JRVM
Journal of Research in Veterinary Medicine

RESEARCH ARTICLE

J Res Vet Med. 2021: 40 (2) 77-87
DOI:10.30782/jrvm.878763

Investigation of Calprotectin Positive Leukocytes in Canine Soft Tissue Tumors

Onur SAVAŞ[1], Volkan İPEK[1*]

[1] Department of Pathology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, Turkey

Received 11-02-2021 Accepted 14-08-2021

Özet

Köpek yumuşak doku sarkomları tedavisi oldukça zorlu, invazyon ve metastaz özellikleri yüksek olan kompleks bir grup tümördür. Malign tümöral dokularda çoğunlukla infiltrasyonları gözlenen yangı hücreleri içerisinde makrofajlar ve nötrofiller bulunmaktadır. Bu iki yangı hücresinin tümörlerdeki malignite ve kötü prognozla ilişkili olan tipleri tümör ilişkili makrofajlar ve tümör ilişkili nötrofiller olarak adlandırılmaktadır. Bu çalışmada köpek yumuşak doku tümörlerinde calprotectin pozitif nötrofil ve makrofajların varlığı immunohistokimyasal yöntemlerle araştırılmıştır. Bu amaçla farklı ırk, yaş ve cinsiyetteki 31 adet köpekten, farklı kökenden mezenkimal tümörler incelendi. Tümörlerin histopatolojik olarak sınıflandırılması sonrasında calprotectin pozitif makrofaj ve nötrofiller açısından benign ve malign tümörler arasında karşılaştırma yapıldı. Pozitiflik gözlenen tüm hücreler bazında yapılan değerlendirmelerde malign yumuşak doku tümörlerinde calprotectin pozitifliği benign tümörlere oranla anlamlı derecede yüksek bulundu. Yalnızca makrofajlar açısından yapılan değerlendirmelerde de malign tümörlerde belirgin bir yükseklik dikkati çekti. Nötrofil varlığı açısından ise benign ve malign tümörler arasında istatistiksel olarak anlamlı bir farklılık bulunamadı. Çalışmamızda elde ettiğimiz sonuçlara göre köpeklerde yumuşak doku tümörlerinde calprotectin pozitif lökosit infiltrasyonlarının malignite ile ilişkili olebileceği gözlenmiştir.

Anahtar Kelimeler: Calprotectin, köpek, makrofaj, nötrofil, yumuşak doku tümörü

Abstract

Soft-tissue sarcomas are a complex group of tumors that are difficult to treat and have high invasion and metastasis potential. Inflammatory cell infiltrations are associated with malignancy in many human and animal tumors. Macrophages and neutrophils are found in inflammatory cells whose infiltration is observed in malignant tumor tissues. Subtypes of these inflammatory cells associated with malignancy and poor prognosis in tumors are called tumor-associated macrophages and tumor-associated neutrophils. In this study, the presence of calprotectin-positive neutrophils and macrophages in canine soft-tissue tumors was investigated using immunohistochemical methods. For this purpose, mesenchymal tumors of different origins from 31 dogs of different breeds, ages, and sexes were examined. After the histopathological classification of the tumors, a comparison was made between benign and malignant tumors in terms of calprotectin-positive macrophages and neutrophils. Calprotectin positivity in malignant soft-tissue tumors was found to be significantly higher than that in benign tumors in the evaluations made on the basis of all positive cells. In the evaluations made only in terms of macrophages, a significant increase in malignant tumors was also noted. No statistically significant difference was found between benign and malignant tumors in terms of neutrophil presence. According to the results in our study, it was observed that calprotectin-positive leukocyte infiltrations could be associated with malignancy in canine soft-tissue tumors.

Keywords: Calprotectin, dog, macrophage, neutrophil, soft-tissue tumor

Introduction

Soft-tissue tumors typically occur in older dogs and constitute 15% of subcutaneous tumors including fibrous connective tissue, blood vessels, lymphatics, nerves, and adipose tissue tumors. They are particularly noticeable in certain parts of the body such as the head and extremities, and metastases typically occur in the lungs and lymph

* Corresponding author: Volkan IPEK Tel: +90 248 213 21 74, volkanipek@mehmetakif.edu.tr

nodes[1]. Despite being a very heterogeneous group of tumors, malignant tumors are together referred to as soft-tissue sarcoma because their biological behavior tends to be very similar. Sarcomas originate from various mesenchymal tissues such as muscle, neurovascular, connective, and adipose tissues. Although the majority of cases are located in the subcutaneous or musculoskeletal system, soft-tissue tumors can develop in other organs such as the lung, heart, liver, spleen, urogenital organs or gastrointestinal organs, as well as retroperitoneal, and mediastinal areas[2].

Inflammation that does not resolve in a tumor microenvironment is the hallmark of cancer[3,4]. Immune cells along with leukocytes, fibroblasts, and vascular endothelial cells form a tumor microenvironment. These immune cells interact with tumor cells to affect the formation, growth, and metastasis of tumors[5]. Tumor-associated macrophages (TAMs) are often prominent immune cells that orchestrate various factors, particularly in the tumor microenvironment[6,7]. Monocytes/macrophages can be polarized to M1 or M2 macrophages. Classically activated macrophages, also known as M1-polarized macrophages, are activated by cytokines such as interferon-gamma, producing proinflammatory and immunostimulatory cytokines (e.g., interleukin [IL]-12 and IL-23). TAMs are thought to be more similar to M2-polarized macrophages and are known as alternatively activated macrophages activated by Th2 cytokines (e.g., IL-4, IL-10, and IL-13). TAMs play an important role in linking inflammation with cancer. They can promote proliferation, invasion, metastasis and angiogenesis of tumors, inhibit T-cell-mediated anti-tumor immune response, and promote tumor progression[8].

The intracellular functions of S100 proteins include the regulation of calcium homeostasis, cell cycle, cell growth and migration, phosphorylation, cytoskeletal components, and transcriptional factors. Unlike their intracellular function, extracellular S100 proteins act in a cytokine-like fashion by binding to cell surface receptors such as enhanced glycation end products and Toll-like receptors[9,10]. Calprotectin (CAL) is a member of the S100s protein family and is a heterodimeric calcium-linked protein11. It is found in neutrophils, monocytes, keratinocytes, and macrophages. After this substance is secreted from the activated granulocytes, it participates in inflammatory processes similar to cytokines and takes part in various cellular processes. These include cell cycle progression, cell survival, proliferation, differentiation, and cell migration. Moreover, they can affect cellular functions involving regulation of calcium hemostasis, rearrangement of cytoskeletal components and cell migration. It can also suppress microbial growth as a zinc and manganese chelator and zinc-dependent enzymes such as matrix metalloproteinases[11–13].

In 1863, Virchow suggested that the origin of cancer was related to chronic inflammation[14]. Several studies point out a strong link between CAL and inflammation, innate immunity, cancer, and malignancies triggered by infections; however, molecular and cellular mechanisms are not fully elucidated[15]. This study was aimed at determining and comparing the presence and scores of CAL-positive leukocyte infiltrates in canine benign and malignant soft-tissue tumors.

Materials and Methods

In this study, 31 canine soft-tissue tumors were included from archives of tissue blocks (Table 1). First, soft-tissue tumors were classified histopathologically as previously reported[16–18]. After these classifications, tumors were evaluated for the presence of CAL-positive macrophages and neutrophils in benign and malignant cases. Density of CAL-positive leukocytes was compared in benign and malignant tumors and evaluated according to the specific tumor diagnosis (e.g., fibrosarcoma and peripheral nerve sheath tumor).

Table 1. Cases and histopathological diagnosis.

No	Species	Race	Gender	Age	Location	Histopathological Diagnosis
1	Dog	Crossbreed	Female	14 year	Mammary	Fibroma
2	Dog	Transylvanian Hound	Male	6 year	Hip	Fibroma
3	Dog	Transylvanian Hound	Male	6 year	Hip	Fibroma
4	Dog	Russian Poodle	Male	10 year	Gum	Fibroma
5	Dog	Crossbreed	-	-	Vagina	Fibroma
6	Dog	-	-	-	-	Fibroma
7	Dog	-	-	-	-	Fibroma
8	Dog	Crossbreed	Male	15 year	-	Fibroma
9	Dog	-	-	-	-	Leiomyoma
10	Dog	Crossbreed	Female	11 year	Vagina	Leiomyoma
11	Dog	Terrier	Female	16 year	Vagina	Leiomyoma
12	Dog	Golden retriever	Male	5.5 year	Neck	Fibrosarcoma
13	Dog	Rottweiler	Male	4 year	Right forearm	Fibrosarcoma
14	Dog	Crossbreed	-	-	Oral	Fibrosarcoma
15	Dog	Setter	Male	3 year	Left forearm	Fibrosarcoma
16	Dog	Crossbreed	Female	16 year	Gum	Fibrosarcoma
17	Dog	Crossbreed	-	1.5 year	-	Fibrosarcoma
18	Dog	Golden retriever	Male	10 year	-	Fibromyxosarcoma
19	Dog	Golden retriever	Female	9 year	Left scapula	Myxosarcoma
20	Dog	Golden retriever	Female	8 year	Spleen	Hemangiosarcoma
21	Dog	Crossbreed	Female	6 year	Larynx	Hemangiosarcoma
22	Dog	Terrier	Male	12 year	Perianal region	PNST
23	Dog	Husky	Female	15 year	Tarsal region	PNST
24	Dog	Crossbreed	-	-	Perianal region	PWT
25	Dog	Crossbreed	Female	7 year	Abdominal muscle	Liposarcoma
26	Dog	Golden retriever	Female	7 year	Intrascapular location	Liposarcoma
27	Dog	Crossbreed	Female	12 year	Lateral femoral muscle	Liposarcoma
28	Dog	Crossbreed	Male	6 year	Lower eyelid	Undifferentiated sarcoma
29	Dog	Crossbreed	Male	13 year	Spleen	Undifferentiated sarcoma
30	Dog	Rottweiler	Male	8 month	Lumbal region	Undifferentiated sarcoma
31	Dog	German Shepherd	Male	6 year	Gum	Undifferentiated sarcoma

PWT: Perivascular wall tumor, PNST: Peripheral nerve sheath tumor

Immunohistochemically, a CAL antibody (Calprotectin, clone MAC387, Thermo Scientific, Fremont, CA, Catalog # MA1-80446) marker was used to determine CAL expression in macrophages and neutrophils. For immunohistochemical staining, 4–5-µm-thick sections were taken from paraffin blocks with microtome (Leica RM 2155) on po-

ly-l-lysine slides, deparaffinized in xylene (Xylene - Merck Millipore: 108661) and dehydrated in an alcohol series. For antigen retrieval, tissues were treated with trypsin (0.25% HyClone Trypsin Solution) for 20 minutes at 37°C. Then, the sections were washed in phosphate buffered saline (PBS) solution for 2 × 5 minutes and incubated in 3% hydrogen peroxide (Merck: 107209) solution for 15 minutes to block the endogenous peroxidase activity. After the incubation was completed, the sections were washed again with a PBS solution for 5 minutes, and normal goat serum (Vectorlab, 2.5% Normal Goat Serum) was applied to the sections for 30 minutes to prevent non-specific antibody binding. Tissues were incubated at 4°C overnight with a primary antibody diluted at the rate of 1/200 with an antibody diluent (Large Volume UltrAb Diluent Plus, Thermo Scientific: TA-125-UDX). After the primary antibody incubation, slides were washed with PBS for 2 × 5 minutes, and biotinylated goat anti-polyvalent (mouse-rabbit) (Thermo Scientific ™ Lab Vision ™ Biotinylated Goat anti-Polyvalent) was applied as a secondary antibody. Washing was carried out with PBS for 2 × 5 minutes. Then, a Streptavidin peroxidase solution (Vectorlab, ImmPRESS Excel Amplified HRP Polymer Reagent) was applied for 30 minutes. DAB (Vectorlab, ImmPACT® DAB EqV Substrate) was used as a chromogen. Counter-staining of sections was made with Mayer's hematoxylin (Merck) for 15 seconds, and slides were then dried, covered with entellan and examined under a light microscope. Normal canine spleen and canine chronic dematitis slides were used as positive controls.

Evaluation of immunohistochemical staining was done semi-quantitatively. Scoring between 0 and 4 was made by counting positively stained neutrophils and macrophages in 10 random high-power fields for each case.

Statistical Analysis

Minitab 16 package program (version 16.1.1) was used for statistical evaluation. The Ryan–Joiner normality test was applied to determine whether the scores obtained showed normal distribution. After scoring, the differences in immune stainings between benign and malignant tumors were evaluated statistically using the paired T test, and whether the results were significant or not was determined. In addition, the Pearson correlation test was used to determine the correlation between neutrophil and macrophage infiltrations in all tumors.

Results

Histopathological Findings

As a result of histopathological examinations, eight fibromas, three leiomyomas, six fibrosarcomas, one fibromyxosarcoma, one myxosarcoma, two hemangiosarcomas, one perivascular wall tumor (PWT), two peripheral nerve sheath tumors (PNSTs), three liposarcomas, and four undifferentiated sarcomas were diagnosed.

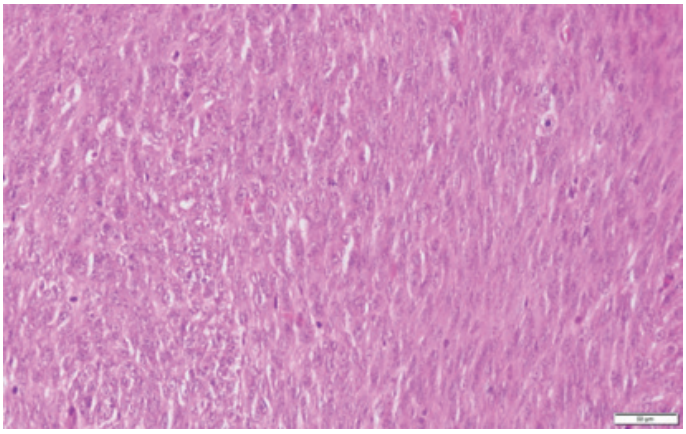

Figure 1. Neoplastic cell proliferations with marked anisokaryosis and multiple mitotic figures in a fibrosarcoma case. H&E. Bar: 50 µm.

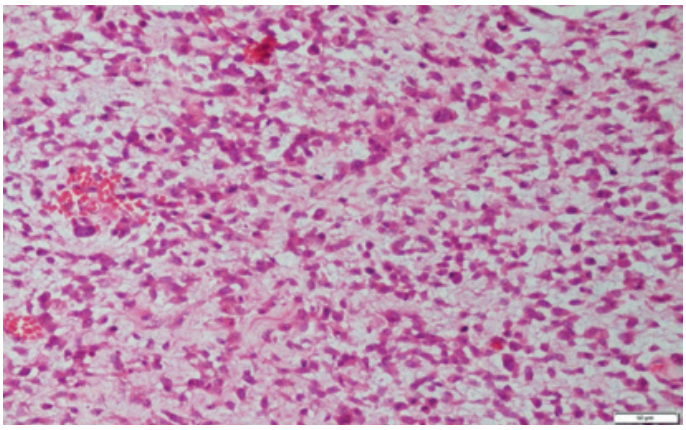

Figure 2. Neoplastic cell proliferations with vacuolar cytoplasm showing significant anisokaryosis in a myxosarcoma case. H&E. Bar: 50 µm.

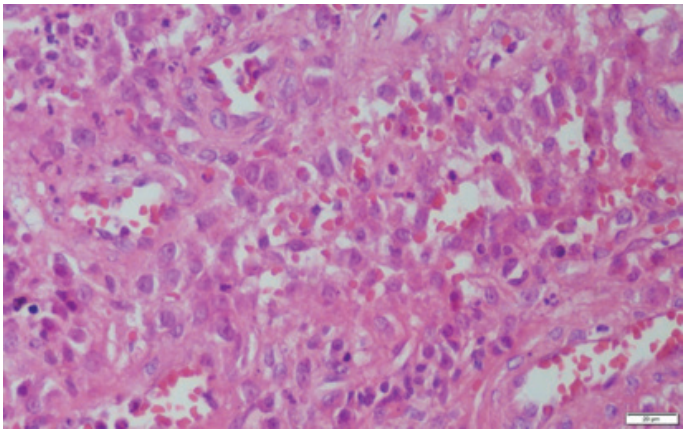

Figure 3. Numerous capillary vessel formations and endothelial cell proliferations with the signs of malignancy in a hemangiosarcoma case. H&E. Bar: 20 µm.

In fibroma cases, fibroblast proliferations showing uniformity within the collagen-rich stroma were observed. Multifocal mononuclear cell infiltrates were noted in the two cases. In the other case, dense vessel formations and superficial inflammation were observed. In the leiomyoma cases, proliferation of neoplastic myocytes with uniform

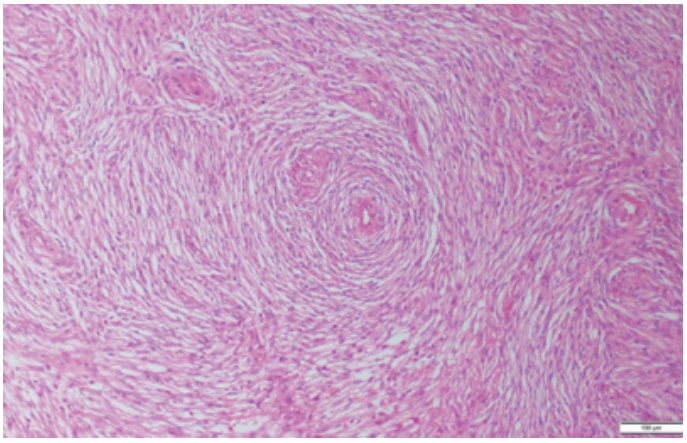

Figure 4. Whirling pattern of proliferating neoplastic cells around the blood vessels in a PWT case. H&E. Bar: 100 µm.

nuclei and prominent large eosinophilic cytoplasms was observed.

In fibrosarcoma cases (Figure 1), there were tumoral cells showing marked anisokaryosis and irregular growth in multiple directions and containing multiple mitotic figures. Leukocyte infiltrations were observed multifocally.

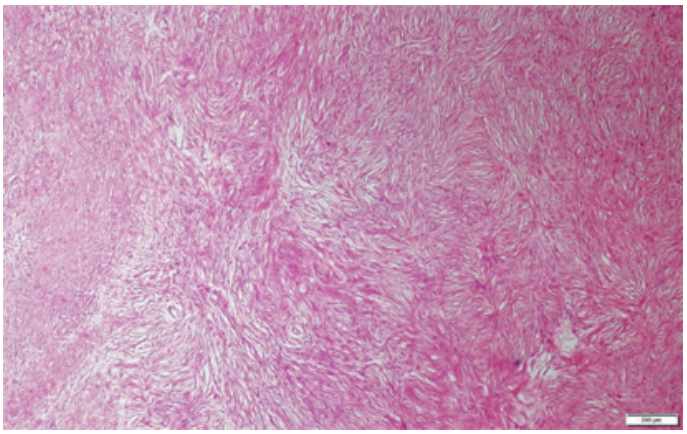

Figure 5. Proliferations in nested bundles and prominent clefts in a PNST case. H&E. Bar: 200 µm.

In the fibromyxosarcoma case, proliferation of neoplastic cells exhibiting marked anisokaryosis with mucinous secretions and numerous large vascular structures were seen.

In the myxosarcoma case (Figure 2), neoplastic cells that have mucinous appearance with moderate anisokaryosis

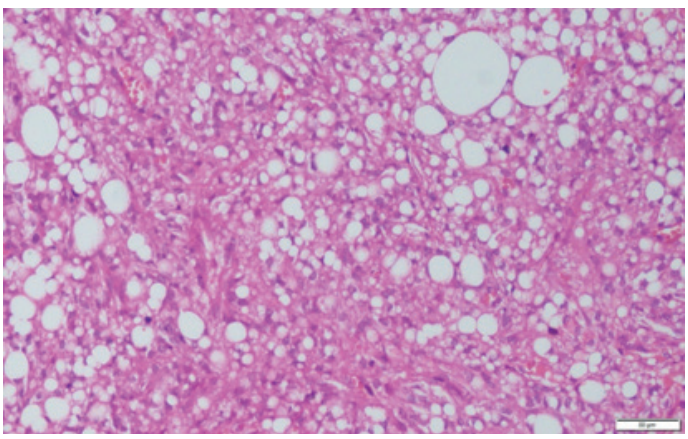

Figure 6. Neoplastic proliferations showing signs of malignancy and containing multiple and different sizes of fat vacuoles in a liposarcoma case. H&E. Bar: 50 µm.

were noted.

In hemangiosarcoma cases (Figure 3), endothelial cell proliferations showing numerous capillary vessel formations and prominent signs of malignancy were observed. In one

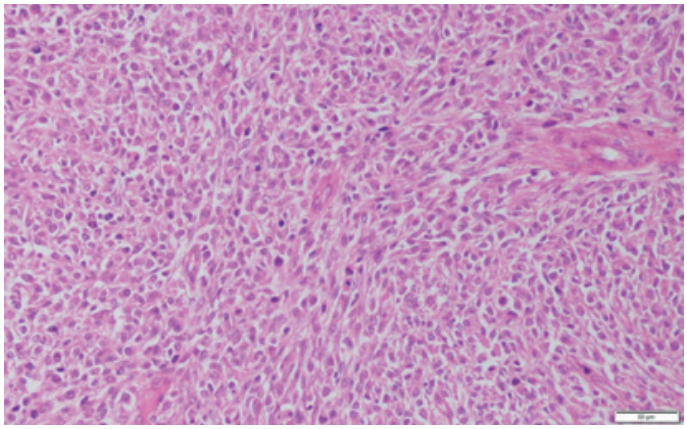

Figure 7. Neoplastic cell proliferations with signs of malignancy and numerous mitotic figures in a case of undifferentiated sarcoma. H&E. Bar: 50 µm.

case, large hemorrhages covered the majority of the tumor parenchyma. In the other case, intense inflammatory cell infiltrates were seen.

In the PWT case (Figure 4), neoplastic cell proliferations that have a spindle or round to oval shape with moderate anisokaryosis showing a whirling pattern in the periphery of the blood vessels and intertwined appearance were noted. Numerous capillary vessels were observed in the tumor tissue. In addition, focal neutrophil infiltration was noted.

In one of the PNST cases, proliferation of the mesenchymal cells forming multiple foci of spiral formations without central vascular structures was noted. In the other case, proliferations in the form of intertwined thin bundles with

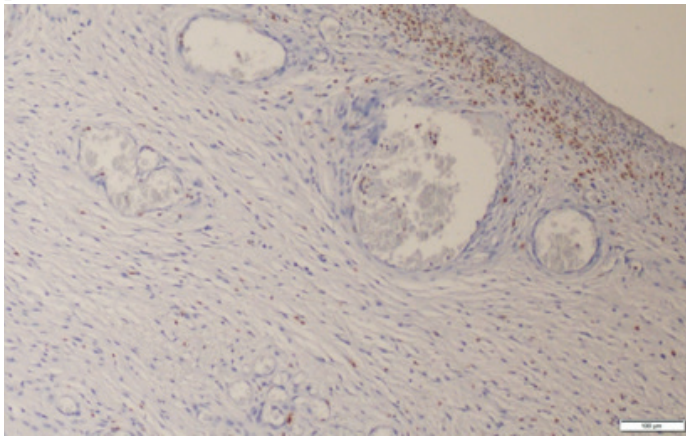

Figure 8. Many CAL-positive neutrophils observed superficially in a fibroma case and scattered macrophages in the parenchyma. Avidin-biotin complex method. DAB. Bar: 100 µm.

prominent clefts (Figure 5) and distinct Antoni A type-like formations were noted.

In liposarcoma cases (Figure 6), neoplastic cell proliferations with prominent and numerous small and large vacuoles in cytoplasms were seen. These cells generally showed

marked anisokaryosis, numerous mitotic figures, and rare multinucleation.

In the undifferentiated sarcoma cases (Figure 7), numerous spindle cell proliferations that showed distinct signs of malignancy in the form of intertwined growths were observed.

Immunohistochemical Findings

CAL positivity was observed in macrophages and neutrophils infiltrating tumor tissues. Macrophage and neutrophil scores and total scores according to the diagnoses are presented in Table 2. In the comparison of benign (Figure 8) and malignant tumors in terms of the total score, it was observed that the scores in malignant tumors were significantly higher than those in benign tumors ($P < 0.01$). No statistically significant difference was observed in the comparison of benign and malignant tumors in terms of neutrophil scores. However, a significant increase in malignant tumors was observed in the comparison of benign–malignant tumors in terms of macrophages ($P < 0.05$).

As the observed benign tumors are mostly fibroma, a comparison of the same type of benign and malignant tumors was considered. To this end, fibroma cases and malignant

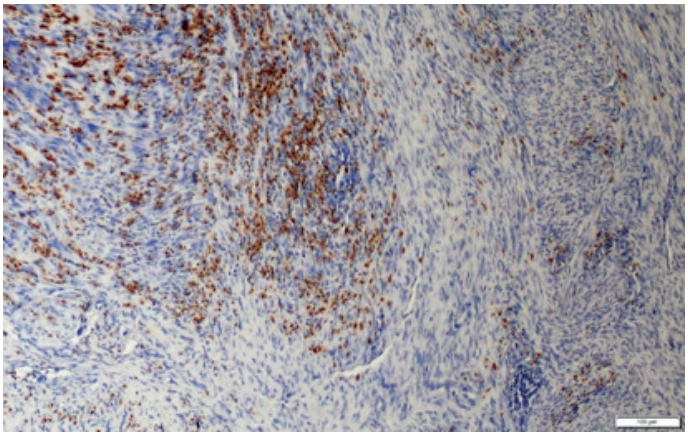

Figure 9. Abundant CAL-positive leukocytes in a fibrosarcoma case. Avidin-biotin complex method. DAB. Bar: 100 µm.

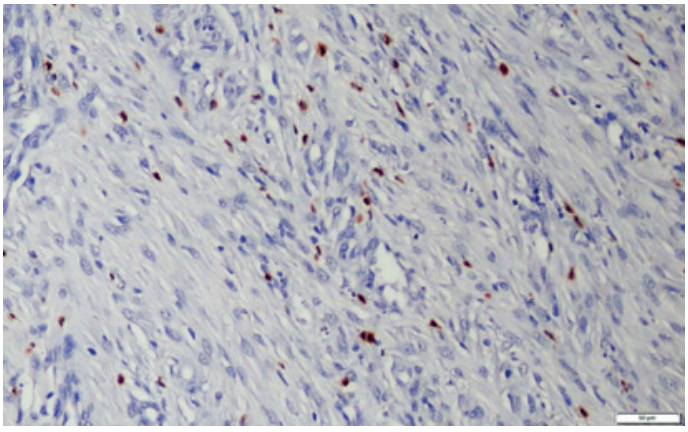

Figure 10. CAL-positive macrophages scattered in the parenchyma in a fibrosarcoma case. Avidin-biotin complex method. DAB. Bar: 50 µm.

Table 2. CAL positive macrophage and neutrophil counts and total scores according to diagnosis.

No	Histopathological Diagnosis	Macrophage Score	Neutrophil Score	Total Score
1	Fibroma	1	1	1
2	Fibroma	0	0	0
3	Fibroma	1	1	1
4	Fibroma	2	1	2
5	Fibroma	3	4	4
6	Fibroma	1	0	1
7	Fibroma	0	0	0
8	Fibroma	1	0	1
9	Leiomyoma	0	0	0
10	Leiomyoma	1	0	1
11	Leiomyoma	2	0	2
12	Fibrosarcoma	1	4	4
13	Fibrosarcoma	4	4	4
14	Fibrosarcoma	4	4	4
15	Fibrosarcoma	2	1	2
16	Fibrosarcoma	4	1	4
17	Fibrosarcoma	3	2	3
18	Fibromyxosarcoma	4	1	4
19	Myxosarcoma	0	0	0
20	Hemangiosarcoma	3	1	3
21	Hemangiosarcoma	1	4	4
22	PNST	0	0	0
23	PNST	0	0	0
24	PWT	2	2	3
25	Liposarcoma	4	1	4
26	Liposarcoma	1	0	1
27	Liposarcoma	4	1	4
28	Undifferentiated sarcoma	1	0	1
29	Undifferentiated sarcoma	1	0	1
30	Undifferentiated sarcoma	4	1	4
31	Undifferentiated sarcoma	4	4	4

PWT: Perivascular wall tumor, PNST: Peripheral nerve sheath tumor

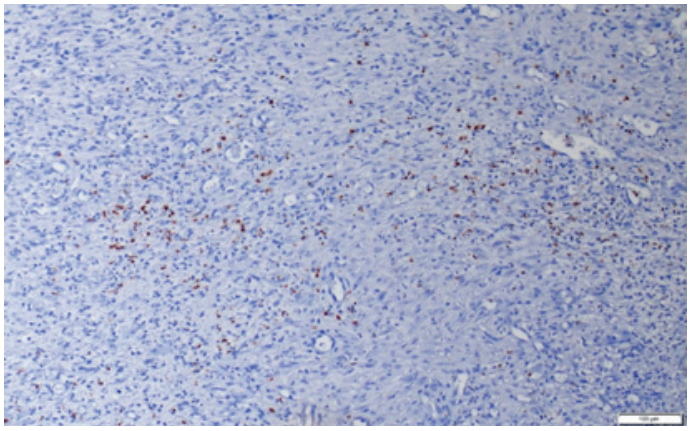

Figure 11. CAL-positive leukocytes in a fibromyxosarcoma case. Avidin-biotin complex method. DAB. Bar: 100 µm.

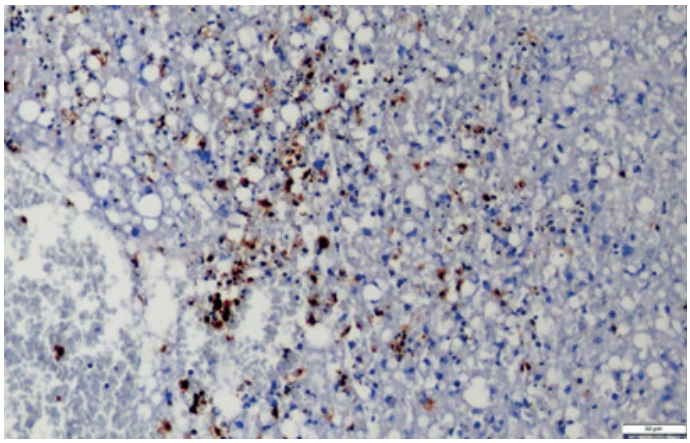

Figure 12. Multiple CAL-positive leukocytes in a liposarcoma case. Avidin-biotin complex method. DAB. Bar: 50 µm.

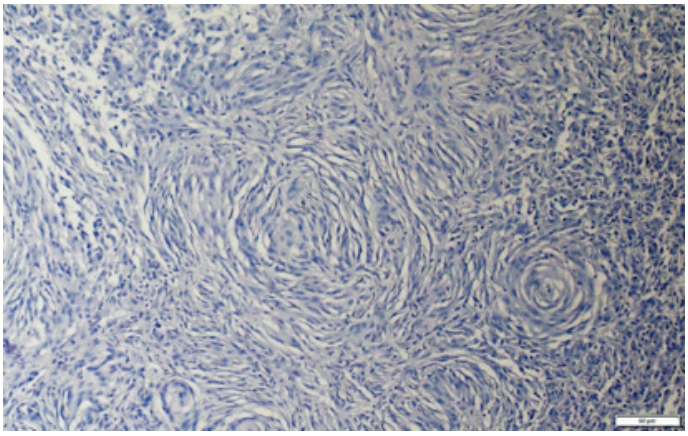

Figure 13. CAL negativity in a PNST case. Avidin-biotin complex method. DAB. Bar: 50 µm.

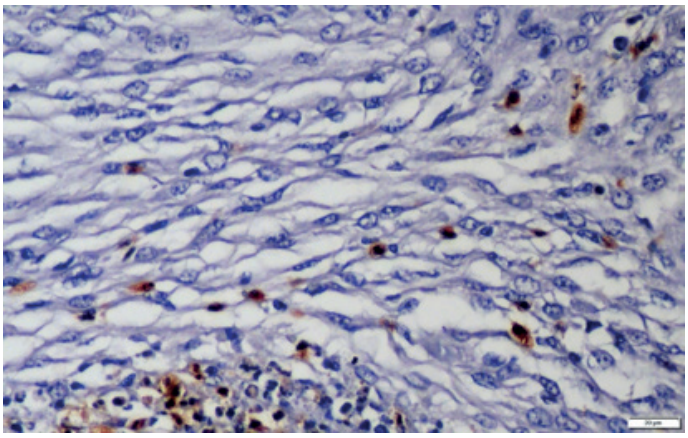

Figure 14. CAL-positive macrophages and neutrophils in a PWT case. Avidin-biotin complex method. DAB. Bar: 20 µm.

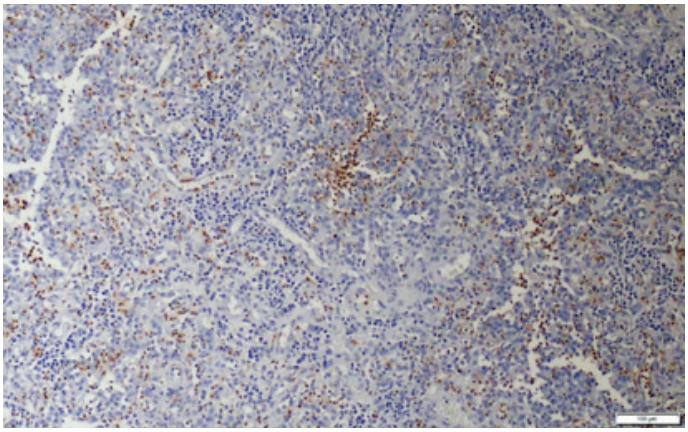

Figure 15. Numerous CAL-positive leukocytes in a hemangiosarcoma case. Avidin-biotin complex method. DAB. Bar: 100 µm.

connective tissue tumors (fibrosarcoma, fibromyxosarcoma and myxosarcoma) were compared in terms of neutrophil, macrophage and total scores (Figures 9–11). In the comparison made in terms of the total score, the total CAL score was found to be significantly higher in malignant connective tissue tumors compared to that in benign ones ($P < 0.05$). Although no significant difference was observed between the two groups in terms of CAL-positive neutrophils, a significant increase in macrophages was noted in malignant connective tissue tumors ($P < 0.05$). A statistical

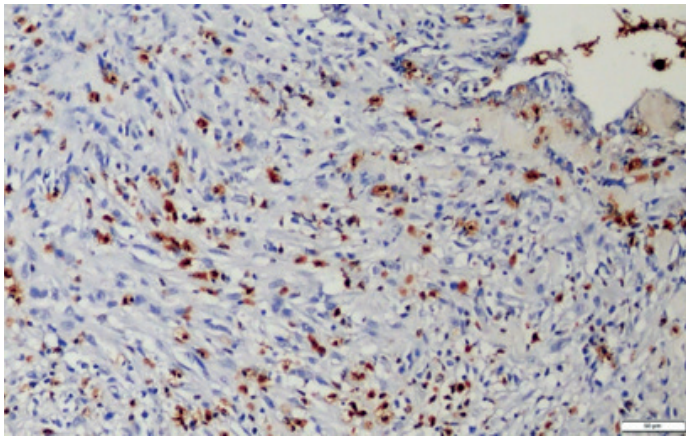

Figure 16. Numerous CAL-positive leukocytes in an undifferentiated sarcoma case. Avidin-biotin complex method. DAB. Bar: 50 µm.

comparison was not made between other specific tumor types (liposarcoma, PWT, PNST, hemangiosarcoma, and undifferentiated sarcoma) (Figures 12–16) because of the low number of cases. The data of statistical analysis are presented in Table 3.

Table 3. Statistical comparison of CAL positivity.

Group	Comparison of the All Benign and Malignant Tumors	Comparison of the Benign and Malignant Connective Tissue Tumors
Benign - Macrophage & Neutrophil	1.182±0.352**	1.250±0.453*
Malignant - Macrophage & Neutrophil	2.700±0.356**	3.125±0.515*
Benign - Macrophage	1.091±0.285*	1.125±0.350*
Malignant - Macrophage	2.350±0.357*	2.750±0.559*

P<0,05; **P<0,01

Pearson's correlation test was also used on the basis of all tumors to reveal the relationship between CAL-positive neutrophils and macrophages, and a positive correlation was found ($P < 0.01$) (Table 4).

Table 4. Pearson's correlation test results between neutrophils and macrophages.

CAL positivity		Neutrophil	Macrophages
Neutrophil	Pearson correlation	1	.510**
	Sig. (2-tailed)		0,003
Macrophages	Pearson correlation	.510**	1
	Sig. (2-tailed)	0,003	

** Correlation is significant at 0.01 level (2-tailed)

Discussion And Conclusion

Inflammatory cells and cytokines contribute to tumor growth, progression and immunosuppression rather than a response to the tumor. Moreover, cancer sensitivity and severity may be related to functional polymorphism of inflammatory cytokine genes, and suppression of inflammatory cytokines in experimental cancer models also suppressed tumor growth[14]. Approximately 15% of global cancer cases can be attributed to infectious agents and inflammation[19]. In the comparison of benign and malignant mesenchymal tumors in our study, a significant increase in CAL-positive inflammatory cells was noted in malignant tumors. Especially in fibrosarcomas, a general increase in

inflammatory cells was observed, but a lower level of inflammatory cells was observed in some other malignant tumors. It is known that the increase in the risk of malignancy is associated with chronic inflammation caused by chemical and physical agents[20], autoimmune diseases, and inflammatory reactions of unknown etiology[21]. In our study, these low CAL-positive leukocyte infiltrates in some tumors were thought to be related to the degree of differentiation of the tumors as stated. As a matter of fact, it was noted that the tumor cells displayed low-grade anaplasia in terms of malignancy findings in the myxosarcoma case with a low inflammatory cell score. The CAL score was also found to be low in one liposarcoma and two PNST cases.

Avallone et al.[17] classified the canine perivascular wall tumors according to their histopathological and immunohistochemical characteristics as angioleiomyoma/sarcoma, myopericytoma, angiofibroma, angiomyofibroblastoma, and hemangiopericytoma. They stated that among these tumors, the whirling pattern is found in myopericytoma-like perivascular myoma, myopericytoma, angiofibroma, and angiomyofibroblastoma and that the distinction between them is possible with a number of markers such as desmin, vimentin, smoothelin, and α-SMA. A distinct whirling pattern was observed in the PWT case observed in our study, and it was thought that the tumor could be one of the PWT types compatible with the whirling pattern mentioned earlier. When evaluated in terms of malignancy, it was observed that the tumor has a characteristic of less differentiation in terms of nuclear malignancy criteria (marked anisokaryosis, high mitosis, prominent nucleolus structure, etc.). Consistent with this, it was observed that abundant CAL-positive inflammatory cell infiltrations were abundant in this case.

It is difficult to distinguish PWT from PNST in dogs. Histologically, PNSTs are seen as intertwined bundles of spindle-shaped cells with whirling patterns. Compared to those in PWTs, the whirls in PNSTs are less prominent, and tumoral cells surround the sclerotic collagen instead of capillaries. In addition, mesh-shaped cells have thinner and more intracellular fibrils or mucinous matrix than PWT[22–25]. Although no spiral pattern was observed in one PNST case observed in our study, it was noticeable in the other case. A distinction is made from PWT by the absence of a central vein in the tumor with a whirling pattern. In the other PNST, palisade-shaped cell nuclei with distinct clefts similar to Verocay bodies were found in some areas. Since no CAL positivity was found in these tumors, it was not possible to evaluate the association of CAL positivity with malignancy for PNSTs. In this regard, it is thought that the relationship between inflammation and malignancy of PNSTs should be investigated in further studies.

With the unraveling of the relationship between TAMs and malignant tumors, TAMs are now recognized as potential biomarkers for the diagnosis and prognosis of cancer and potential therapeutic targets for cancer. TAMs produce growth factors and angiogenic factors in the tumor microenviroment and release protease enzymes that degrade the extracellular matrix. Therefore, TAMs can support tumor invasion and metastasis by increasing tumor cell proliferation and angiogenesis and degrading of surrounded tissues[26]. There are reports on the direct importance of proteases produced from TAMs, neutrophils, and mast cells in experimental carcinogenesis models[27]. The presence of macrophages in smooth muscle tumors in humans has been investigated, and it has been determined that macrophage infiltrates have a significant relationship with survival in non-gynecological leiomyosarcomas[28]. It is also known that TAMs are significantly associated with poor prognosis in breast, cervical, bladder and gastric cancers in humans[29,30]. Numerous previous studies in dogs have demonstrated the presence of TAMs in malignant mammary tumors and their association with poor prognosis[31–33]. The presence of TAMs in colorectal cancers and malignant melanocytic tumors in dogs has been demonstrated, and it has been demonstrated that their numbers increase compared to that of benign tumors[34,35]. In our study, as in CAL-positive macrophage and neutrophils, a significant increase was observed in malignant tumors compared to benign tumors in scoring made only for macrophages. These macrophages have been found to be dispersed and widespread, particularly in the tumor parenchyma. Although CAL-positive macrophages are statistically high in malignant tumors, their absence in some malignant tumors may be related to the differentiation degree of tumors. Additionally, the presence of mild to moderate macrophages has been observed in some benign tumors. The reason for these infiltrations may be the stimulation of macrophage response associated with environmental effects such as trauma or infections.

There are predictions that damage-related molecular pattern molecules such as CAL may lead to tumor formation and progression of malignancy by regulating the host immune response. CAL stimulates Nf-kB activation through which various genes (Cxc11, Cc15, Cc17, Slc39a10, Lcn2, Zc3h12a, and Enpp2) that are involved in angiogenesis, tumor migration, wound healing and pre-metastatic formations are produced[36,37]. Recent studies have demonstrated that malignancy and tumor invasion are reduced in CAL-suppressed animals and CAL induces cancer cell migration[38,39]. In our study, we observed intensive CAL positivity in both neutrophils and macrophages that were more prominent in malignant tumors, and these cells were distinguished by their morphological features. Neutrophils

are reported to have important roles in cancer and inflammation[40]. CXC chemokines such as interleukin-8 are frequently secreted in human and rodent tumors, and these chemokines have a strong chemotactic effect for neutrophils41. Neutrophils are classified as N1 and N2 according to their capacity to affect T lymphocytes[42]. N2 neutrophils have been reported to suppress anti-tumor CD8+ T cell functions through iNOS production in a rodent breast cancer model[43]. N1 neutrophils have been demonstrated to have anti-tumor activities through direct antibody-dependent cytotoxicity and indirectly through T cell activation and proinflammatory cytokine production[44]. Indeed, neutrophil infiltrations have been associated with poor prognosis in many tumors (hepatocellular carcinoma, bronchoalveolar carcinoma, colorectal carcinoma, and head and neck squamous cell carcinomas) in humans[45–49]. However, it has been reported that neutrophils are associated with a better prognosis in some other tumor types such as gastric carcinoma[50]. Although a numerical increase in malignant tumors was observed in the comparison of benign and malignant neutrophils in our study, no statistically significant increase was observed. The observed variation in neutrophil infiltration in tumors may be related to the level of synthesis of CXC and similar factors in different tumor types. Moreover, neutrophils may not be directly associated with malignancy in canine soft-tissue tumors.

Well-known relationships exist between neutrophils and monocytes/macrophages[51]. Neutrophils and monocytes/macrophages express similar antigens and produce effector molecules such as granular proteins, oxidants, chemokines, and cytokines[52–54]. Chemotactic substances for neutrophils such as CXCL1, CXCL2, and interleukin 1 alpha are produced by tissue macrophages following microbial exposure[55–57]. On the contrary, with substances such as cathepsin G and azurocidin released by neutrophils, other immune cells, especially monocytes/macrophages, are attracted to the region[58]. In our study, although macrophage infiltrations were found to be higher in malignant tumors, no significant difference was observed regarding neutrophil infiltrations. However, a significant correlation was found between these two inflammatory cells when evaluated on the basis of all tumors. This indicates that TAMs and neutrophils can play an important role in tumor malignancy in relation to each other. Thus, it has been demonstrated that in mice whose neutrophils are suppressed, infiltration of mononuclear inflammatory cells, especially monocytes, is decreased[59,60]. Reconstitution of inflammatory monocyte migration has also been observed with the restoration of neutrophils[59]. In another study, a decrease in the distribution of surface markers of monocytes was observed in mice with neutrophil-specific granule deficiency[61]. In our study, it was predicted that these cells may interact with each other in the tumor microenvironment related to cancer. However, since neutrophil infiltrates were observed to be more localized in tumor tissues compared to macrophages, it was thought that the increase of these cells in acute trauma and/or contamination sites could be possible. As a matter of fact, dense localized neutrophil infiltrates that are formed only superficially in a vaginal fibroma case also support this idea. However, it is also obvious that neutrophil infiltrations may cause macrophage migration in cancer tissue.

In conclusion, in our study, it was determined that CAL-positive leukocytes in canine soft-tissue tumors were generally increased in malignant tumors compared to those in benign tumors. Similarly, macrophages were found to be higher, but neutrophils did not significantly increase in malignant tumors. Our findings indicate that these cells may play an important role in the malignancy of canine soft-tissue tumors. However, it is possible to investigate in detail the relationship of these cells with malignancy and prognosis in canine soft-tissue tumors with further studies to be carried out with larger sample sizes and more specific tumor types and by demonstration of subtypes of both macrophages and neutrophils.

Acknowledgements

This research supported by a grant received from Burdur Mehmet Akif Ersoy University, Scientific Research Project Office (Master of Science-Project No. 0645-YL-20).

References

1. Meuten DJ. Tumors in Domestic Animals. 4th ed. Iowa State Press; 2002. doi:10.1139/W07-062
2. Bray J. Soft tissue sarcoma in the dog: An update. In Pract. 2018;40(9):383-395. doi:10.1136/inp.k707
3. Tu Z, Xiao R, Xiong J, et al. CCR9 in cancer: Oncogenic role and therapeutic targeting. J Hematol Oncol. 2016;9(1). doi:10.1186/s13045-016-0236-7
4. Caso G, Barry C, Patejunas G. Dysregulation of CXCL9 and reduced tumor growth in Egr-1 deficient Mice. J Hematol Oncol. 2009;2(1):7. doi:10.1186/1756-8722-2-7
5. Teng F, Tian WY, Wang YM, et al. Cancer-associated fibroblasts promote the progression of endometrial cancer via the SDF-1/CXCR4 axis. J Hematol Oncol. 2016;9(1). doi:10.1186/s13045-015-0231-4
6. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423-1437. doi:10.1038/nm.3394
7. Chen Y, Zhang S, Wang Q, Zhang X. Tumor-recruited

M2 macrophages promote gastric and breast cancer metastasis via M2 macrophage-secreted CHI3L1 protein. J Hematol Oncol. 2017;10(1):1-13. doi:10.1186/s13045-017-0408-0
8. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. Cell. 2010;140(6):883-899. doi:10.1016/j.cell.2010.01.025
9. Donato R. S100: A multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int J Biochem Cell Biol. 2001;33(7):637-668. doi:10.1016/S1357-2725(01)00046-2
10. Heizmann CW, Fritz G, Schäfer BW. S100 proteins: structure, functions and pathology. Front Biosci. 2002;7:d1356-68. doi:10.2741/a846
11. Koy M, Hambruch N, Hussen J, Pfarrer C, Seyfert HM, Schuberth HJ. Recombinant bovine S100A8 and A9 enhance IL-1β secretion of interferon-gamma primed monocytes. Vet Immunol Immunopathol. 2013;155(3):162-170. doi:10.1016/j.vetimm.2013.07.002
12. Loser K, Vogl T, Voskort M, et al. The toll-like receptor 4 ligands Mrp8 and Mrp14 are crucial in the development of autoreactive CD8+ T cells. Nat Med. 2010;16(6):713-717. doi:10.1038/nm.2150
13. Chen H, Xu C, Jin Q, Liu Z. S100 protein family in human cancer. Am J Cancer Res. 2014;4(2):89-115. Accessed January 9, 2021. http://www.ncbi.nlm.nih.gov/pubmed/24660101
14. Balkwill F, Mantovani A. Inflammation and cancer: Back to Virchow? Lancet. 2001;357(9255):539-545. doi:10.1016/S0140-6736(00)04046-0
15. Kang JH, Hwang SM, Chung IY. S100A8, S100A9 and S100A12 activate airway epithelial cells to produce MUC5AC via extracellular signal-regulated kinase and nuclear factor-κB pathways. Immunology. 2015;144(1):79-90. doi:10.1111/imm.12352
16. Hendrick M, Mahaffeey E, Moore F, Vos J, Walder E. Histological Classification of Mesenchymal Tumors of Skin and Soft Tissues of Domestic Animals, Vol. 2. Armed Forces Institute of Pathology; 1998.
17. Avallone G, Helmbold P, Caniatti M, Stefanello D, Nayak RC, Roccabianca P. The spectrum of canine cutaneous perivascular wall tumors: morphologic, phenotypic and clinical characterization. Vet Pathol. 2007;44(5):607-620. doi:10.1354/vp.44-5-607
18. Gross T, Ihrke P, Walder E, Affolter V. Skin Diseases of the Dog and Cat: Clinical and Histopathologic Diagnosis. 2nd ed. Blackwell; 2005.
19. Parkin D, Pisani P, Munoz N, Ferlay J. Infections and human cancer. In: Newton R, Beral V, Weiss R, eds. Cold Spring Harbor Laboratory Press; 1999.
20. Gulumian M. The role of oxidative stress in diseases caused by mineral dusts and fibres: Current status and future of prophylaxis and treatment. Mol Cell Biochem. 1999;196(1-2):69-77. doi:10.1023/A:1006918212866
21. Ekbom A, Helmick C, Zack M, Adami H. Ulcerative colitis and colorectal cancer. A population-based study. New Engl J Med. 1990;323(18):1228-1233.
22. Brooks J. Disorders of soft tissue. In: Sternberg S, ed. Diagnostic Surgical Pathology. 3rd ed. Lippincott Williams & Wilkins; 1999:131-221.
23. Middleton LP, Duray PH, Merino MJ. The histological spectrum of hemangiopericytoma: Application of immunohistochemical analysis including proliferative markers to facilitate diagnosis and predict prognosis. Hum Pathol. 1998;29(6):636-640. doi:10.1016/S0046-8177(98)80015-4
24. Perry A, Scheithauer BW, Nascimento AG. The immunophenotypic spectrum of meningeal hemangiopericytoma: A comparison with fibrous meningioma and solitary fibrous tumor of meninges. Am J Surg Pathol. 1997;21(11):1354-1360. doi:10.1097/00000478-199711000-00010
25. Rosai J. Soft Tissues. In: Rosai J, ed. Ackerman's Surgical Pathology. 8th ed. Mosby; 1996:2021-2034.
26. Mantovani A, Bussolino F, Dejana E. Cytokine regulation of endothelial cell function. FASEB J. 1992;6(8):2591-2599. doi:10.1096/fasebj.6.8.1592209
27. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell. 2000;103(3):481-490. doi:10.1016/S0092-8674(00)00139-2
28. Lee CH, Espinosa I, Vrijaldenhoven S, et al. Prognostic significance of macrophage infiltration in leiomyosarcomas. Clin Cancer Res. 2008;14(5):1423-1430. doi:10.1158/1078-0432.CCR-07-1712
29. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: Implications for new anticancer therapies. J Pathol. 2002;196(3):254-265. doi:10.1002/path.1027
30. Qian BZ, Pollard JW. Macrophage Diversity Enhances Tumor Progression and Metastasis. Cell. 2010;141(1):39-51. doi:10.1016/j.cell.2010.03.014
31. Raposo T, Gregório H, Pires I, Prada J, Queiroga FL. Prognostic value of tumour-associated macrophages in canine mammary tumours. Vet Comp Oncol. 2014;12(1):10-19. doi:10.1111/j.1476-5829.2012.00326.x
32. Monteiro LN, Rodrigues MA, Gomes DA, Salgado BS, Cassali GD. Tumour-associated macrophages: Relation with progression and invasiveness, and assessment of

M1/M2 macrophages in canine mammary tumours. Vet J. 2018;234:119-125. doi:10.1016/j.tvjl.2018.02.016
33. Monteiro LN, dos Reis DC, Salgado BS, Cassali GD. Clinical significance and prognostic role of tumor-associated macrophages infiltration according to histologic location in canine mammary carcinomas. Res Vet Sci. Published online October 17, 2020. doi:10.1016/j.rvsc.2020.10.010
34. Woldemeskel M, Hawkins I, Whittington L. Ki-67 protein expression and tumor associated inflammatory cells (macrophages and mast cells) in canine colorectal carcinoma. BMC Vet Res. 2017;13(1). doi:10.1186/s12917-017-1030-7
35. Pires I, Prada J, Coelho L, Garcia A, Luisa F. Tumour-Associated Macrophages (TAMs) and Cox-2 Expression in Canine Melanocytic Lesions. In: Murph M, ed. Melanoma in the Clinic - Diagnosis, Management and Complications of Malignancy. InTech; 2011:163-180.
36. Ichikawa M, Williams R, Wang L, Vogl T, Srikrishna G. S100A8/A9 activate key genes and pathways in colon tumor progression. Mol Cancer Res. 2011;9(2):133-148. doi:10.1158/1541-7786.MCR-10-0394
37. Zhong JM, Li J, Kang AD, et al. Protein S100-A8: A potential metastasis-associated protein for breast cancer determined via iTRAQ quantitative proteomic and clinicopathological analysis. Oncol Lett. 2018;15(4):5285-5293. doi:10.3892/ol.2018.7958
38. Srikrishna G. S100A8 and S100A9: New insights into their roles in malignancy. J Innate Immun. 2011;4(1):31-40. doi:10.1159/000330095
39. Rajendran P, Chen YF, Chen YF, et al. The multifaceted link between inflammation and human diseases. J Cell Physiol. 2018;233(9):6458-6471. doi:10.1002/jcp.26479
40. Leliefeld PHC, Koenderman L, Pillay J. How neutrophils shape adaptive immune responses. Front Immunol. 2015;6(SEP). doi:10.3389/fimmu.2015.00471
41. Negus RPM, Stamp GWH, Hadley J, Balkwill FR. Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. Am J Pathol. 1997;150(5):1723-1734. Accessed January 12, 2021. /pmc/articles/PMC1858213/?report=abstract
42. Fridlender ZG, Sun J, Kim S, et al. Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β: "N1" versus "N2" TAN. Cancer Cell. 2009;16(3):183-194. doi:10.1016/j.ccr.2009.06.017
43. Coffelt SB, Kersten K, Doornebal CW, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. Nature. 2015;522(7556):345-348. doi:10.1038/nature14282
44. Brandau S, Dumitru CA, Lang S. Protumor and antitumor functions of neutrophil granulocytes. Semin Immunopathol. 2013;35(2):163-176. doi:10.1007/s00281-012-0344-6
45. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, Von Der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. J Clin Oncol. 2009;27(28):4709-4717. doi:10.1200/JCO.2008.18.9498
46. Kuang DM, Zhao Q, Wu Y, et al. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. J Hepatol. 2011;54(5):948-955. doi:10.1016/j.jhep.2010.08.041
47. Rao H-L, Chen J-W, Li M, et al. Increased Intratumoral Neutrophil in Colorectal Carcinomas Correlates Closely with Malignant Phenotype and Predicts Patients' Adverse Prognosis. Hold GL, ed. PLoS One. 2012;7(1):e30806. doi:10.1371/journal.pone.0030806
48. Trellakis S, Bruderek K, Dumitru CA, et al. Polymorphonuclear granulocytes in human head and neck cancer: Enhanced inflammatory activity, modulation by cancer cells and expansion in advanced disease. Int J Cancer. 2011;129(9):2183-2193. doi:10.1002/ijc.25892
49. Wislez M, Rabbe N, Marchal J, et al. Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: Role in tumor progression and death. Cancer Res. 2003;63(6):1405-1412.
50. Caruso RA, Bellocco R, Pagano M, Bertoli G, Rigoli L, Inferrera C. Prognostic value of intratumoral neutrophils in advanced gastric carcinoma in a high-risk area in Northern Italy. Mod Pathol. 2002;15(8):831-837. doi:10.1097/01.MP.0000020391.98998.6B
51. Prame Kumar K, Nicholls AJ, Wong CHY. Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. Cell Tissue Res. 2018;371(3):551-565. doi:10.1007/s00441-017-2753-2
52. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J Leukoc Biol. 2008;83(1):64-70. doi:10.1189/jlb.0407247
53. Nauseef WM. How human neutrophils kill and degrade microbes: An integrated view. Immunol Rev. 2007;219(1):88-102. doi:10.1111/j.1600-065X.2007.00550.x
54. Sunderkötter C, Nikolic T, Dillon MJ, et al. Subpopulations of Mouse Blood Monocytes Differ in Maturation Stage and Inflammatory Response. J

Immunol. 2004;172(7):4410-4417. doi:10.4049/jimmunol.172.7.4410

55. Barry KC, Fontana MF, Portman JL, Dugan AS, Vance RE. IL-1α Signaling Initiates the Inflammatory Response to Virulent Legionella pneumophila In Vivo . J Immunol. 2013;190(12):6329-6339. doi:10.4049/jimmunol.1300100
56. Beck-Schimmer B, Schwendener R, Pasch T, Reyes L, Booy C, Schimmer RC. Alveolar macrophages regulate neutrophil recruitment in endotoxin-induced lung injury. Respir Res. 2005;6(1). doi:10.1186/1465-9921-6-61
57. De Filippo K, Henderson RB, Laschinger M, Hogg N. Neutrophil Chemokines KC and Macrophage-Inflammatory Protein-2 Are Newly Synthesized by Tissue Macrophages Using Distinct TLR Signaling Pathways. J Immunol. 2008;180(6):4308-4315. doi:10.4049/jimmunol.180.6.4308
58. Chertov O, Ueda H, Xu LL, et al. Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. J Exp Med. 1997;186(5):739-747. doi:10.1084/jem.186.5.739
59. Soehnlein O, Zernecke A, Eriksson EE, et al. Neutrophil secretion products pave the way for inflammatory monocytes. Blood. 2008;112(4):1461-1471. doi:10.1182/blood-2008-02-139634
60. Zhou J, Stohlman SA, Hinton DR, Marten NW. Neutrophils Promote Mononuclear Cell Infiltration During Viral-Induced Encephalitis. J Immunol. 2003;170(6):3331-3336. doi:10.4049/jimmunol.170.6.3331
61. Shiohara M, Gombart AF, Sekiguchi Y, et al. Phenotypic and functional alterations of peripheral blood monocytes in neutrophil-specific granule deficiency. J Leukoc Biol. 2004;75(2):190-197. doi:10.1189/jlb.0203063