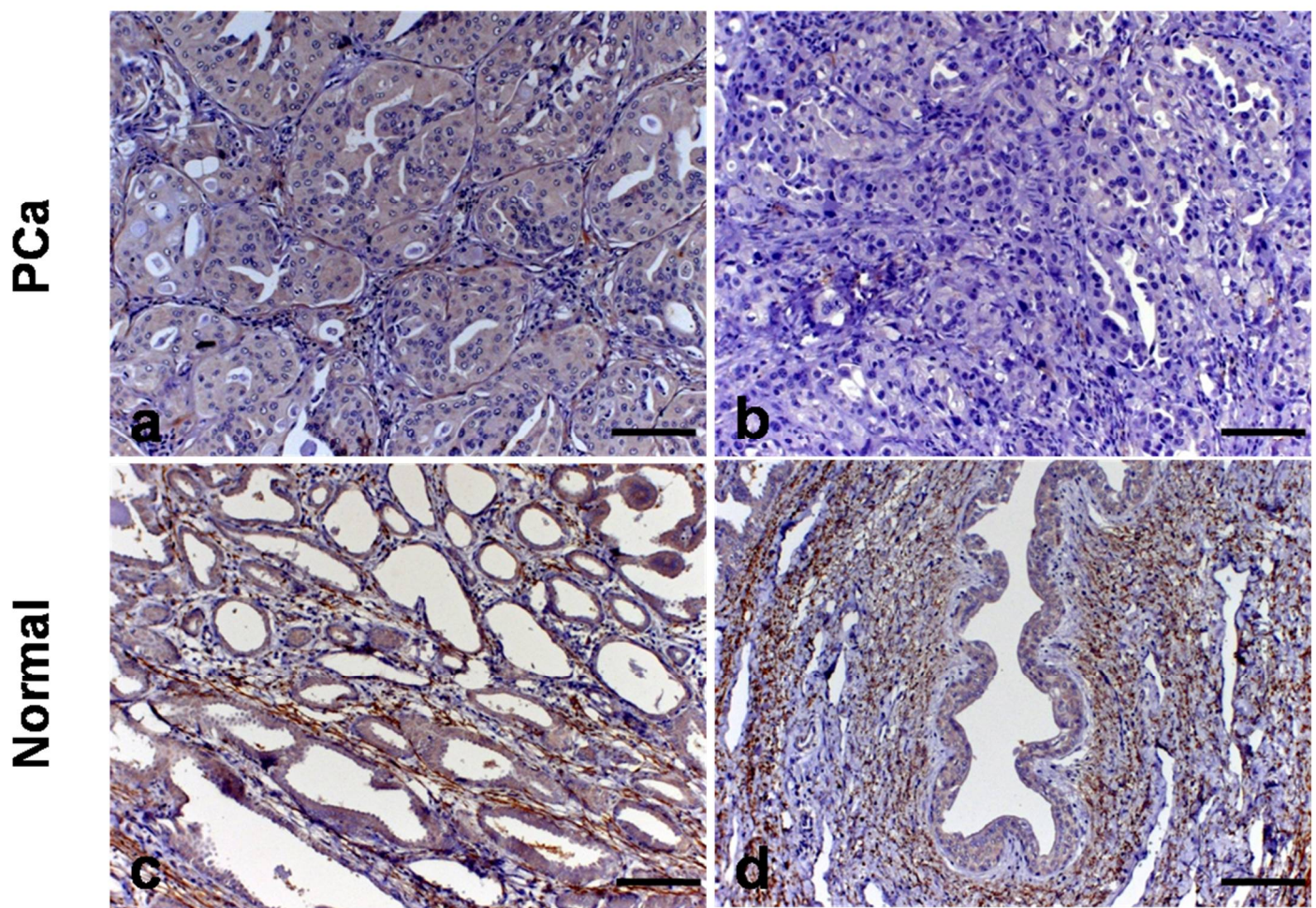
Figure 2. Immunostaining for Coll-IV and elastin in canine prostatic tissue. a: Coll-IV immunostaining in the basal membrane of canine PC with cribirforme pattern (case No. 15). b: Absence of Coll-IV immunostaining in canine PC with solid pattern (case No. 12). c: Elastin fibers around the prostatic acini of normal samples (case No. 5). d. High amount of elastin fibers around the urethra (case No. 5).

4. Discussion

In cancer, the EMC is a network of macromolecules that allow the cellular evasion towards the defense of the organism, besides helping in their metastasis process [10]. The collagens fibers are important components of remodeling of ECM in the TME, actually is know that their degradation and redeposition promote tumor infiltration, angiogenesis, invasion and migration [11]. In this study, we identified and characterized the collagen and elastin fibers in the normal prostate and canine PC using PSR and IHC test.

The PSR birefringent color proportions (red-orange and green) were not statistically different when compared normal prostates and canine PC samples. In humans, Bauman et al., [24] evaluated the collagen content in normal prostates and benign prostatic hyperplasia (BPH) by PSR staining, with no statistical difference. We did not find studies performed in human and canine PC evaluating the collagens fibers with PSR and IHC.

In one study gene and protein expression of Col-I and Col-III were evaluated in human PC, in relation to Gleason score, using RT-qPCR and IHC [25]. No correlation was found between protein and gene expression for both collagens. However, the IHC analysis showed that Col-I and Col-III was significantly reduced in PC, in all Gleason scores, when compared to benign areas. These authors suggest that collagen reduction in PC could be the result from high metalloproteinase activity. In a

study from Faleiro et al. [26] in canine PC, MMP-2 and MMP-9 were overexpressed, when compared to normal prostate, but the authors did not compare the results with collagen expression.

Wegner et al., [27] performed a study with fluorescent PSR in prostate of C57BL/6J mice. They found that fluorescent PSR imaging was more sensitive than polarized light for identify the collagen fibers. In addition, Fluorescent PSR imaging was compatible with the collagen expression by IHC test. The fluorescent PSR imaging method seems to be promising, but it must still be studied in the comparative oncology.

Fewer studies with formalin-fixed paraffin-embedded (FFPE) samples of canine tumors were conducted to analyze the collagens fibers [19, 28, 29]. Bedoya et al., [30] used PSR staining in canine squamous cell carcinomas (SCC), classified in well and poorly differentiated. The percentage of Col-I was approximately 30% for low and high grade SCC. Their results showed higher percentage of collagen fibers than observed in our study, comparing normal and canine PC, but these are different tumors, with different patterns and locations. However, the percentage of Col-III in these SCC was similar when compared to canine PC.

The pattern of Col-IV expression was similar to normal human prostate and PC [31]. An interesting finding was that solid canine PC had lower/absent Coll-IV expression, than other PC patterns and normal tissue. Sinha et al., [31] found that Coll-IV immunostaining was less uniform or absent in the acinar BM of poorly-differentiated PC when compared to acinar BM of well-differentiated PC, HBP and normal human prostate. Similar results also were observed in feline and canine mammary tumors as well as in canine hemangiosarcoma [28, 29].

We also found elastic fibers in the septa dividing the lobules, around the alveoli, ducts and the urethra. Marettová et al. [32] performed an immunohistochemical localization of elastic fibers in the canine prostate. Just like our study, these authors observed elastic fibers around blood vessels, in the septa supporting the lobules and between the secretory alveoli, as well as, a concentration of fibers around the ducts and in the area of the urethra. In canine PC, there was a tendency to higher median of elastin fibers, than normal tissue. Elastin fibers are involved in tumor invasion and metastasis [33], cell proliferation, adhesion, apoptosis and angiogenesis [34].

5. Conclusions

The distribution and percentage area of collagen are similar in normal and neoplastic canine prostate, when analyzed with PSR and IHC tests. In addition, the immunohistochemical localization of elastic system fibers is similar in both groups. Only, in canine PC with solid pattern was identified loss of Col-IV compared to others tumor patterns and normal prostate samples.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Samples used for PSR and IHC test, S2: Histological type of 10 canine prostatic carcinomas, according to Eble et al., 2004 and Palmieri et al., 2014.

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