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Original Full Article

Quantification of the collagen fibers of healthy and neoplastic mammary tissue in cats by the Picrossirius Red histochemical method

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Abstract

Although feline mammary carcinoma is not the most prevalent among the species, its aggressive behavior represents a low life expectancy, compared with most undifferentiated types of breast cancer. Tissue stiffness induced by the accumulation of collagen fibers is related to a risk factor for carcinogenesis in healthy women and aggressiveness in those with breast cancer, which can also occur in cats. The objective of this work is to identify the relationship between stromal collagen density and aggressiveness of mammary carcinoma in cats, according to the peripheral and central tissue distribution by the Picrossirius Red histochemical method. Image.J® and MatLab® software were used for digital image processing. The mean values of kurtosis and entropy attributes were grouped into a control group, and low and high-grade carcinoma groups, analyzed with one-way ANOVA and Bonferroni's multiple comparison test ($p < 0.01$). Interpretation of stromal dynamics is important to evaluate both central and peripheral locations. According to entropy, there was a significant increase in the peripheral density in the carcinoma groups in relation to the control group, which can be justified by blood support. The same can be said of the central region, with a significant gain in collagen fibers from the tumors, indicated by kurtosis. The results suggest the presence of increases in stromal density in mammary carcinomas of cats, regardless of their graduation, and occurring in both regions.

Key words: carcinoma, feline, breast density, extracellular matrix, breast cancer.

Feline mammary carcinoma is not the most frequent in the species (23), but approximately 80% are malignant (12). According to histopathological classification, graduation has a better prognostic value for mammary cancer in different species (29). In cats, aggressive behavior is similar to that of basal-type carcinomas that affect women's breasts (3,20,30).

The increase in stromal density due to the excess deposition of collagen fibers is considered a risk factor for breast cancer (33) and can be detected by follow-up exams, helping in the early diagnosis of the disease (26). Thus, molecular interactions with the adjacent stroma are involved in the stages of initiation, progression, metastasis, and refractoriness (32). Since the parenchyma is glandular, analyzing the degree of peripheral or central distribution enables comparison with normal tissue (1).

The periphery has rich vascularization, high biological activity, and greater viability for the invasion process (8,18). In the tumor center, there is tissue hypoxia, which gives force to cancer cells, contributing to the selection of aggressive clones (7, 10), and stimulating chemotactic factors to the defense cells that benefit the invasion process after suffering the action of tumor promoters (27).

Image analysis through computational operations is more effective when combining different attributes (19). Kurtosis indicates flatness of pixel distribution, presenting good statistical reliability when positive (13). In the binary images analyzed in the current work, the light points represent the collagen fibers that, with respect to the dark background, were in lesser quantity. Thus, the value of the kurtosis attribute is inversely proportional to the stromal density. Entropy assesses the irregularity in the pixel distribution, directly proportional to its value (28), with reliable results obtained when evaluating breast neoplasms (34) and prostatic injuries (14).

The control group included four samples of mammary tissue from non-neutered cats without reproductive disorders, destined for the Pathology Service of the Faculty of Veterinary Medicine and Zootechnics at UNESP - Botucatu Campus. A total of 31 samples of feline mammary carcinoma from the histopathological archive of the Veterinary Pathology Service of Unimar - Marília - SP and the private laboratory VetPat - Campinas - SP were analyzed, from mastectomized cats, as a therapeutic approach, including only tumors with the highest histopathological frequency, in the case of tumors of the simple subtype (29). Benign tumors, or those with extensive areas of necrosis, as well as carcinomas without agreement between pathologists, were not included. The research was approved by CEUA of FMVZ - Unesp Botucatu, with protocol number 0245/2017.

The samples were stained with Hematoxylin-Eosin (HE) enabling classification according to graduation by the Nottingham system, which is based on women (9), and is also suitable for cats (4). To obtain a more balanced sample percentage, the samples were classified as low (grades I and II) or high (grade III) graduation, a methodology already used in studies with mammary carcinoma of cats (5, 6) and women (24), with 16 samples (51.6%) of low grade, and 15 samples (48.4%) of high grade.

The analysis of connective tissue by the PSR method has proven to be effective in assessing the healing process (1) in malignant lesions, to identify collagen fibers in canine prostatic carcinoma (2), and breast cancer (15), however, for cats, there is no mention of this use in the stromal analysis of mammary parenchyma in cats. Using the Picrossirius Red technique (PSR), 5 µm thick histological sections were dewaxed and subjected to successive immersion in 0.2% phosphomolybdic acid, followed by Direct Red® 0.1% (Direct Red 36554-8, Sigma Chemical CO) and 0.1 N hydrochloric acid. Finally, the samples were treated with 70% alcohol solution, dehydration, diaphanization, and assembled in synthetic resin.

For each region, periphery, and center, five different image fields were analyzed on polarized light in a dark field with a 20x magnification lens (Imager A1 microscope® - Carl Zeiss, Germany). In normal tissue, the periphery was delimited below the epidermis, and in neoplastic tissue by the edges of the tumor. The central portion of the healthy tissue was represented by the fields centrally adjacent to the periphery, and of the carcinomas, the intralobular portions. High stromal regions of the dermis, as well as regions rich in arteries, were not included in the digital analysis of the images.

The images were processed by both software packages converted into 8 bits of gray levels to minimize background noise and improve the quality of statistical results (Fig. 1). Through the ImageJ® software, attributes with first-order statistics (mean intensity of gray levels, standard deviation, and mode) were extracted, such as multivariate skewness and kurtosis (16). To extract the texture attribute, entropy, based on the pixel correlation matrix (11), an algorithm developed in Matlab® was used.

Once quality in capturing images is guaranteed, the attributes can vary, as well as their interpretation. (25). (Fig. 2) demonstrated that Kurtosis in the central region and entropy in the periphery were better at predicting the stromal density of the cat's mammary tissue. Due to the homogeneity of the images obtained in normal tissue, the attributes were chosen based on the control group. There was a predominance of darker gray levels, which guaranteed discrepant values of mode, median, average, and standard deviation, which reduces the statistical reliability of these data. These characteristics strengthened the choice of the

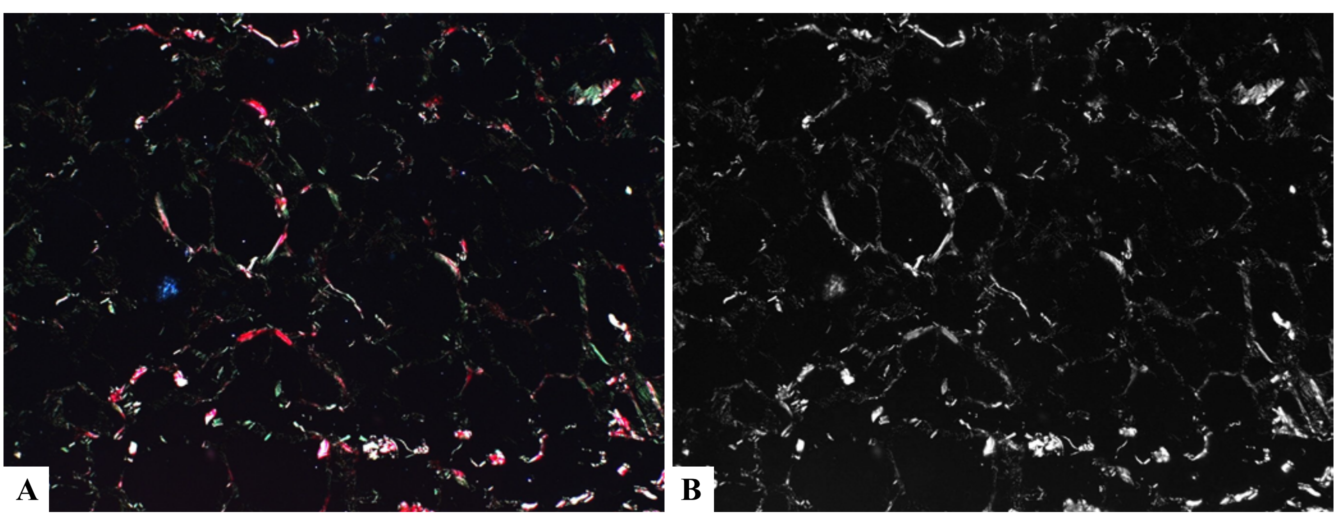

Figure 1. Sample of the control group. Image captured in dark field under polarized light of histological section stained by PSR (A). The same image after being transformed into 8 bits (B). (20x magnification)

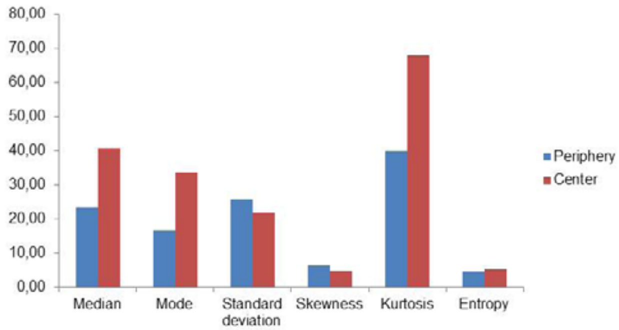

Figure 2. Mean values for each of the variables in the control group regarding their location.

Table 1. Mean values obtained from kurtosis and entropy in healthy tissue and in mammary carcinomas of cats grouped according to low (grade I and II) and high (grade III) histopathological grades.

Sample	Periphery		Center	
	Kurt/ Ent		Kurt/ Ent	
Control	39.84[a]	4.62[a]	67.95[a]	5.53[a]
Low Grade	30.13[a]	5.26[b]	27.80[b]	5.71[a]
High Grade	30.12[a]	5.30[b]	27.90[b]	5.64[a]

* The same letters indicate that there was no statistical difference between the rows of the same column (p <0.01).

kurtosis attribute to improve the statistical analysis by the flat distribution in the T-test (17). In addition, there was a large difference between the mean values of periphery and center. Another important factor is that the values of this descriptor were inversely proportional to the values presented by entropy, which demonstrates the greater statistical reliability of kurtosis. The fact that all samples showed positive values fits the high distribution analysis, reinforcing the good statistical performance (13).

The texture attributes are ideal for evaluating the edges of the image, with entropy indicated for improving the details of the tumor periphery (34). For the images evaluated in this work, this descriptor was interpreted as fiber disorganization, thus the lower numerical value represents the low dispersion of collagen fibers and, consequently, low density.

After establishing the attributes, average values of kurtosis and entropy were obtained for the carcinomas group for both regions (Table 1). Statistical analysis was performed with the aid of the GraphPad 5 software (GraphPad Software Inc®, La Jolla, CA). One-way ANOVA, followed by the Bonferroni multiple comparison tests were performed, with a p-value <0.01.

The carcinomas did not differ from each other, but did differ from healthy tissue. There was evident gain of collagen fibers in mammary carcinomas of cats, both in the central area, identified by the kurtosis attribute (Fig. 3), and in the periphery, according to entropy (Fig. 4), regardless of the degree of differentiation. The effectiveness of entropy to analyze the tumor periphery in comparison to kurtosis corroborates with the literature (28).

The significant increase in stromal density in the central region of feline mammary carcinomas can be justified by the structural function of collagen fibers (31), which are modeled according to the need for glandular tissue. With respect to the peripheral region of the 31 samples analyzed, the collagen fibers are in greater quantity and with a more organized pattern than in the center, a characteristic that has already been related to aggressiveness in breast cancer (21, 22).

(Fig. 5) shows images without digital treatment to illustrate both control and carcinoma groups, according to the location.

From the results of the current study, it is possible to conclude that the kurtosis and entropy attributes, extracted from the images of the histological sections stained by PSR of the mammary tissue of cats, were effective to estimate the stromal density of the center and periphery, respectively, and indicated that the increase in stromal density of carcinomas, regardless of their degree, occurs in both regions. Further studies are needed, but it is suggested that, like in women, the increase in the density of the breast stroma may be related to the aggressiveness of mammary cancer in cats.

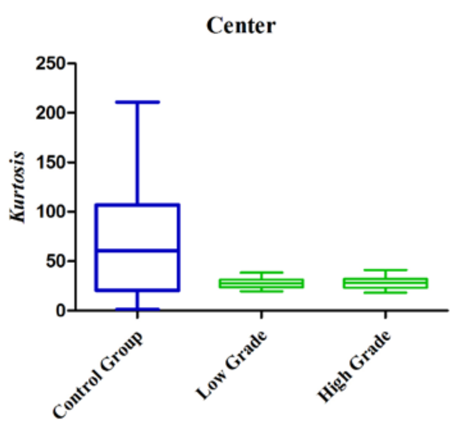

Figure 3. Comparison between control group and low- and high-grade carcinomas in terms of central location according to mean kurtosis values. P-value <0.01.

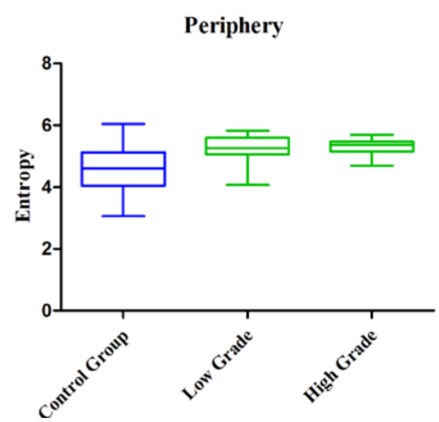

Figure 4. Comparison between the control group and low- and high-grade carcinomas, regarding the periphery location, of the entropy attribute. P-value <0.01.

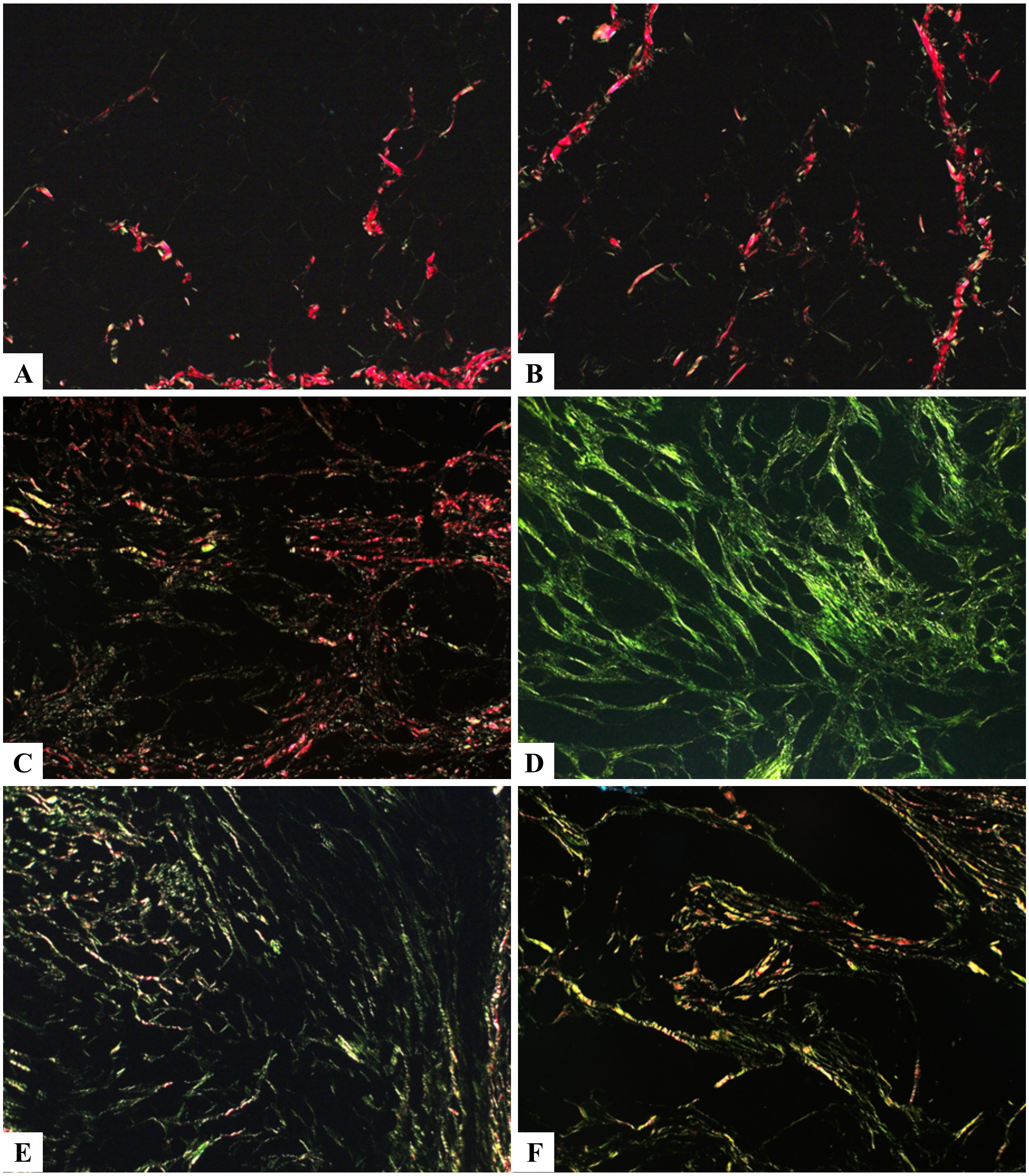

Figure 5. Dark background images of the histological sections stained by the PSR method, according to the location that illustrates the differences between the groups. Peripheral **(A)** and central **(B)** regions of healthy breast tissue**,** peripheral **(C)** and central **(D)** regions of low-grade carcinoma, and peripheral **(E)** and central **(F)** regions of high-grade carcinoma. (20x magnification)

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